# Inhibition of the serine protease HtrA1 by SerpinE2 suggests an extracellular proteolytic pathway in the control of neural crest migration

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**ABSTRACT**

We previously showed that SerpinE2 and the serine protease HtrA1 modulate fibroblast growth factor (FGF) signaling in germ layer specification and head-to-tail development of *Xenopus* embryos. Here we present an extracellular proteolytic mechanism involving this serpin-protease system in the developing neural crest (NC). Knockdown of SerpinE2 by injected antisense morpholino oligonucleotides did not affect the specification of NC progenitors but instead inhibited the migration of NC cells, causing defects in dorsal fin, melanocyte and craniofacial cartilage formation. Similarly, overexpression of the HtrA1 protease impaired NC cell migration and the formation of NC-derived structures. The phenotype of SerpinE2 knockdown was overcome by concomitant downregulation of HtrA1, indicating that SerpinE2 stimulates NC migration by inhibiting endogenous HtrA1 activity. SerpinE2 binds to HtrA1, and the HtrA1 protease triggers degradation of the cell surface proteoglycan Syndecan-4 (Sdc4). Microinjection of *Sdc4* mRNA partially rescued NC migration defects induced both by HtrA1 upregulation and SerpinE2 downregulation. These epistatic experiments suggest a proteolytic pathway by a double inhibition mechanism:

SerpinE2 ┤ HtrA1 protease ┤ Syndecan-4 → NC cell migration

## Introduction

Collective cell migration is a fundamental process in the development and maintenance of multicellular organisms (Friedl and Gilmour, 2009; Shellard and Mayor, 2020). Embryonic development relies on the coordinated movement of cells to specific locations, and aberrant cell migration is linked to cancer metastasis. The acquisition of cell motility is associated with an epithelial-mesenchymal transition (EMT), in which cells disrupt epithelial adhesions and remodel junctional complexes in favor of cell-matrix adhesions to adopt a migratory behavior (Nieto et al., 2016; Piacentino et al., 2020). The EMT does not have to be complete, and cells can be motile whilst maintaining contact with one another. A prime example for the study of collective cell migration is the neural crest (NC) in vertebrate embryos (Szabó and Mayor, 2018). The NC is a multipotent cell population that is specified at the border of the neural plate (Le Douarin and Kalcheim, 1999; Schock et al., 2023). EMT initiates migration streams of NC cells towards their targets where they differentiate into diverse cell types and tissues, including peripheral nervous system, melanocytes and craniofacial skeleton. The behavior of NC cells recapitulates certain stages of cancer progression and metastasis (Nieto et al., 2016). Defects in NC development can lead to many congenital syndromes and tumors of the NC lineage (Medina-Cuadra and Monsoro-Burq, 2021). These neurocristopathies highlight the need to better understand the molecular basis of key processes in NC development, including EMT and collective migration.

HtrA1 belongs to a conserved family of serine proteases that are homologous to the heat shock-induced HtrA (High temperature requirement A) peptidase from bacteria and primarily involved in protein quality control and degradation (Zurawa-Janicka et al., 2017). Vertebrate HtrA proteases, comprising the four members HtrA1-4, share a trypsin serine protease domain and a carboxy-terminal PDZ domain with their bacterial counterpart. The HtrA family is implicated in various pathological conditions including cancer, arthritis, neurodegenerative diseases, and

pregnancy disorders. HtrA1 modulates the extracellular matrix and cell signaling as a secreted protein but was found to be active in the cytoplasm and nucleus, too (Clawson et al., 2008; Chien et al., 2009a,b; Campioni et al., 2010). The function of HtrA1 in cell migration has been studied *in vitro* to reveal mainly a negative role, although also a positive role has been reported (Pei et al., 2015). How HtrA1 activities are regulated is poorly understood, and the mechanism by which this protease affects cell migration *in vivo* remains elusive.

Members of the serpin superfamily contain a carboxyterminal reactive center loop that covalently binds to and inhibits target serine proteases inside and outside of cells (Olson and Gettins, 2011). Serpin peptidase inhibitor clade E member 2 (SerpinE2), also known as Protease Nexin-1 (PN1) or Glia-Derived Nexin (GDN) has important roles in the nervous, blood and reproductive systems (Arocas and Bouton, 2015; Monard, 2017). SerpinE2 also functions as a key factor in tumor dissemination, but the molecular mechanism by which this protease inhibitor governs cell migration and metastasis is largely unknown.

We previously showed that HtrA1 and SerpinE2 are transcriptionally induced by fibroblast growth factor (FGF) signals and act as feedback regulators of FGF/Erk signaling in germ layer and anteroposterior axis formation in the early *Xenopus* embryo (Hou et al., 2007; Acosta et al., 2015). HtrA1 activates Erk (extracellular signal-regulated kinase) and expression of the transcription factor Brachyury in the posterior mesoderm, whereas SerpinE2 suppresses Erk activation and *Brachyury* expression in the anterior ectoderm. The HtrA1 protease releases FGF ligands by triggering the cleavage of cell surface proteoglycans such as Syndecan-4 (Sdc4), thereby stimulating FGF/Erk signaling in mesoderm and trunk/tail formation (Hou et al., 2007). SerpinE2 binds to and inhibits HtrA1, thus restricting FGF/Erk signaling and allowing ectoderm and head formation to occur (Acosta et al., 2015). Since SerpinE2 and HtrA1 exhibit overlapping

gene expression in the NC, we now asked whether these proteins might have a role in NC development.

Here, we introduce SerpinE2 as a key player in collective NC cell migration. We show that SerpinE2 promotes NC cell migration via inhibition of the extracellular serine protease HtrA1. SerpinE2 de-represses the HtrA1-mediated block of NC migration in mRNA-injected embryos, and the ability of this protease inhibitor to rescue cell migration depends on its extracellular location and intact reactive center loop. In epistatic experiments, Sdc4 mRNA can partly revert the NC migration defects that are induced by SerpinE2 knockdown or HtrA1 overexpression. We conclude that the SerpinE2/HtrA1/Sdc4 pathway regulates neural crest cell migration in the developing embryo.

## Results

### *SerpinE2* and *HtrA1* are expressed in neural crest cells

The expression of *SerpinE2* and *HtrA1* in early *Xenopus* embryos was previously reported by us and others (Pera et al., 2005; Onuma et al., 2006; Hou et al., 2007; Acosta et al., 2015). To investigate whether these genes are expressed in the developing NC, we performed whole-mount *in situ* hybridization analysis of these genes side by side with the NC marker *Twist* (Figure 1). At neurula stage 17, *Twist* expression labelled pre-migratory NC cells in the deep layer of the ectoderm (Figure 1A,A'; Hopwood et al., 1989). *SerpinE2* transcripts were found in the deep layer of the neural plate and adjacent NC cells (Figure 1B-B'). *HtrA1* was transcribed in the superficial layer of the neural plate and in the NC (Figure 1C,C'). At stage 26, *SerpinE2* and *HtrA1* were co-expressed with *Twist* in ventrally migrating NC cells in the mandibular, hyoid, anterior branchial and posterior branchial streams (numbered as 1 to 4 in Figure 1D-F; see also Figure 1-figure supplement 1A-D). Importantly, *SerpinE2* transcripts accumulated at the ventral

leading front, while *HtrA1* expression was abundant in more dorsal follower cells within the migrating NC cohorts (Figure 1D'-F'; see also Figure 1-figure supplement 1E-G). In addition, *SerpinE2* and *HtrA1* shared overlapping expression in the brain, eye vesicles and otic placodes (Figure 1E,F; see also Figure 1-figure supplement 1F,G). These results showed that *SerpinE2* and *HtrA1* are expressed in pre-migratory NC cells and adjacent tissues (Figure 1G), and that the SerpinE2 inhibitor transcripts prevails in the leading edge and the HtrA1 protease expression is predominant in the following migratory NC cells (Figure 1H).

**SerpinE2 knockdown reproduces the phenotype of neural crest extirpation in *Xenopus* embryos**

Classical extirpation experiments carried out in the urodele *Amblystoma* demonstrated that the cranial NC is important for the formation of the head tissue (Stone, 1922,1926) and the trunk NC for dorsal fin and melanocyte development (Du Shane, 1936). We reproduced these findings in the *Xenopus* embryo and showed that bilateral removal of the neural folds (anlagen of the NC) in the cranial and anterior trunk region at mid-neurula stage (stage 17) resulted in larvae displaying a reduced head size, missing dorsal fin (open arrowheads) and reduced melanocyte pigmentation (arrow) (Figure 2A-C).

The expression pattern prompted us to investigate the function of SerpinE2 in NC development. *Xenopus laevis* is allotetraploid (Session et al., 2016) and contains two *SerpinE2* genes, namely *SerpinE2.L* (*PN1.a*) and *SerpinE2.S* (*PN1.b*), which encode two proteins that share 96% amino acid identity. A combination of two antisense morpholino oligonucleotides (MOs) that target the translation initiation site of *SerpinE2.L* and one MO directed against *SerpinE2.S* (collectively termed *SerpinE2*-MO) efficiently blocks SerpinE2 protein biosynthesis in *Xenopus* embryos (Acosta et al., 2015). We previously noted reduction of head structures in tadpoles upon

microinjection of *SerpinE2*-MO into the animal pole blastomeres at the 8-cell stage. A closer analysis now revealed that these *SerpinE2*-morphant embryos also exhibited a loss of dorsal fin structures (open arrowheads) and a decreased number of melanocytes (arrow) at stage 40 (Figure 2E). These phenotypes were specific, since a standard control-MO had no phenotypic effect (Figure 2D), and co-injection of *SerpinE2*-MO together with a *Flag-SerpinE2* mRNA that is not targeted by the MO, rescued normal development (Figure 2F). The striking similarity of the phenotype obtained after knockdown of SerpinE2 with that of NC extirpation (Figure 2A-C) and supporting evidence from fate mapping studies that NC cells contribute to head mesenchyme, dorsal fin structures, and melanocytes (Tucker, 1986; Sadaghiani and Thiébaud, 1987; Tucker and Slack, 2004) suggest that SerpinE2 might be important for NC development.

**SerpinE2 is dispensable for the specification but essential for the migration of neural crest cells**

We used single injections into the animal pole at 8-cell stage embryos of MOs together with *nlacZ* mRNA as lineage tracer (red nuclei) to identify the injected side and ensure that the MO is properly targeted (Figure 2G). *SerpinE2*-MO does not appear to affect NC specification in the head and trunk, as the expression of *Twist*, *Sox9*, *cMyc*, *Foxd3*, *Snail1* and *Snail2* in pre-migratory NC cells remained unchanged in mid-neurula embryos at stage 18 (Figure 2H-K; see also Figure 2-figure supplement 1).

Since migration of the NC is initiated progressively from anterior to posterior in the neural folds of the closing neural tube (Sadaghiani and Thiébaud, 1987), we chose two distinct stages to monitor the EMT and migration of this cell population. At stage 20, NC cells of the mandibular crest segment are migrating from the mesencephalon around the eye primordium, while cells of the hyoid and branchial crest segments are undergoing EMT in the rhombencephalon (Figure

2L,O). At stage 26, NC cell migration occurs in the hyoid segment anterior to the otic vesicle, and in two split branchial segments posterior to the ear primordium (Figure 2R). A single dorsal injection of *SerpinE2*-MO caused a delay or failure of these NC cells to undergo EMT and migration (open arrowheads) in advanced neurula (Figure 2M,P) and tailbud embryos (Figure 2S). These knockdown effects were specific, because a *SerpinE2*-5MM-MO, which contains five mismatches with the *SerpinE2.L* and *SerpinE2.S* target mRNA sequences, as well as a combination of *SerpinE2*-MO and non-targeted *Flag-SerpinE2* mRNA failed to disrupt NC migration (Figure 2N,Q,T; see also Figure 8B-E' and Figure 8-figure supplement 1A,B below). Ventrally injected *SerpinE2*-MO was less efficient in reducing NC migration (Figure 8-figure supplement 1C,D), because expression of the *SerpinE2* is highest in dorsal regions of post-gastrula embryos (Figure 1B). We conclude that SerpinE2 is dispensable for the initial specification but necessary for the migration of NC cells in *Xenopus* embryos.

**HtrA1 inhibits the development and migration of neural crest cells**

Next, we investigated whether HtrA1 affects NC development (Figure 3). Injection of *HtrA1* mRNA into the animal pole blastomeres led to a decrease of head and eye structures in early tadpole embryos (Figure 3A,B) in accordance with the previously reported axis posteriorizing activity of this protease (Hou et al., 2007). *HtrA1* overexpression also caused a reduction of dorsal fin tissue (open arrowheads) and fewer melanocytes (arrow), suggesting that HtrA1 inhibits the formation of NC-derived structures.

A single dorsal injection of *HtrA1* mRNA did not affect the expression of cranial and trunk NC markers (Figure 3E,F) but blocked EMT and migration of NC cells (Figure 3H-K and Figure 6B-C' below). Quantitative analysis showed that HtrA1 overexpression caused migratory defects at stages 20 and 26 in a concentration-dependent manner (Figure 6-figure supplement 1A,B).

Ventral mRNA injection of *HtrA1*-derived constructs does not properly target the NC and therefore was less effective in inhibiting the migration of this cell population (Figure 6-figure supplement 1C,D).

Using Western blot analysis with a polyclonal anti-HtrA1 antibody, we previously showed that a morpholino oligonucleotide directed against *HtrA1.L* and *HtrA1.S*, designated *HtrA1*-MO, efficiently blocks endogenous HtrA1 protein expression in *Xenopus* embryos (Hou et al., 2007). Dorsal injection of *HtrA1*-MO failed both to affect the induction of the NC marker *Twist* (Figure 3G) and to alter the migration of *Twist*$^+$ NC cells (Figure 3L), suggesting that knockdown of HtrA1 appears not to affect NC development. We conclude that upregulation of HtrA1 protease activity inhibits the migration but not the specification of NC cells.

**HtrA1 and SerpinE2 control the development of the head cartilage skeleton**

Cranial neural crest cells contribute to the craniofacial skeleton (Sadaghiani and Thiébaud, 1987). The mandibular crest stream gives rise to the ethmoid-traberculum (upper jaw), palatoquadrate and Meckel's cartilage (lower jaw); the hyoid crest stream supplies the ceratohyal cartilage; and the branchial crest streams forms the paired ceratobranchial cartilages (gills) (Figure 4L). The transcription factor Sox9 is a master regulator of chondrogenesis that mediates the condensation of chondrogenic mesenchyme and chondrocyte differentiation (Suzuki et al., 2012). In early tadpoles at stages 40/41, *Sox9* expression demarcates chondrogenic precursors in the head mesenchyme (Figure 4A,A'). Upon microinjection of *HtrA1* mRNA into the animal pole of all blastomeres at the 4-cell stage, *Sox9* expression was reduced in developing cartilage structures (Figure 4B,B'). A similar reduction of *Sox9*$^+$ cartilaginous elements was induced by *SerpinE2*-MO, whereas the control-MO had no effect (Figure 4C-D'). The results suggest that HtrA1 overexpression and SerpinE2 knockdown impair with craniofacial cartilage formation.

Alcian Blue staining was used to visualize the cartilaginous skeleton in *Xenopus* tadpoles at stages 46 (Figure 4E). For quantification of the skeleton size, we measured the width at the level of the anterior ceratobranchial cartilages (Figure 4-figure supplement 1A). *HtrA1* mRNA injection reduced all cartilage structures and lowered the size of the head skeleton by 21% (Figure 4F, Figure 4-figure supplement 1B,E). Similarly, *SerpinE2*-MO decreased the skeleton size by 22% compared to control-morphant siblings (Figure 4I,J, Figure 4-figure supplement 2A,B,D). The coiling pattern of the gut was not affected in *SerpinE2*-morphant tadpoles (Figure 4-figure supplement 2B, arrowhead), ruling out an overall delay of embryonic development. Co-injection of *SerpinE2*-MO together with non-targeted *Flag-SerpinE2* mRNA rescued craniofacial skeleton development (Figure 4K, Figure 4-figure supplement 2C,D). We conclude that HtrA1 and SerpinE2 regulate the formation of NC-derived cartilage structures.

**HtrA1 and SerpinE2 act in the neural crest to regulate cell adhesion on fibronectin**

Since HtrA1 and SerpinE2 are expressed in both NC cells and surrounding tissue, we asked whether the proteins affect cell migration in a NC-autonomous or non-autonomous manner. The cranial NC can be dissected from *Xenopus* embryos at stage 17 and cultured on fibronectin *in vitro* to investigate collective cell migration in relative isolation, allowing for the identification of extrinsic versus intrinsic mechanisms (Figure 5A; Alfandari et al., 2003). After 4 hours, cells from uninjected explants spread as a coherent sheet towards one side and thereby doubled the surface area compared to the time of plating (Figure 5B-B'',F, filled arrowhead). In contrast, little cell migration was observed in explants upon injection with *HtrA1* mRNA, contributing to only a 20% increase in surface area within this time frame (Figure 5C-C'',F, open arrowhead). Similarly, *SerpinE2*-MO inhibited cell migration, leading to only one tenth of the increase in total surface area that was observed in control-morphant explants (Figure 5D-E'',F). At 7 hours after plating,

distinct segments were seen in uninjected and control-MO-injected explants (Figure 5B’’’,D’’’), resembling the mandibular, hyoid, and branchial streams seen in sibling control embryos at the tailbud stage (Figure 1D). In contrast, explants injected with either *HtrA1* mRNA or *SerpinE2*-MO failed to display segmentation into distinct streams (Figure 5C’’’,E’’’). In time-lapse video microscopy, control-MO-injected explants exhibited lamellipodia and filopodia at the leading front of the migratory NC cell clusters (Figure 5-video 1). *SerpinE2*-morphant NC cells lost polarity and took on a spherical appearance (Figure 5-video 2). These results show that both HtrA1 overexpression and SerpinE2 knockdown inhibit collective cell migration *in vitro*, providing evidence that HtrA1 and SerpinE2 regulate cell migration in the isolated NC.

To examine cell-matrix adhesion, we dissociated cranial NC explants in $Ca^{2+}$/$Mg^{2+}$-free medium and cultured them as single cells on fibronectin (Figure 5G). For better visibility of the NC cells, the donor embryos were injected with mRNA for enhanced green-fluorescent protein (eGFP). At 1 hour after plating, $eGFP^{+}$ cells adhered to fibronectin and extended cytoplasmic processes to this extracellular matrix protein (Figure 5H, filled arrowhead). Upon injection of *HtrA1* mRNA, nearly 80% of cells failed to attach and acquired a round morphology (Figure 5I and Figure5L, open arrowhead). Similarly, *SerpinE2*-MO-injected cells lost adherence and cytoplasmic extensions, whereas the control-MO had no significant effect (Figure 5J-L). We conclude from these *in vitro* explant and single cell data that HtrA1 and SerpinE2 regulate NC cell adhesion and migration on fibronectin.

**HtrA1 controls neural crest migration as a secreted protease**

HtrA1 contains a cleavable N-terminal signal peptide (SP), a trypsin-like serine protease domain and a carboxyterminal PDZ (Post-synaptic density of 95kD, Discs large, Zona occludens-1) protein-protein interaction domain (Figure 6A, top left; Zurawa-Janicka et al., 2017). The SP is a

short hydrophobic peptide sequence that destines proteins normally to the secretory pathway (Owji et al., 2018). We previously identified *Xenopus* HtrA1 as a protein in the supernatant of cDNA-transfected and metabolically labelled HEK 293T cells (Hou et al., 2007), suggesting that the protease might act in the extracellular space. On the other hand, human HtrA1 has been shown to associate with and stabilize microtubules in a PDZ domain-dependent manner (Chien et al., 2009b), raising the possibility for a non-proteolytic function of cytosolic HtrA1 in the regulation of cell motility. In a structure-function analysis, we investigated (1) whether HtrA1 acts as a protease in NC development, (2) whether HtrA1 operates in the extracellular space or inside the cell, and (3) whether its PDZ domain is involved in regulating NC migration. To this end, we compared with their cognate wild type HtrA1 constructs three mutant derivatives, including HtrA1(S307A), in which the catalytic serine residue in amino acid position 307 is replaced by alanine (Hou et al., 2007), a newly generated Flag-tagged construct that lacks the secretory signal peptide (Flag-HtrA1ΔSP), and a myc-tagged construct that is devoid of the PDZ domain (HtrA1ΔPDZ-myc; Acosta et al., 2015) (Figure 6A).

Using Western blot analysis, we previously reported that HtrA1(S307A) (Hou et al., 2007) and HtrA1ΔPDZ-myc (Acosta et al., 2015) generate proteins of the expected size in mRNA-injected *Xenopus* embryos. These mutant HtrA1 constructs co-immunoprecipitate with Flag-SerpinE2 at protein levels similar to their corresponding wild type HtrA1 and HtrA1-myc constructs (Acosta et al., 2015). Here we used Western blot analysis of lysates to show that the Flag-HtrA1 and Flag-HtrA1ΔSP protein constructs were expressed with the expected molecular weights in both cDNA-transfected HEK293T cells and mRNA-injected *Xenopus* embryos (Figure 6-figure supplement 2A,B,D). However, while the Flag-HtrA1 protein accumulated at high levels in the supernatant of transfected cells, the Flag-HtrA1ΔSP protein failed to be efficiently secreted into the culture medium (Figure 6-figure supplement 2C). We also showed that wild type Flag-HtrA1 and

cytosolic Flag-HtrA1ΔSP degraded αTubulin, but not βActin, which validates the proteolytic activity and target specificity of these constructs (Figure 6-figure supplement 2B,E-G).

Neither *HtrA1(S307A)* nor *Flag-HtrA1ΔSP* affected NC migration in mRNA-injected embryos (Figure 6D-E'; see also Figure 6-figure supplement 1A,B). These two mutant constructs also failed to induce any defects in dorsal fin, melanocyte (Figure 3C,D) and craniofacial skeleton structures (Figure 4G,H; Figure 4-figure supplement 1C-E), suggesting that HtrA1 relies on an intact protease domain and a transport to the secretory pathway to regulate the development of these NC derivatives. *HtrA1ΔPDZ-myc* efficiently inhibited EMT and NC migration to a degree comparable to that induced by *HtrA1-myc* control mRNA (Figure 6F-G'; Figure 6-figure supplement 1A,B). These results support the conclusions that HtrA1 acts as an extracellular protease and that an association of HtrA1 via its PDZ domain to microtubuli appears not to regulate the migratory behavior of NC cells in the *Xenopus* embryo.

**SerpinE2 interacts with HtrA1 in neural crest cell migration**

The serpin superfamily comprises extracellular and intracellular members with an exposed reactive center loop (RCL) that is cleaved by a target protease and irreversibly inhibits the attacking enzyme by forming a covalent serpin-protease complex (Olson and Gettins, 2011). SerpinE2 contains an N-terminal signal peptide and a C-terminal RCL (Figure 7A). We previously showed that SerpinE2 via its RCL binds to the trypsin domain of HtrA1 (Acosta et al., 2015). Do SerpinE2 and HtrA1 interact in NC cell migration? To this end, we used Flag-tagged SerpinE2 (Flag-SerpinE2), a point mutant Flag-SerpinE2pm derivative, in which two proline residues replace the critical arginine and serine residues (R362P and S363P) at the process site of the RCL, and a newly generated truncated construct that lacks the N-terminal signal peptide (Flag-SerpinE2ΔSP) (Figure 7A). In previous Western blot studies, we showed that Flag-

SerpinE2 and Flag-SerpinE2pm are synthesized in similar protein amounts and at the expected sizes, but that overexpressed HtrA1 immunopreciptates Flag-SerpinE2pm less efficiently than Flag-SerpinE2 (Acosta et al., 2015). Here we use Western blotting to validate that Flag-SerpinE2ΔSP protein is generated in expected amount and size upon mRNA injection in *Xenopus* embryos (Figure 6-figure supplement 2A,D,E). However, unlike wild type Flag-SerpinE2, the signal peptide-deficient Flag-SerpinE2ΔSP construct is not efficiently secreted in cDNA-transfected HEK293 cells (Figure 6-figure supplement 2B,C).

Microinjection of *Flag-SerpinE2* mRNA at doses of up to 4 ng did not affect the migration of *Twist*$^{+}$ NC cells (marked with nuclear lacZ lineage tracer) at stages 20 and 26 (Figure 7B,B'; see also Figure 7-figure supplement 1). However, co-injection of *Flag-HtrA1* and *Flag-SerpinE2* mRNAs reverted the EMT and NC migration defects that were induced by *Flag-HtrA1* mRNA alone (Figure 7C-D'; Figure 7-figure supplement 1), suggesting that SerpinE2 can relieve HtrA1-mediated suppression of NC migration. *Flag-SerpinE2ΔSP* failed to rescue the *Flag-HtrA1*-induced NC migration defects (Figure 7E,E'; Figure 7-figure supplement 1), underscoring that entry into the secretory pathway is important for the function of SerpinE2. Moreover, *Flag-SerpinE2pm* did not inhibit *Flag-HtrA1* mRNA from blocking NC migration (Figure 7F,F'; Figure 7-figure supplement 1). We conclude that SerpinE2 functions in the extracellular space and requires an intact RCL for efficient interaction with HtrA1 to modulate NC cell migration.

**Double knockdown of SerpinE2 and HtrA1 rescues neural crest migration**

We next asked whether the NC migration defects induced by SerpinE2 depletion are dependent on endogenous HtrA1 protein. In loss-of-function experiments, we co-injected *SerpinE2*-MO and *HtrA1*-MO into a single dorsal blastomere and assessed the effects on NC cells. Importantly, knockdown of HtrA1 significantly reduced the EMT and migration defects in SerpinE2-depleted

embryos (Figure 8D,D',F,F'; see also Figure 8-figure supplement 1A,B). These epistatic experiments support the existence of an extracellular proteolytic regulatory system, in which SerpinE2 stimulates NC cell migration through the inhibition of HtrA1.

**HtrA1 and SerpinE2 regulate cranial neural crest migration via Syndecan-4**

Syndecans comprise a family of single-pass transmembrane proteoglycans with four members in vertebrates (Keller-Pinter et al., 2021). Syndecan-4 (Sdc4) is a major component of focal adhesions and interacts with fibronectin during cell-matrix adhesion and cell movement (Woods and Couchman, 2001). We previously showed that HtrA1 triggers the proteolytic cleavage of Sdc4 and that SerpinE2 protects this proteoglycan from degradation by HtrA1 (Hou et al., 2007; Acosta et al., 2015). In *Xenopus* embryos, Sdc4 is abundant in the NC; knockdown of Sdc4 does not affect the induction of the NC but reduces its cell migration (Matthews et al., 2008). The effects of HtrA1 overexpression and SerpinE2 downregulation described in this study mimic the effects of Sdc4 depletion (Matthews et al., 2008). To investigate the relationship between the SerpinE2 and HtrA1 axis with Sdc4 in the NC we dorsally injected *Sdc4* mRNA and found it reduced in a concentration-dependent manner the EMT and NC migration defects induced by *Flag-HtrA1* mRNA (Figure 7C,C',G,G'; see also Figure 7-figure supplement 1A,B). Ventral co-injection of *Sdc4* and *Flag-HtrA1* mRNAs was less efficient in rescuing NC cell migration (Figure 7-figure supplement 1C,D), because the transmembrane Sdc4 protein - unlike the secreted HtrA1 protease - remains anchored to ventral regions of the embryo and does not reach the dorsally located NC cells. Importantly, dorsally injected *Sdc4* mRNA partially restored normal EMT and cell migration in *SerpinE2*-morphant embryos (Figure 8D,D',G,G'; see also Figure 8-figure supplement 1A,B). Therefore, our epistatic studies in *Xenopus* embryos suggest that SerpinE2 promotes NC cell migration by inhibiting HtrA1-mediated degradation of Syndecan-4.

## Discussion

This study reveals, for the first time, a role for the SerpinE2 and HtrA1 proteolytic pathway in embryonic cell migration. Several lines of evidence support the conclusion that this inhibitor/protease pair functionally interact in the control of NC cell motility. (1) SerpinE2 and HtrA1 were co-expressed in pre-migratory and migrating NC cells in the *Xenopus* embryo. (2) In gain-of-function studies, wild-type HtrA1, but not a protease-defective construct, inhibited NC migration and development of NC-derived structures, such as branchial arch cartilage, dorsal fin tissue, and melanocytes. (3) SerpinE2 reverted the HtrA1-induced migration defects in mRNA-injected embryos. (4) In loss-of-function studies, SerpinE2 knockdown inhibited NC migration and development of NC-derived structures. (5) Concomitant knockdown of SerpinE2 and HtrA1 restored normal NC migration in morpholino-oligonucleotide-injected embryos. Additional epistatic experiments showed that *Sdc4* mRNA partially rescues the migration defects induced by HtrA1 overexpression or SerpinE2 knockdown. Thus, SerpinE2, HtrA1 and Sdc4 form an important regulatory axis to control NC development. SerpinE2 promotes NC cell migration by inhibiting endogenous HtrA1 and preventing this protease from degrading the transmembrane proteoglycan Sdc4 in the *Xenopus* embryo. Hence, our study reveals a critical role for the SerpinE2-HtrA1-Sdc4 axis of extracellular proteins to regulate collective cell migration *in vivo* (Figure 8A).

### Proteolytic control of morphogens

Secreted proteases, particularly of the astacin class of zinc metalloproteases are important for the formation of morphogen gradients. In Hydra, the HAS-7 protease normally processes Wnt3 and restricts head organizer formation; its knockdown results in ectopic organizers (Ziegler et al., 2021; Holstein, 2022). In *Xenopus*, Tolloid regulates Spemann's organizer function by cleaving

Chordin and de-repressing BMP signaling (Piccolo et al., 1997). The secreted Frizzled-related protein Sizzled binds to and inhibits Tolloid (Lee et al., 2006) and controls patterning of the dorsoventral axis through the following pathway:

Sizzled ┤ Tolloid protease ┤ Chordin ┤ BMP

Our group previously showed that the serine protease HtrA1 induces mesoderm and ectopic tail formation by triggering the cleavage of Syndecan-4 and releasing active FGF messages (Hou et al., 2005). SerpinE2 (previously named protease nexin-1, PN1) binds to and inhibits HtrA1 (Acosta et al., 2015) and controls germ layer formation and patterning of the anteroposterior axis as follows:

SerpinE2 ┤ HtrA1 protease ┤ Syndecan-4 ┤ FGF

**Extracellular proteases in cell migration**

Migrating NC cells face major challenges to overcome physical barriers, such as the basal membrane or the extracellular matrix, suggesting proteolysis as an important mechanism for these cells to invade other tissues and reach their destined targets in the embryo. Matrix metalloproteases have been well-studied in extracellular matrix remodeling during NC development (Gouignard et al., 2018). Less understood are other classes of proteases in the control of NC cell migration and invasion. The laboratory of Nicole Le Douarin was first to show that migrating NC cells produce serine proteases (Valinsky and Le Douarin, 1985). Using interspecific quail-chick grafting and enzyme-specific zymography, the group demonstrated high plasminogen activator activity in lysates of cranial NC compared to adjacent embryonic tissues.

Subsequent studies by other laboratories showed that mouse NC cells secrete urokinase and tissue plasminogen activators (uPA and tPA) into the culture medium (Menoud et al., 1989) and that uPA promotes chick NC migration *in vitro* via activation of plasmin and TGFβ signaling (Brauer

and Yee, 1993; Agrawal and Brauer, 1996). Mutations in the lectin complement pathway gene MASP1/3, encoding for Mannose-associated serine protease-1 and -3, cause 3MC (Mingarelli, Malpuech, Michels and Carnevale) syndrome, a rare autosomal recessive disorder that is characterized by a spectrum of developmental features including craniofacial abnormalities (Rooryck et al., 2011). Zebrafish morphants exhibit craniofacial cartilage and pigment defects as well as abnormal NC migration, suggesting that MASP1 is a guidance cue that directs the migration of NC cells in the early embryo. Of note, all secreted serine proteases listed above have a positive role in NC migration. As we now demonstrate, HtrA1 is the only serine protease identified so far that acts as a negative regulator of NC migration.

SerpinE2 has a broad spectrum of target serine proteases that it binds to and inhibits, including uPA, tPA and plasmin (Arocas and Bouton, 2015). Given the pro-migratory properties of these serine proteases, one should expect that SerpinE2 would inhibit NC migration by antagonizing their activity. However, overexpression of *SerpinE2* at mRNA doses of up to 4 ng did not affect the migration of NC cells in the *Xenopus* embryo. Instead, knockdown by morpholino oligonucleotides that block endogenous SerpinE2 protein biosynthesis (Acosta et al., 2015), efficiently inhibited NC migration and the development of NC-derived structures, as shown in this study. The finding that microinjection of *SerpinE2* mRNA reverted migration defects in these morphant embryos supports the view that SerpinE2 specifically promotes NC migration. It therefore appears that uPA, tPA and plasmin are not target proteases of SerpinE2 in the modulation of NC migration in the *Xenopus* embryo.

**SerpinE2 and HtrA1 form a proteolytic pathway in neural crest migration**

In *Xenopus* embryos at the neurula stage, *HtrA1* transcripts were most abundant in the superficial (ependymal) layer of the ectoderm containing non-motile epithelial cells, whereas *SerpinE2*

expression was confined to the deep (sensorial) layer of the ectoderm that gives rise to motile mesenchymal NC cells (Figure 1G). In post-neurula embryos, transcripts of these genes appeared in the collective of migrating NC cells, with *SerpinE2* accumulating near the front of the cell streams and *HtrA1* being enriched at their rear ends (Figure 1H). We propose that only in regions where the SerpinE2 concentration is sufficiently high, HtrA1-mediated repression of cell motility is relieved so that NC cell migration can occur. In support of this conclusion, microinjection of *HtrA1* mRNA reduced in a concentration-dependent manner EMT and migration of NC cells, leading to defects in craniofacial skeleton structures. A key experiment was that EMT and migration of NC cells were restored by HtrA1 knockdown in SerpinE2-depleted embryos, suggesting that SerpinE2 promotes NC migration via inhibiting endogenous HtrA1 protease activity in the embryo. Our finding that *HtrA1* mRNA and *SerpinE2*-MO diminished cell migration in isolated NC explants provided evidence that the two proteins act in a NC-autonomous manner. HtrA1 overexpression and SerpinE2 knockdown also reduced *Sox9*-expressing chondrogenic precursors in the NC-derived head mesenchyme and caused defects in the cartilaginous skull and hyobranchial skeleton in *Xenopus* tadpoles. Interestingly, increased *HtrA1* expression has been detected in cranial sutures of mice with thyroid hormone-induced craniofacial disruptions (Howie et al., 2016). Since proper migration of NC cells is essential for the formation of bones, cartilage and soft tissue in the head (Minoux and Rijli, 2010), elevated HtrA1 levels might disturb NC migration not only in *Xenopus*, but also in mammalian embryos.

**SerpinE2 and HtrA1 regulate neural crest migration as extracellular proteins**

The expression patterns were consistent with loss- and gain-of-function data that SerpinE2 promoted EMT and NC cell migration, whereas HtrA1 had the opposite effect. The actin cytoskeleton and microtubules play important roles in cell migration (Seetharaman and Etienne-Manneville, 2020). Two cytoskeletal proteins with functions in cell movement, i.e. fascin (actin

bundling) and talin1 (regulation of actin assembly in focal adhesions), were previously identified as proteolytic substrates of HtrA1 (An et al., 2010). It has also been shown that the protease cleaves tubulins (Chien et al., 2009a) and that HtrA1 binds to and stabilizes microtubules via its PDZ domain (Chien et al., 2009b). However, the significance of the degradation of cytoskeletal proteins by HtrA1 remains unclear and it is unknown whether the association between HtrA1 and microtubules is important for cell motility. Here we showed that wild-type HtrA1, but not a derived construct that was lacking a secretory signal peptide (HtrA1ΔSP), inhibited NC migration as well as craniofacial, dorsal fin, and melanocyte development. An HtrA1 construct with a deletion of the PDZ domain (HtrA1ΔPDZ) efficiently reduced EMT and migration of NC cells. The results strongly suggest that HtrA1 acts primarily as an extracellular protease during NC collective cell migration.

SerpinE2 shares with other members of the Serpin family a reactive center loop (RCL) that is cleaved by target proteases at the process site and forms a covalent acyl-enzyme complex leading to irreversible inhibition of the protease (Olson and Gettins, 2011; Arocas and Bouton, 2015). The introduction of prolines to residues P1 and P1' at the RCL cleavage site reduces the ability of SerpinE2 to physically interact with the catalytic trypsin domain of HtrA1 and inhibits its protease activity (Acosta et al., 2015). Here we demonstrated that wild-type SerpinE2 restored normal EMT and migration of NC cells in *HtrA1* mRNA-injected embryos, whereas a point mutant SerpinE2 construct with the mutations Arg362Pro and Ser363Pro at P1 and P1' of the scissile site (SerpinE2pm) failed to show any effect. In addition, a SerpinE2 construct without a secretory signal peptide (SerpinE2ΔSP) did not revert the HtrA1-induced NC migration defects. The results underscore that SerpinE2 promotes NC migration as an extracellular protease inhibitor.

**The SerpinE2-HtrA1-Syndecan-4 axis functions in neural crest migration**

Syndecan-4 (Sdc4) is a central component of focal adhesion complexes that regulate cell-matrix adhesion and cell migration in cooperation with members of the integrin family of transmembrane proteins (Keller-Pinter et al., 2021). In zebrafish and *Xenopus* embryos, Sdc4 is expressed in migrating NC cells and promotes directional NC cell migration via regulation of the small GTPase Rac1 and activation of non-canonical Wnt signaling in the planar cell polarity pathway (Matthews et al. 2008). Similarly, human SDC4 favors cell migration and invasion through activation of Wnt5A signals in melanoma (O'Connell et al., 2009), indicating conserved signaling downstream of this proteoglycan in NC cell migration and malignant progression of a NC-derived cancer.

Several metalloproteases (MMP-3, -9 and -14) and serine proteases (plasmin, thrombin) cleave Sdc4 preferentially at the juxtamembrane site, so that its ectodomain can be released from the cell surface (Manon-Jensen et al., 2013). We previously showed that HtrA1 triggers the proteolytic cleavage of *Xenopus* Sdc4 (Hou et al., 2007). Whether HtrA1 directly cleaves this transmembrane protein or induces its cleavage through activation of other proteases remains to be shown. We further reported that SerpinE2 through physical interaction prevents HtrA1-mediated Sdc4 degradation, and that endogenous SerpinE2 is needed to protect the integrity of Sdc4 in embryos (Acosta et al., 2015). Here we showed that *Sdc4* mRNA rescued NC migration defects that were induced by co-injection of *HtrA1* mRNA, indicating that Sdc4 is a relevant target of HtrA1 *in vivo*. The finding that *Sdc4* mRNA also rescued migration defects in *SerpinE2*-morphant embryos suggests a proteolytic pathway by the following double inhibition mechanism:

SerpinE2 ┤ HtrA1 protease ┤ Syndecan-4 → NC cell migration

**SerpinE2 and HtrA1 might modulate FGF signals in directed neural crest migration**

FGF signals including FGF8 stimulate NC migration by increasing cell motility and guiding cell movement (Kubota and Ito, 2000; Sato et al., 2011). In *Xenopus* embryos, the target tissues of NC streams secrete FGF8 and other FGF ligands, while the migrating NC cells express FGF receptors, including FGFR1 and FGFR4 (Brändli and Kirschner, 1995; Lea et al., 2009). Once released from the cells, FGFs are bound by heparan sulfate (HS) chains of cell surface proteoglycans such as the transmembrane Sdc4, which limit their diffusion (Matsuo and Kimura-Yoshida, 2013). We previously reported that the HtrA1-mediated proteolytic cleavage of Sdc4 mobilizes FGFs complexed to its soluble ectodomain, allowing these FGF-proteoglycan messages to activate FGFRs at distance (Hou et al., 2007). HS protects FGFs against cleavage by serine proteases (Saksela et al., 1988, Sommer and Rifkin, 1989), and HtrA1 can degrade free FGF8 protein (Kim et al., 2012). We showed that the HtrA1 inhibitor SerpinE2 stabilizes Sdc4 and regulates long-range FGF signaling in the *Xenopus* embryo (Acosta et al., 2015). The expression data presented here suggest that opposing gradients of SerpinE2 and HtrA1 form in the collective of migrating NC cells (Figure 1H). By controlling the mobilization of FGF signals, the SerpinE2-HtrA1 pair might help to establish a chemotactic FGF gradient and thereby facilitate collective migration of NC cells (Figure 9B).

**Fibronectin is a possible target of the SerpinE2-HtrA1 axis in neural crest cell adhesion and migration**

Binding of fibronectin to the HS chains of Sdc4 and to the extracellular domain of the integrin adhesion receptors is critical for the activation of intracellular signaling that affects actin polymerization and contraction (Keller-Pinter et al., 2021). Fibronectin is ubiquitously expressed

along NC migration pathways in the *Xenopus* embryo (Davidson et al., 2004). This glycoprotein is the only known extracellular matrix component that promotes *Xenopus* cranial NC cell migration as an adhesive substrate (Alfandari et al., 2003). Our study showed that HtrA1 overexpression and SerpinE2 knockdown reduced adhesion of cranial NC cells to fibronectin *in vitro*. Since fibronectin is a proteolytic substrate of HtrA1 (Grau et al., 2006; Hadfield et al., 2008), degradation of this matrix component might contribute to the HtrA1-mediated inhibition of NC migration.

**A gradient of serine protease activity may act in collective NC migration**

We are proposing a mechanism, in which SerpinE2 and HtrA1 constitute a proteolytic pathway that regulates collective NC migration by targeting the focal adhesion protein Sdc4 and the matrix protein fibronectin (Figure 9A). Our structure-function analyses and epistatic experiments led us suggest a model in which SerpinE2 and HtrA1 regulate directed migration of the NC collective via remodeling of cell-matrix adhesions (Figure 9C). Since SerpinE2 has a high affinity for HS through its heparin binding domain (Li and Huntington, 2012), the transmembrane Sdc4 proteoglycan likely recruits this protease inhibitor to the pericellular space. Elevated SerpinE2 concentration keeps HtrA1 activity low near the leading edge of the NC collective; this protects the integrity of both Sdc4 and fibronectin in focal adhesions, which is critical for the leader cells to attach to the matrix and drive cell migration (left side in Figure 9C). Gradually decreasing SerpinE2 protein levels and a concomitant increase in HtrA1 protease activity behind the leader cells triggers degradation of Sdc4 and cleavage of fibronectin, causing disruption of focal adhesion complexes in follower cells and loss of cell-matrix binding at the rear end of the NC cluster (right side in Figure 9C).

**SERPINE2, HTRA1 and SDC4 might interact in human placenta development and pre-eclampsia**

An interaction of SerpinE2, HtrA1 and Sdc4 in cell migration might not be confined to the NC as shown in this study but could also occur in other aspects of development and disease. In the developing placenta, extravillous trophoblast (EVT) cells from the human embryo migrate into the inner uterus wall (endometrium) and invade the uterine spiral arteries, converting them into large blood vessels so that the blood flow to the embryo is enhanced. Inadequate EVT migration results in insufficient maternal artery remodeling, causing hypoxia of the placenta and hypertension in a pathological condition called pre-eclampsia that is potentially life-threatening for both fetus and mother. The placenta expresses the highest levels of SERPINE2 among all probed human tissues (The Human Protein Atlas; https://www.proteinatlas.org/), with abundant protein expression in migratory EVTs and spiral arteries (Chern et al., 2011). HTRA1 expression is low in EVTs, high in less motile villous trophoblasts and upregulated in the placenta of patients with pre-eclampsia (Ajayi et al., 2008). Interestingly, the migration and invasion of cultured EVTs is inhibited by silencing of SERPINE2 (Chern et al., 2011), overexpression of HTRA1 (Ajayi et al., 2008) and knockdown of SDC4 (Jeyarajah et al., 2019). The results suggest that the SERPINE2-HTRA1-SDC4 axis might regulate trophoblast motility during placental development and a serious pregnancy condition in humans.

**The SERPINE2-HTRA1-SDC4 regulatory axis might act in cancer**

Cell migration is a hallmark of tumor malignancy and essential for metastasis formation (Hanahan and Weinberg, 2011). SERPINE2 is upregulated in aggressive variants of several cancer types, and high expression levels correlate with poor prognosis in patients (Arocas and Bouton, 2015). Downregulation of SERPINE2 impairs the metastatic spread of melanoma and breast cancer cells in xenografted mice (Wagenblast et al., 2015; Wu et al., 2016; Tang et al., 2019), suggesting a

positive role of SERPINE2 in cancer cell migration and metastasis. On the other hand, HTRA1 is downregulated in many primary tumors and metastatic foci, while low levels correlate with poor clinical outcome (Zurawa-Janicka et al., 2017). Overexpression of HTRA1 inhibits the invasion and migration of melanoma and breast cancer cells, respectively (Baldi et al., 2002; Wang et al., 2012). SDC4 is overexpressed in several tumor types such as melanoma, and high expression levels are associated with poor prognosis in breast cancer (Keller-Pinter et al., 2021). Gene silencing confirms the requirement of SDC4 in the metastasis of human breast carcinoma cells in mice (Leblanc et al. 2018). Together, the double-inhibitory mechanism involving the molecular interaction of SERPINE2, HTRA1 and SDC4 as suggested here for NC cell migration in the *Xenopus* embryo might also regulate cancer cell migration in human metastasis.

**Conclusions**

This is the first study to demonstrate an interaction of SerpinE2 and HtrA1 in cell migration *in vivo*. We showed that SerpinE2 and HtrA1 acted in the extracellular space at least partly through the cell surface proteoglycan Sdc4 to govern NC migration in *Xenopus* embryos. The secreted SerpinE2 and HtrA1 proteins, as well as the ectodomain of Sdc4, are amenable to neutralizing antibodies. Our structure-function analysis unraveled important roles for the RCL of SerpinE2 and the catalytic serine residue of its target protease, whereas the PDZ domain of HtrA1 was not involved in the control of NC cell migration. The identification of critical domains in SerpinE2 and HtrA1 provides an important basis for the development of effective therapeutics in craniofacial anomalies and other neurocristopathies. Given the roles of these proteins in trophoblast migration in the developing placenta, together with their implication in metastasis of numerous tumor types, the SERPINE2-HTRA1-SDC4 axis constitutes a potential therapeutic target for the treatment of pre-eclampsia and various aggressive cancers.

## Materials and Methods

| Key Resources Table | | | | |
|---|---|---|---|---|
| **Reagent type (species) or resource** | **Designation** | **Source or reference** | **Identifiers** | **Additional information** |
| Cell line (*Homo-sapiens*) | HEK293T | TakaraBio | Cat# 632180 | Used for transient transfection and protein production |
| Biological sample (*Xenopus laevis*, male) | Sperm | Nasco | Cat# LM00715M | Used for *in vitro* fertilization |
| Biological sample (*Xenopus laevis*, male) | Sperm | Xenopus 1 | Cat# 5215 | Albino; used for *in vitro* fertilization |
| Biological sample (*Xenopus laevis*, female) | Egg | Nasco | Cat# LM00535M | Used for *in vitro* fertilization |
| Biological sample (*Xenopus laevis*, female) | Egg | Nasco | Cat# LM00510M | Albino; used for *in vitro* fertilization |
| Biological sample (*Xenopus laevis*, female) | Egg | Xenopus 1 | Cat# 4280 | Used for *in vitro* fertilization |
| Antibody | anti-flag M2-Peroxidase (HRP) (Mouse monoclonal) | Sigma-Aldrich | Cat# A8592 | WB (1:1000) |
| Antibody | anti-$\alpha$-Tubulin (Mouse monoclonal) | Sigma-Aldrich | Cat# T5168 clone B-5-1-2 | WB (1:1000) |

| | | | | |
|---|---|---|---|---|
| Antibody | anti-β-Actin (Mouse monoclonal) | Sigma-Aldrich | Cat# A5441 clone AC-15 | WB (1:10,000) |
| Antibody | anti-HtrA1 (Rabbit polyclonal) | Hou et al. (2007) PMID: 17681134 | Immuno-purified GST5057, G22-7 | WB (1:2500) |
| Antibody | Mouse IgG HRP (Goat polyclonal) | R&D Systems | Cat# AF007 | WB (1:2000) |
| Antibody | Rabbit IgG HRP (Goat polyclonal) | R&D Systems | Cat# HAF008 | WB (1:2000) |
| Commercial assay or kit | NucleoBond PC 100, Midi kit | Macherey-Nagel | Cat# 740573.100 | DNA purification |
| Commercial assay or kit | NucleoSpin Gel and PCR Clean-up, Mini kit | Macherey-Nagel | Cat# 740609.50 | DNA purification |
| Commercial assay or kit | mMessage mMachine SP6 Transcriptio n kit | Invitrogen | Cat# AM1340 | mRNA synthesis |
| Commercial assay or kit | RNeasy Mini Kit | Qiagen | Cat# 74104 | RNA purification |
| Commercial assay or kit | Pierce BCA protein assay kit | Thermo Fisher Scientific | Cat# 23225 | Protein measuremen t |
| Chemical compound, drug | Pfu DNA Polymerase | Thermo Fisher Scientific | Cat# EP0572 | DNA synthesis |

| | | | | |
|---|---|---|---|---|
| Chemical compound, drug | Lipofectamin 3000 Transfection Reagent | Thermo Fisher Scientific | Cat# L3000001 | Transfection |
| Chemical compound, drug | RIPA Lysis and Extraction Buffer | Thermo Fisher Scientific | Cat# 89901 | Protein purification |
| Chemical compound, drug | Halt Protease and Phosphatase Inhibitor Cocktail | Thermo Fisher Scientific | Cat# 78442 | Protein purification |
| Chemical compound, drug | cOmplete, EDTA-free Protease Inhibitor Cocktail | Roche | Cat# 11873580001 | Protein purification |
| Chemical compound, drug | Bolt Bis-Tris Plus Mini Protein Gels, 4-12% | Thermo Fisher Scientific | Cat# NW04125BOX | Protein elektrophoresis |
| Chemical compound, drug | Bolt Sample Reducing Agent (10x) | Thermo Fisher Scientific | Cat# B0009 | Protein Elektrophoresis |
| Chemical compound, drug | Bolt LDS Sample Buffer (4x) | Thermo Fisher Scientific | Cat# B0007 | Protein elektrophoresis |
| Chemical compound, drug | Bolt MES SDS Running Buffer (20x) | Thermo Fisher Scientific | Cat# B0002 | Protein elektrophoresis |
| Chemical compound, drug | PageRuler Prestained Protein Ladder, 10 to 180 kDa | Thermo Fisher Scientific | Cat# 26616 | Protein elektrophoresis |
| Chemical compound, drug | Ponceau S Solution | Sigma-Aldrich | Cat# P7170 | Western blotting |

| | | | | |
|---|---|---|---|---|
| Chemical compound, drug | Pierce ECL Western Blotting Substrate | Thermo Fisher Scientific | Cat# 32106 | Western blotting |
| Chemical compound, drug | Restore PLUS Western Blot Stripping Buffer | Thermo Fisher Scientific | Cat# 46430 | Western blotting |
| Chemical compound, drug | Human Plasma Fibronectin Purified Protein | Sigma-Aldrich | Cat# FC010 | Neural crest explant culture |
| Chemical compound, drug | Gentamycin Solution | Sigma-Aldrich | Cat# G1272 | Embryo culture |
| Software, algorithm | ImageJ | NIH | https://imagej.net/ij/ | Neural crest explant measurement |
| Software, algorithm | AxioVision 4.8 | Zeiss | https://www.zeiss.com/ | Neural crest explant imaging |
| Other | 35 mm Dish, No. 0 Coverslip, 10 mm Glass Diameter, uncoated | MatTek | Cat# P35G-0-10-C | Neural crest explant culture, time lapse imaging |
| Other | Vivaspin 2 MWCO 10,000 | Cytiva | Cat# 28932247 | Protein concentration |
| Other | iBlot Transfer Stack, PVDF, mini | Thermo Fisher Scientific | Cat# IB401002 | Western blotting |

**Materials availability statement**

Requests for resources and further information should be directed and will be fulfilled by the corresponding author, Edgar M. Pera.

**Constructs**

pCS2-*Flag-HtrA1* (identical with pCS2-*xHtrA1**; Hou et al., 2007) and pCS2-*Flag-SerpinE2* (identical with pCS2-*Flag-PN1*; Acosta et al., 2015) were previously described. Briefly, N-terminally truncated open reading frames of *Xenopus laevis* HtrA1.S (amino acids 17-459) and SerpinE2.L (amino acids 20-395) were each introduced into a modified pCS2 expression vector in frame and downstream of a secretion cassette with the sequence

MQCPPILLVWTLWIMAVDCSRPKVFLPIQPEQEPLQSKT(DYKDDDDK)LE

that contains a cleavable signal peptide (underlined) and N-terminus until Thr39 of *Xenopus laevis* Chordin, followed by a Flag-tag (in brackets) and two amino acids (LE) representing an *Xho*I cloning site (pCS2-Chd*SP-Flag*, constructed by Stefano Piccolo in the laboratory of Eddy De Robertis, UCLA). Using pCS2-*Flag-HtrA1* and pCS2-*Flag-SerpinE2* as templates, the N-bterminally truncated open reading frames of HtrA1 and SerpinE2 were PCR-amplified, using a forward primer that inserts a start codon before the N-terminal Flag-tag, and subcloned into the expression vector pCS2 to generate pCS2-*Flag-HtrA1ΔSP* and pCS2-*Flag-SerpinE2ΔSP*. The PCR reactions were performed with high fidelity *Pfu* DNA polymerase (Thermo Fisher, EP0572), and correct sequences were validated by sequencing in sense and antisense directions (Eurofins, Germany). Protein bands were quantified using Image Lab (BioRad).

**Antisense morpholino oligonucleotides** (purchased from Gene Tools LLC.)

| Name | Forward | Reference |
|---|---|---|
| Standard control-MO | 5'- CCT CTT ACC TCA GTT ACA ATT TAT A | Gene Tools LLC. |
| *SerpinE2.L* (*PN1.a*)-MO1 | 5'- GAA GTC AAG TAA GAA TAC TCC CGG C | Acosta et al., 2015 |
| *SerpinE2.L* (*PN1.a*)-MO2 | 5'- ACT AGT CGC CTC ATG ATC GTA CAA C | Acosta et al., 2015 |
| *SerpinE2.S* (*PN1.b*)-MO | 5'- CAT GAT CGT AGA ACT GGA TAG AAG T | Acosta et al., 2015 |
| *SerpinE2-5MM*-MO | 5'-ACT ACT CAC CTA ATG ATA GTA AAA C | Acosta et al., 2015 |
| *HtrA1*-MO | 5'- ACA CCG CCA GCC ACA ACA TGG TCA T | Hou et al., 2007 |

***Xenopus* embryo microinjection**

To prepare mRNA, pCS2 constructs containing *HtrA1*, *HtrA1-S307A*, and *Flag-HtrA1* (Hou et al., 2007), *HtrA1-myc*, *HtrA1ΔPDZ-myc, Flag-SerpinE2* and *Flag-SerpinE2pm* (Acosta et al., 2015), *Flag-HtrA1ΔSP* and *Flag-SerpinE2ΔSP* (this study), *nlacZ* (a kind gift from Dr. Tomas Pieler, University Göttingen, Germany), *eGFP* (a kind gift from Dr. Eric Bellefroid, Université Libre de Bruxelles, Belgium) and *Flag-Sdc4* (Muñoz et al., 2006) were linearized with NotI and transcribed with Sp6 RNA polymerase (mMessage mMachine™, Invitrogen). Embryos were injected in 1xMBS (Modified Barth's saline) and cultured in 0,1X MBS. Pigmented embryos were injected into all four animal blastomeres at the 8-cell stage. Albino embryos were injected

into a single animal blastomere with *nlacZ* mRNA as a lineage tracer and stained with Magenta-Red (Sigma B8931, red nuclei) after fixation. Dorsally and ventrally injected embryos were identified based on the lineage tracer pattern. Embryos were prepared, fixed and processed by whole-mount *in situ* hybridization as described (Pera et al., 2015).

**Neural crest explantation, *in vitro* culture and time-lapse imaging**

For NC extirpation, the vitelline membrane was removed with fine forceps from pigmented embryos at stage 17 and the neural folds were extracted in 1xMMR (Marc's Minimal Ringers) buffer, using eye lashes mounted with nail polisher to pipet tips. Operated embryos were cultured in 0,3xMMR and 50 ug/ml Gentamycin (Sigma G1272) until stage 41.

CNC explants and single fluorescently labelled cells from *eGFP* mRNA-injected embryos were cultured in Danilchik's for Amy media (53 mM NaCl, 5 mM $Na_2CO_3$, 4.5 K gluconate, 32 mM Na gluconate, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1% bovine serum albumine, adjusted with 1 M bicine to pH 8.3) in fibronectin-coated plastic dishes as described (Gouignard et al., 2016).

For video time-lapse imaging, CNC explants were cultured in 23% Leibovitz's L15 medium in fibronectin-coated glass-bottom culture dishes (MatTek Corp. P35G-0-10-C). The video was taken after 7 hours in culture. Filming was done with a Zeiss inverted microscope, and AxioVision 4.8.2 SP3 software was used for imaging.

**Cell culture and transfection**

HEK293T cells were cultured in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptavidin. The cells were seeded at a density of 20-30% in Opti-MEM (Thermo Fisher Scientific) with penicillin/streptavidin in 6-well plates, and transient transfection with plasmid DNAs was

performed using Lipofectamin 3000 (Thermo Fisher Scientific), when cells reached between 60-70% confluency. Following a medium exchange 6 hours after transfection, the supernatant was harvested, and the cells were lysed 70 hours after transfection.

**Sample preparations and Western blotting**

Microinjected embryos and transfected HEK293T cells were lysed using RIPA-buffer (Thermo Fisher Scientific, Cat# 89901) supplemented with protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, Cat# 78442). The cell lysate was sonicated, using the Sonicator Bioruptor Plus (Diagenode) with 40 cycles for 15 seconds on and 15 seconds off. The supernatant was cleared from cells by centrifugations first at 2500 rpm and then at 8000 rpm for each 5 minutes at 4°C. After addition of a protease inhibitor cocktail (Roche, Cat#11873580001), proteins in the supernatant were concentrated, using Vivaspin2 10kDa molecular weight cut-off columns (Cytiva, Cat# 28932247). Proteins were measured in the lysates and supernatants, using the Pierce BCA Protein assay kit (Thermo Fisher Scientific, Cat# 23225). Gel electrophoresis was performed with 20 μg protein per lane, using Bolt Bis-Tris Plus Mini Protein gels (4-12%, Thermo Fisher Scientific, Cat# NW04125BOX). Western blots were performed, using the following primary antibodies: anti-flag HRP-conjugated (1:1000; Sigma-Aldrich, A8592), anti-αTubulin (1:1000; Sigma-Aldrich, T5168), anti-βActin (1:10,000; Sigma-Aldrich, A5441), immunopurified anti-HtrA1 (5 μg/ml; Hou et al., 2007).

**Statistical analysis**

Results are shown as mean ± standard deviation (SD). Unpaired t-test was employed for comparing means of two treatment groups. For multiple comparisons, one-way ANOVA followed

by Tukey's post hoc test was used. Statistical significance was defined as **p<0,01, ***p<0,001 and ****p<0,0001.

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## Ethics

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All *Xenopus laevis* experiments reported in this study were approved by the Lund/Malmö regional ethical committee (Dnr. 5.8.18-15884/2019).

## References


Acosta H, Iliev D, Grahn TH, et al. The serpin PN1 is a feedback regulator of FGF signaling in germ layer and primary axis formation. *Development*. 2015;142(6):1146-1158. doi:10.1242/dev.113886.

Agrawal M, Brauer PR. Urokinase-type plasminogen activator regulates cranial neural crest cell migration in vitro. *Dev Dyn*. 1996;207(3):281-290. doi:10.1002/(SICI)1097-0177(199611)207:3<281::AID-AJA5>3.0.CO;2-D.

Ajayi F, Kongoasa N, Gaffey T, Asmann YW, Watson WJ, Baldi A, Lala P, Shridhar V, Brost B, Chien J. Elevated expression of serine protease HtrA1 in preeclampsia and its role in trophoblast cell migration and invasion. *Am J Obstet Gynecol.* 2008;199(5):557.e1-10. doi: 10.1016/j.ajog.2008.04.046.

Alfandari D, Cousin H, Gaultier A, Hoffstrom BG, DeSimone DW. Integrin alpha5beta1 supports the migration of Xenopus cranial neural crest on fibronectin. *Dev Biol*. 2003;260(2):449-464. doi:10.1016/s0012-1606(03)00277-x.

An E, Sen S, Park SK, Gordish-Dressman H, Hathout Y. Identification of novel substrates for the serine protease HTRA1 in the human RPE secretome. *Invest Ophthalmol Vis Sci*. 2010;51(7):3379-3386. doi:10.1167/iovs.09-4853.

Arocas V, Bouton MC. Protease nexin-1: a serpin involved in pathophysiology. M. Geiger, F. Wahlmüller, M. Furtmüller (Eds.), The Serpin Family, Springer International Publishing, Cham. (2015), pp. 179-196, 10.1007/978-3-319-22711-5_11.

Baldi A, De Luca A, Morini M, Battista T, Felsani A, Baldi F, Catricalà C, Amantea A, Noonan DM, Albini A, Natali PG, Lombardi D, Paggi MG. The HtrA1 serine protease is down-regulated during human melanoma progression and represses growth of metastatic melanoma cells. *Oncogene.* 2002;21(43):6684-8. doi: 10.1038/sj.onc.1205911.

Brändli AW, Kirschner MW. Molecular cloning of tyrosine kinases in the early Xenopus embryo: identification of Eck-related genes expressed in cranial neural crest cells of the second (hyoid) arch. *Dev Dyn.* 1995;203(2):119-40. doi: 10.1002/aja.1002030202.

Brauer PR, Yee JA. Cranial neural crest cells synthesize and secrete a latent form of transforming growth factor beta that can be activated by neural crest cell proteolysis. *Dev Biol*. 1993;155(1):281-285. doi:10.1006/dbio.1993.1026.

Campioni M, Severino A, Manente L, Tuduce IL, Toldo S, Caraglia M, Crispi S, Ehrmann M, He X, Maguire J, De Falco M, De Luca A, Shridhar V, Baldi A. The serine protease HtrA1 specifically interacts and degrades the tuberous sclerosis complex 2 protein. *Mol Cancer Res.* 2010;8(9):1248-60. doi: 10.1158/1541-7786.MCR-09-0473.

Chern SR, Li SH, Chiu CL, Chang HH, Chen CP, Tsuen Chen EI. Spatiotemporal expression of SERPINE2 in the human placenta and its role in extravillous trophoblast migration and invasion. *Reprod Biol Endocrinol*. 2011;9:106. doi:10.1186/1477-7827-9-106.

Chien J, He X, Shridhar V. Identification of tubulins as substrates of serine protease HtrA1 by mixture-based oriented peptide library screening. *J Cell Biochem*. 2009a;107(2):253-263. doi:10.1002/jcb.22121.

Chien J, Ota T, Aletti G, Shridhar R, Boccellino M, Quagliuolo L, Baldi A, Shridhar V. Serine protease HtrA1 associates with microtubules and inhibits cell migration. *Mol Cell Biol.* 2009b;29(15):4177-87. doi: 10.1128/MCB.00035-09.

Clawson GA, Bui V, Xin P, Wang N, Pan W. Intracellular localization of the tumor suppressor HtrA1/Prss11 and its association with HPV16 E6 and E7 proteins. *J Cell Biochem*. 2008;105(1):81-88. doi:10.1002/jcb.21804.

Davidson LA, Keller R, DeSimone DW. Assembly and remodeling of the fibrillar fibronectin extracellular matrix during gastrulation and neurulation in Xenopus laevis. *Dev Dyn.* 2004;231(4):888-95. doi: 10.1002/dvdy.20217.

Du Shane GP. An experimental study of the origin of pigment cells in amphibia. *J Exp Zool*. 1935;72(1):1-31.

Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol*. 2009;10(7):445-457. doi:10.1038/nrm2720.

Gouignard N, Maccarana M, Strate I, von Stedingk K, Malmström A, Pera EM. Musculocontractural Ehlers-Danlos syndrome and neurocristopathies: dermatan sulfate is required for Xenopus neural crest cells to migrate and adhere to fibronectin. *Dis Model Mech*. 2016;9(6):607-20. doi: 10.1242/dmm.024661.

Gouignard N, Andrieu C, Theveneau E. Neural crest delamination and migration: Looking forward to the next 150 years. *Genesis*. 2018;56(6-7):e23107. doi:10.1002/dvg.23107.

Grau S, Richards PJ, Kerr B, Hughes C, Caterson B, Williams AS, Junker U, Jones SA, Clausen T, Ehrmann M. The role of human HtrA1 in arthritic disease. *J Biol Chem.* 2006;281(10):6124-9. doi: 10.1074/jbc.M500361200.

Hadfield KD, Rock CF, Inkson CA, Dallas SL, Sudre L, Wallis GA, Boot-Handford RP, Canfield AE. HtrA1 inhibits mineral deposition by osteoblasts: requirement for the protease and PDZ domains. *J Biol Chem.* 2008;283(9):5928-38. doi: 10.1074/jbc.M709299200.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013.

Holstein TW. The role of cnidarian developmental biology in unraveling axis formation and Wnt signaling. *Dev Biol*. 2022;487:74-98. doi: 10.1016/j.ydbio.2022.04.005.

Hopwood ND, Pluck A, Gurdon JB. A Xenopus mRNA related to Drosophila twist is expressed in response to induction in the mesoderm and the neural crest. *Cell*. 1989;59(5):893-903.

Hou S, Maccarana M, Min TH, Strate I, Pera EM. The secreted serine protease xHtrA1 stimulates long-range FGF signaling in the early Xenopus embryo. *Dev Cell*. 2007;13(2):226-241. doi:10.1016/j.devcel.2007.07.001.

Howie RN, Durham EL, Black L, Bennfors G, Parsons TE, Elsalanty ME, Yu JC, Weinberg SM, Cray JJ Jr. Effects of In Utero Thyroxine Exposure on Murine Cranial Suture Growth. *PLoS One.* 2016;11(12):e0167805. doi: 10.1371/journal.pone.0167805.

Jeyarajah MJ, Jaju Bhattad G, Kops BF, Renaud SJ. Syndecan-4 regulates extravillous trophoblast migration by coordinating protein kinase C activation. *Sci Rep*. 2019;9(1):10175. doi:10.1038/s41598-019-46599-6.

Keller-Pinter A, Gyulai-Nagy S, Becsky D, Dux L, Rovo L. Syndecan-4 in Tumor Cell Motility. *Cancers (Basel)*. 2021;13(13):3322. doi:10.3390/cancers13133322.

Kim GY, Kim HY, Kim HT, Moon JM, Kim CH, Kang S, Rhim H. HtrA1 is a novel antagonist controlling fibroblast growth factor (FGF) signaling via cleavage of FGF8. *Mol Cell Biol.* 2012;32(21):4482-92. doi: 10.1128/MCB.00872-12.

Kubota Y, Ito K. Chemotactic migration of mesencephalic neural crest cells in the mouse. *Dev Dyn.* 2000;217(2):170-9. doi: 10.1002/(SICI)1097-0177(200002)217:2<170::AID-DVDY4>3.0.CO;2-9.

Le Douarin N, Kalcheim C. The neural crest. 2nd edition. J.B.L. Bard, P.W. Barlow, D.L. Kirk (Eds.) Developmental and cell biology series. Cambridge University Press, Cambridge (1999).

Lea R, Papalopulu N, Amaya E, Dorey K. Temporal and spatial expression of FGF ligands and receptors during Xenopus development. *Dev Dyn.* 2009;238(6):1467-79. doi: 10.1002/dvdy.21913.

Leblanc R, Sahay D, Houssin A, Machuca-Gayet I, Peyruchaud O. Autotaxin-β interaction with the cell surface via syndecan-4 impacts on cancer cell proliferation and metastasis. *Oncotarget*. 2018;9(69):33170-33185. doi:10.18632/oncotarget.26039.

Lee HX, Ambrosio AL, Reversade B, De Robertis EM. Embryonic dorsal-ventral signaling: secreted frizzled-related proteins as inhibitors of tolloid proteinases. *Cell*. 2006;124(1):147-59. doi: 10.1016/j.cell.2005.12.018.

Li W, Huntington JA. Crystal structures of protease nexin-1 in complex with heparin and thrombin suggest a 2-step recognition mechanism. *Blood*. 2012;120(2):459-467. doi:10.1182/blood-2012-03-415869.

Manon-Jensen T, Multhaupt HA, Couchman JR. Mapping of matrix metalloproteinase cleavage sites on syndecan-1 and syndecan-4 ectodomains. *FEBS J*. 2013;280(10):2320-2331. doi:10.1111/febs.12174.

Matsuo I, Kimura-Yoshida C. Extracellular modulation of Fibroblast Growth Factor signaling through heparan sulfate proteoglycans in mammalian development. *Curr Opin Genet Dev*. 2013;23(4):399-407. doi: 10.1016/j.gde.2013.02.004.

Matthews HK, Marchant L, Carmona-Fontaine C, Kuriyama S, Larraín J, Holt MR, Parsons M, Mayor R. Directional migration of neural crest cells in vivo is regulated by Syndecan-4/Rac1 and

non-canonical Wnt signaling/RhoA. *Development.* 2008;135(10):1771-80. doi: 10.1242/dev.017350.

Medina-Cuadra L, Monsoro-Burq AH. Xenopus, an emerging model for studying pathologies of the neural crest. *Curr Top Dev Biol*. 2021;145:313-348. doi: 10.1016/bs.ctdb.2021.03.002.

Menoud PA, Debrot S, Schowing J. Mouse neural crest cells secrete both urokinase-type and tissue-type plasminogen activators in vitro. *Development*. 1989;106(4):685-690. doi:10.1242/dev.106.4.685.

Minoux M, Rijli FM. Molecular mechanisms of cranial neural crest cell migration and patterning in craniofacial development. *Development*. 2010;137(16):2605-2621. doi:10.1242/dev.040048.

Monard D. SERPINE2/Protease Nexin-1 in vivo multiple functions: Does the puzzle make sense?. *Semin Cell Dev Biol*. 2017;62:160-169. doi:10.1016/j.semcdb.2016.08.012.

Nieto MA, Huang RY, Jackson RA, Thiery JP. EMT: 2016. *Cell*. 2016;166(1):21-45. doi:10.1016/j.cell.2016.06.028.

O'Connell MP, Fiori JL, Kershner EK, Frank BP, Indig FE, Taub DD, Hoek KS, Weeraratna AT. Heparan sulfate proteoglycan modulation of Wnt5A signal transduction in metastatic melanoma cells. *J Biol Chem.* 2009;284(42):28704-12. doi: 10.1074/jbc.M109.028498.

Olson ST, Gettins PG. Regulation of proteases by protein inhibitors of the serpin superfamily. *Prog Mol Biol Transl Sci*. 2011;99:185-240. doi:10.1016/B978-0-12-385504-6.00005-1.

Onuma Y, Asashima M, Whitman M. A Serpin family gene, protease nexin-1 has an activity distinct from protease inhibition in early Xenopus embryos. *Mech Dev*. 2006;123(6):463-471. doi:10.1016/j.mod.2006.04.005.

Owji H, Nezafat N, Negahdaripour M, Hajiebrahimi A, Ghasemi Y. A comprehensive review of signal peptides: Structure, roles, and applications. *Eur J Cell Biol.* 2018;97(6):422-441. doi: 10.1016/j.ejcb.2018.06.003.

Pei X, Ma K, Xu J, Wang N, Liu N. Inhibition of cell proliferation and migration after HTRA1 knockdown in retinal pigment epithelial cells. *Graefes Arch Clin Exp Ophthalmol*. 2015;253(4):565-572. doi:10.1007/s00417-014-2901-2.

Pera EM, Hou S, Strate I, Wessely O, De Robertis EM. Exploration of the extracellular space by a large-scale secretion screen in the early Xenopus embryo. *Int J Dev Biol*. 2005;49(7):781-796. doi:10.1387/ijdb.052003ep.

Pera EM, Acosta H, Gouignard N, Climent M. Whole-Mount In Situ Hybridization and Immunohistochemistry in *Xenopus* Embryos. In: G. Hauptmann (Ed.) In Situ Hybridization Methods. Neuromethods, vol 99. Humana Press, New York, NY. (2015). https://doi.org/10.1007/978-1-4939-2303-8_8.

Piacentino ML, Li Y, Bronner ME. Epithelial-to-mesenchymal transition and different migration strategies as viewed from the neural crest. *Curr Opin Cell Biol*. 2020;66:43-50. doi:10.1016/j.ceb.2020.05.001.

Piccolo S, Agius E, Lu B, Goodman S, Dale L, De Robertis EM. Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell*. 1997;91(3):407-16. doi: 10.1016/s0092-8674(00)80424-9.

Rooryck C, Diaz-Font A, Osborn DP, Chabchoub E, Hernandez-Hernandez V, Shamseldin H, Kenny J, Waters A, Jenkins D, Kaissi AA, Leal GF, Dallapiccola B, Carnevale F, Bitner-Glindzicz M, Lees M, Hennekam R, Stanier P, Burns AJ, Peeters H, Alkuraya FS, Beales PL. Mutations in lectin complement pathway genes COLEC11 and MASP1 cause 3MC syndrome. *Nat Genet.* 2011;43(3):197-203. doi: 10.1038/ng.757.

Sadaghiani B, Thiébaud CH. Neural crest development in the Xenopus laevis embryo, studied by interspecific transplantation and scanning electron microscopy. *Dev Biol*. 1987;124(1):91-110. doi:10.1016/0012-1606(87)90463-5.

Saksela O, Moscatelli D, Sommer A, Rifkin DB. Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. *J Cell Biol.* 1988;107(2):743-51. doi: 10.1083/jcb.107.2.743.

Sato A, Scholl AM, Kuhn EN, Stadt HA, Decker JR, Pegram K, Hutson MR, Kirby ML. FGF8 signaling is chemotactic for cardiac neural crest cells. *Dev Biol.* 2011;354(1):18-30. doi: 10.1016/j.ydbio.2011.03.010.

Schock EN, York JR, LaBonne C. The developmental and evolutionary origins of cellular pluripotency in the vertebrate neural crest. *Semin Cell Dev Biol*. 2023;138:36-44. doi: 10.1016/j.semcdb.2022.04.008.

Seetharaman S, Etienne-Manneville S. Cytoskeletal Crosstalk in Cell Migration. *Trends Cell Biol*. 2020;30(9):720-735. doi:10.1016/j.tcb.2020.06.004

Session AM, Uno Y, Kwon T, Chapman JA, Toyoda A, Takahashi S, Fukui A, Hikosaka A, Suzuki A, Kondo M, van Heeringen SJ, Quigley I, Heinz S, Ogino H, Ochi H, Hellsten U, Lyons JB, Simakov O, Putnam N, Stites J, Kuroki Y, Tanaka T, Michiue T, Watanabe M, Bogdanovic O, Lister R, Georgiou G, Paranjpe SS, van Kruijsbergen I, Shu S, Carlson J, Kinoshita T, Ohta Y, Mawaribuchi S, Jenkins J, Grimwood J, Schmutz J, Mitros T, Mozaffari SV, Suzuki Y, Haramoto Y, Yamamoto TS, Takagi C, Heald R, Miller K, Haudenschild C, Kitzman J, Nakayama T, Izutsu Y, Robert J, Fortriede J, Burns K, Lotay V, Karimi K, Yasuoka Y, Dichmann DS, Flajnik MF, Houston DW, Shendure J, DuPasquier L, Vize PD, Zorn AM, Ito M, Marcotte EM, Wallingford JB, Ito Y, Asashima M, Ueno N, Matsuda Y, Veenstra GJ, Fujiyama A, Harland RM, Taira M, Rokhsar DS. Genome evolution in the allotetraploid frog Xenopus laevis. *Nature.* 2016;538(7625):336-343. doi: 10.1038/nature19840.

Shellard A, Mayor R. Rules of collective migration: from the wildebeest to the neural crest. *Philos Trans R Soc Lond B Biol Sci*. 2020;375(1807):20190387. doi:10.1098/rstb.2019.0387.

Sommer A, Rifkin DB. Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. *J Cell Physiol.* 1989;138(1):215-20. doi: 10.1002/jcp.1041380129.

Stone LS. Experiments on the development of the cranial ganglia and the lateral line sense organs in Amblystoma. *J Exp Zool*. 1922;35:421-495.

Stone LS. Further experiments on the extirpation and transplantation of mesectoderm in Amblystoma punctatum. *J Exp Zool*. 1926;44:95-131.

Suzuki T, Kusakabe M, Nakayama K, Nishida E. The protein kinase MLTK regulates chondrogenesis by inducing the transcription factor Sox6. *Development.* 2012;139(16):2988-98. doi: 10.1242/dev.078675.

Szabó A, Mayor R. Mechanisms of Neural Crest Migration. *Annu Rev Genet*. 2018;52:43-63. doi: 10.1146/annurev-genet-120417-031559.

Tang T, Zhu Q, Li X, Zhu G, Deng S, Wang Y, Ni L, Chen X, Zhang Y, Xia T, Zen K, Pan Y, Jin L. Protease Nexin I is a feedback regulator of EGF/PKC/MAPK/EGR1 signaling in breast cancer cells metastasis and stemness. *Cell Death Dis.* 2019;10(9):649. doi: 10.1038/s41419-019-1882-9. Erratum in: *Cell Death Dis.* 2020;11(1):13.

Tucker RP. The role of glycosaminoglycans in anuran pigment cell migration. *J Embryol Exp Morphol*. 1986;92:145-164

Tucker AS, Slack JM. Independent induction and formation of the dorsal and ventral fins in Xenopus laevis. *Dev Dyn*. 2004;230(3):461-467. doi:10.1002/dvdy.20071

Valinsky JE, Le Douarin NM. Production of plasminogen activator by migrating cephalic neural crest cells. *EMBO J.* 1985;4(6):1403-1406. doi:10.1002/j.1460-2075.1985.tb03793.x

Wagenblast E, Soto M, Gutiérrez-Ángel S, Hartl CA, Gable AL, Maceli AR, Erard N, Williams AM, Kim SY, Dickopf S, Harrell JC, Smith AD, Perou CM, Wilkinson JE, Hannon GJ, Knott SR. A model of breast cancer heterogeneity reveals vascular mimicry as a driver of metastasis. *Nature.* 2015;520(7547):358-62. doi: 10.1038/nature14403.

Wang N, Eckert KA, Zomorrodi AR, Xin P, Pan W, Shearer DA, Weisz J, Maranus CD, Clawson GA. Down-regulation of HtrA1 activates the epithelial-mesenchymal transition and ATM DNA damage response pathways. *PLoS One.* 2012;7(6):e39446. doi: 10.1371/journal.pone.0039446.

Woods A, Couchman JR. Syndecan-4 and focal adhesion function. *Curr Opin Cell Biol*. 2001;13(5):578-583. doi:10.1016/s0955-0674(00)00254-4.

Wu QW. Serpine2, a potential novel target for combating melanoma metastasis. *Am J Transl Res*. 2016;8(5):1985-1997.

Ziegler B, Yiallouros I, Trageser B, Kumar S, Mercker M, Kling S, Fath M, Warnken U, Schnölzer M, Holstein TW, Hartl M, Marciniak-Czochra A, Stetefeld J, Stöcker W, Özbek S. The Wnt-specific astacin proteinase HAS-7 restricts head organizer formation in Hydra. *BMC Biol.* 2021;19(1):120. doi: 10.1186/s12915-021-01046-9.

Zurawa-Janicka D, Wenta T, Jarzab M, Skorko-Glonek J, Glaza P, Gieldon A, Ciarkowski J, Lipinska B. Structural insights into the activation mechanisms of human HtrA serine proteases. *Arch Biochem Biophys.* 2017;621:6-23. doi: 10.1016/j.abb.2017.04.004.

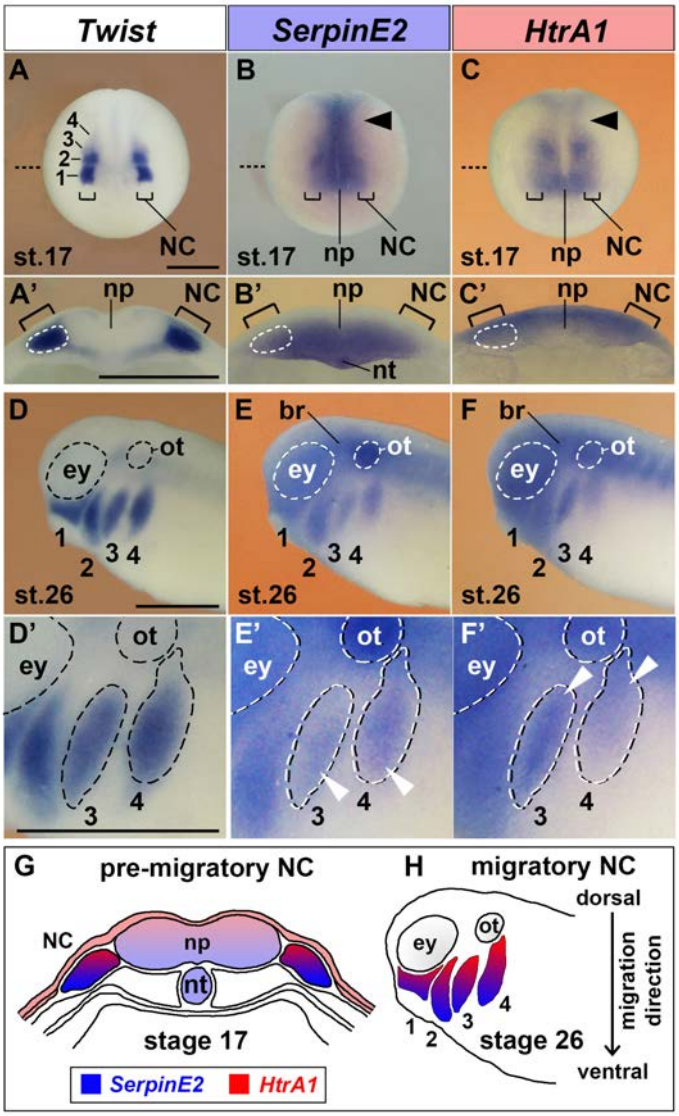


**Figure 1. SerpinE2 and HtrA1 are expressed in the neural crest**
*Xenopus* embryos were analyzed by whole-mount *in situ* hybridization.
**(A-C)** Anterior view of embryos at stage 17. The brackets point to pre-migratory NC cells on each side of the neural plate. The numbers label the *Twist*-expressing cranial NC segments: 1, mandibular; 2, hyoid; 3, anterior branchial; 4, posterior branchial. Arrowheads show *SerpinE2* and *HtrA1* transcripts in the trunk NC. The stippled lines indicate the level of sections in A'-C'.
**(A'-C')** Transversally hemisectioned embryos. *SerpinE2* and *HtrA1* signals appear in the NC (strippled circle lines). Note that *SerpinE2* is also expressed in the inner sensorial layer of the neural plate and underlying notochord, whereas *HtrA1* expression is more abundant in the outer ependymal layer of the neural plate.
**(D-F)** Lateral view of embryos at stage 26. *SerpinE2* and *HtrA1* are expressed in *Twist*$^+$ NC cell streams (1-4). Transcripts of both genes can also be seen in the brain, eye and otic placode.
**(D'-F')** Magnification of embryos. Arrowheads demarcate *SerpinE2* transcripts near the front (E') and *HtrA1* transcripts at the rear end (F') of the migrating NC cell collectives in the anterior branchial arch (3) and posterior branchial arch (4).
**(G, H)** Summary of gene expression domains. At stage 17, *SerpinE2* is transcribed in ventral and *HtrA1* in dorsal cells of the pre-migratory NC (G). At stage 26, *SerpinE2* is expressed in leader cells and *HtrA1* in follower cells of migrating NC streams (H).
ey, eye; hb, hindbrain; NC, neural crest; np, neural plate; nt, notochord; ot, otic placode. Scale bar, 0.5 mm.

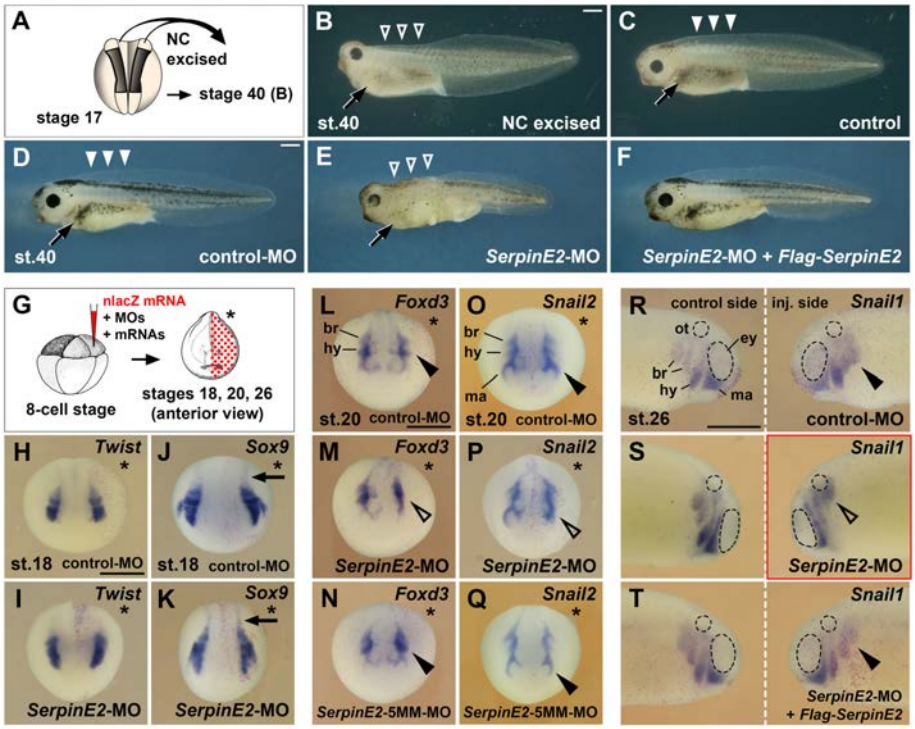


**Figure 2. Knockdown of SerpinE2 mimics the phenotype of neural excision and inhibits migration of neural crest cells**

**(A)** Scheme of extirpation. Dorsal view of *Xenopus* embryo at stage 17, from which NC tissue was removed on both sides.

**(B,C)** Tadpole embryo at stage 40 following NC excision (B) and sibling control (C). Note the small head, absence of dorsal fin tissue (open arrowheads) and the reduced number of melanocytes (arrow) resulting from NC extirpation in A.

**(D)** Unaffected tadpole after microinjection with control-MO into all animal blastomeres at the 8-cell stage. Highlighted are the pigmented melanocytes (arrow) and the intact dorsal fin (filled arrowheads).

**(E)** *SerpinE2*-MO causes a reduction of head tissue, dorsal fin structures (open arrowheads) and melanocytes (arrow).

**(F)** Co-injection of *SerpinE2*-MO and 2 ng non-targeted *Flag-SerpinE2* mRNA restores a normal phenotype.

**(G)** Scheme for microinjections in K-W. MOs and mRNAs were injected together with 100 pg *nlacZ* mRNA as lineage tracer (red nuclei) into one dorsal animal blastomere of embryos at the 8-cell stage. The injected side is marked with a star.

**(H-Q)** Anterior view of neurula embryos. Neither control-MO nor *SerpinE2*-MO affect *Twist* and *Sox9* expression in NC cells in the head and trunk (arrows) at stage 18 (H-K). *SerpinE2*-MO inhibits the EMT of *Foxd3*$^+$ and *Snail2*$^+$ NC cells (open arrowheads) at stage 20, whereas the control-MO and *SerpinE2*-5MM-MO have no effect (filled arrowheads) (L-Q).

**(R-T)** Lateral view of stage 26 embryos. A single injection of control-MO does not affect the migration of *Snail1*$^+$ NC cells (filled arrowhead). *SerpinE2*-MO leads to a migration defect on the injected side (open arrowhead). 333 pg *Flag-SerpinE2* mRNA rescues NC migration in the *SerpinE2*-morphant embryo.

br, branchial crest segment; ey, eye primordium; hy, hyoid crest segment; ma, mandibular crest segment; MO, morpholino oligonucleotide; NC, neural crest; ot, otic vesicle. Doses of injected MOs per embryo were 40 ng (D-I) and 10 ng (H-T). Indicated phenotypes were shown in B, 10/11; D, 89/90; E, 64/83; F, 122/132; H, 29/29; I, 24/26; J, 7/7; K, 10/11; L, 9/9; M, 7/7; N, 7/9; O, 7/8; P, 8/9; Q, 9/11; R, 10/10; S, 13/15; T, 9/9. Scale bar, 0.5 mm.

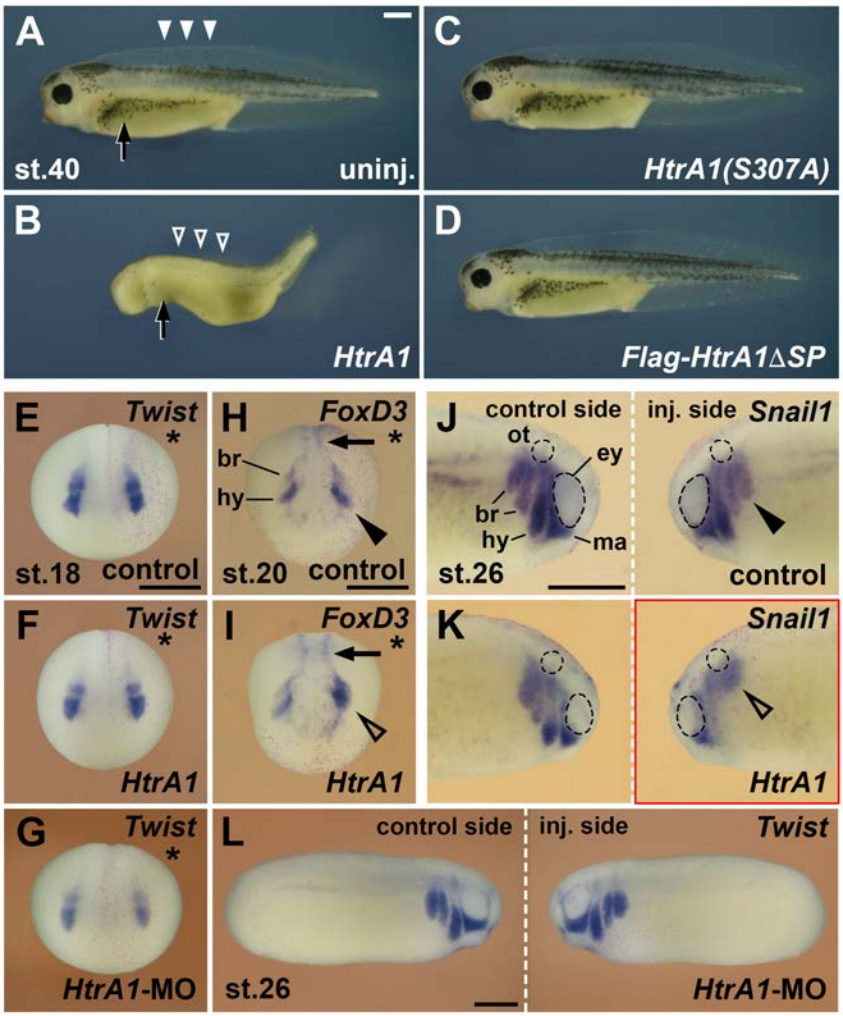


**Figure 3. HtrA1 protease inhibits the formation of neural crest-derived structures and reduces neural crest migration**

Embryos were injected into all animal blastomeres (A-H) or a single dorsal animal blastomere (I-P) at the 8-cell stage.

**(A-D)** Tadpoles at stage 40. *HtrA1* mRNA causes reduction of head tissue, dorsal fin structures (arrowheads) and melanocytes (arrow), whereas *HtrA1(S307A)* and *Flag-HtrA1ΔSP* mRNA have no effect.

**(E-G)** Anterior view of embryos at stage 18. Stars demarcate the injected sides. Neither 65 pg *HtrA1* mRNA nor 10 ng *HtrA1*-MO do affect the specification of *Twist*$^+$ cranial NC cells.

**(H,I)** Anterior view of embryos at stage 20. 65 pg *HtrA1* mRNA reduces the EMT of *Foxd3*$^+$ cranial NC cells (arrowheads) but does not affect the specification of trunk NC cells (arrows).

**(J-L)** Lateral view of embryos at stage 26. The migration of NC cells (arrowheads) is reduced by 65 pg *HtrA1* mRNA but not affected by 10 ng *HtrA1*-MO.

br, branchial crest segment; hy, hyoid crest segment; ma, mandibular crest segment. Unless otherwise noted, the mRNA doses of *HtrA1* and derived constructs per embryo were 100 pg. Indicated phenotypes were shown in B, 98/100; C, 74/83; D, 84/87; E, 31/31; F, 51/53; G, 59/60; H, 28/31; I, 23/26; J, 9/10; K, 21/21; L, 58/79 embryos; at least two independent experiments. Scale bar, 0.5 mm.

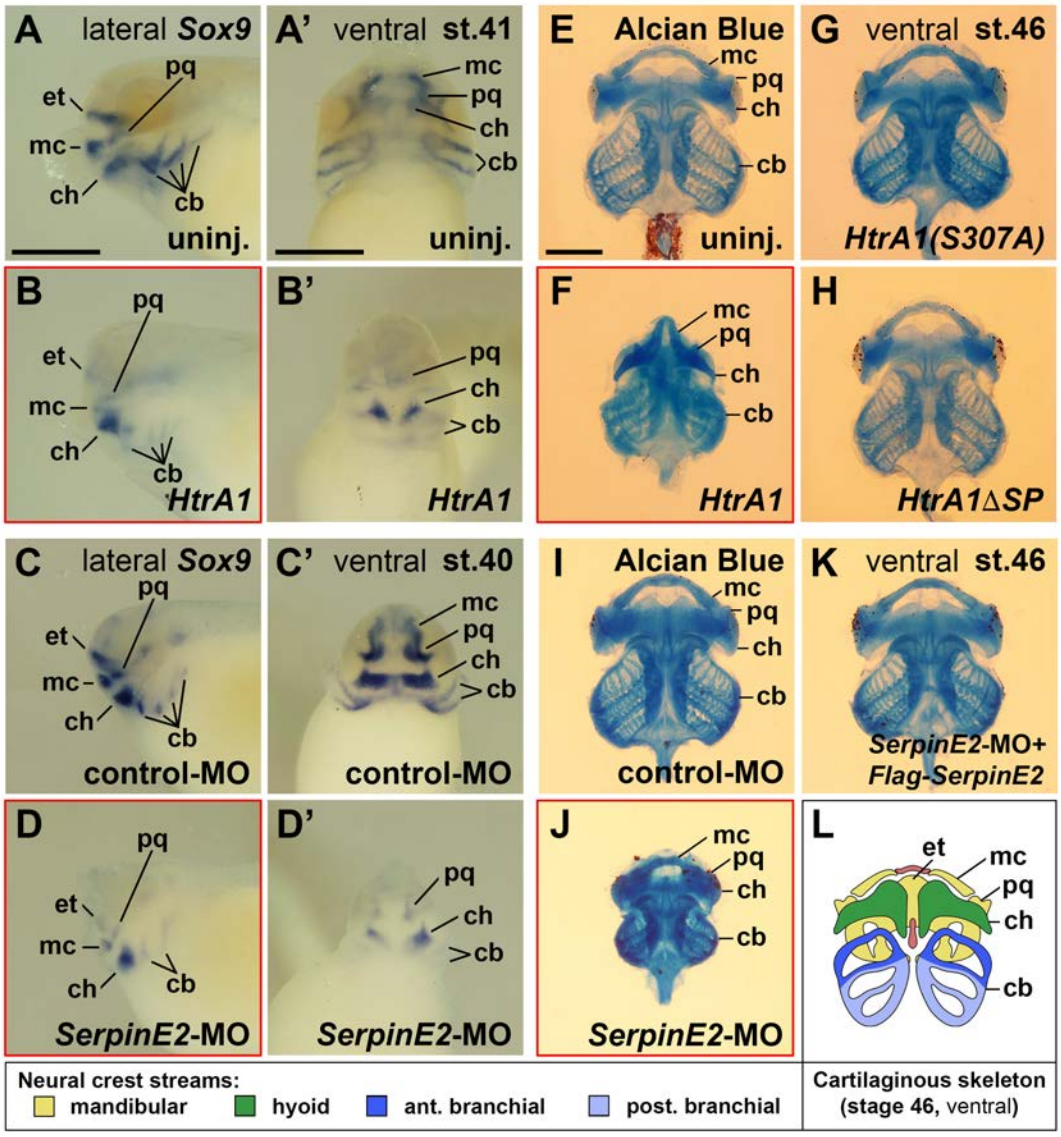


**Figure 4. HtrA1 overexpression and SerpinE2 knockdown decrease cartilaginous elements and craniofacial structures**

*Xenopus* embryos were injected into four animal blastomeres at the 8-cell stage with a total of 100 pg mRNA and 40 ng morpholino oligonucleotides (MOs).

**(A-D')** Tadpoles at stages 40/41 after whole-mount *in situ* hybridization in lateral (A-D) and ventral view (A'-D'). Note that *HtrA1* mRNA and *SerpinE2*-MO reduce *Sox9* expression, whereas control-MO has no effect on the labelled cartilaginous elements.

**(E-K)** Ventral view of cartilaginous skeleton extracted from embryos at stage 46 after Alcian Blue staining. The dorsal ethmoid-trabecular cartilage was removed for better visibility. Note that *HtrA1* mRNA, but not *HtrA1(S307A)* and *Flag-HtrA1ΔSP* mRNAs, diminishes craniofacial structures (E-H). *SerpinE2*-MO, but not control-MO nor a combination of *SerpinE2*-MO and *Flag-SerpinE2* mRNA, reduce head skeleton structures (I-K).

**(L)** Scheme of the cartilaginous skeleton at stage 46 in ventral view. Indicated is the contribution of neural crest streams to the craniofacial skeleton elements. cb, ceratobranchial; ch, ceratohyal; et, ethmoid-trabecular; mc, Meckel's cartilage; pq, palatoquadrate.

Indicated phenotypes were shown in A, 11/11; B, 17/19; C, 12/13; D, 15/17; E, 57/58; F, 17/21; G, 72/77; H, 67/78; I, 83/88; J, 73/77; K, 77/87 embryos; at least two independent experiments. Scale bar, 0.5 mm.

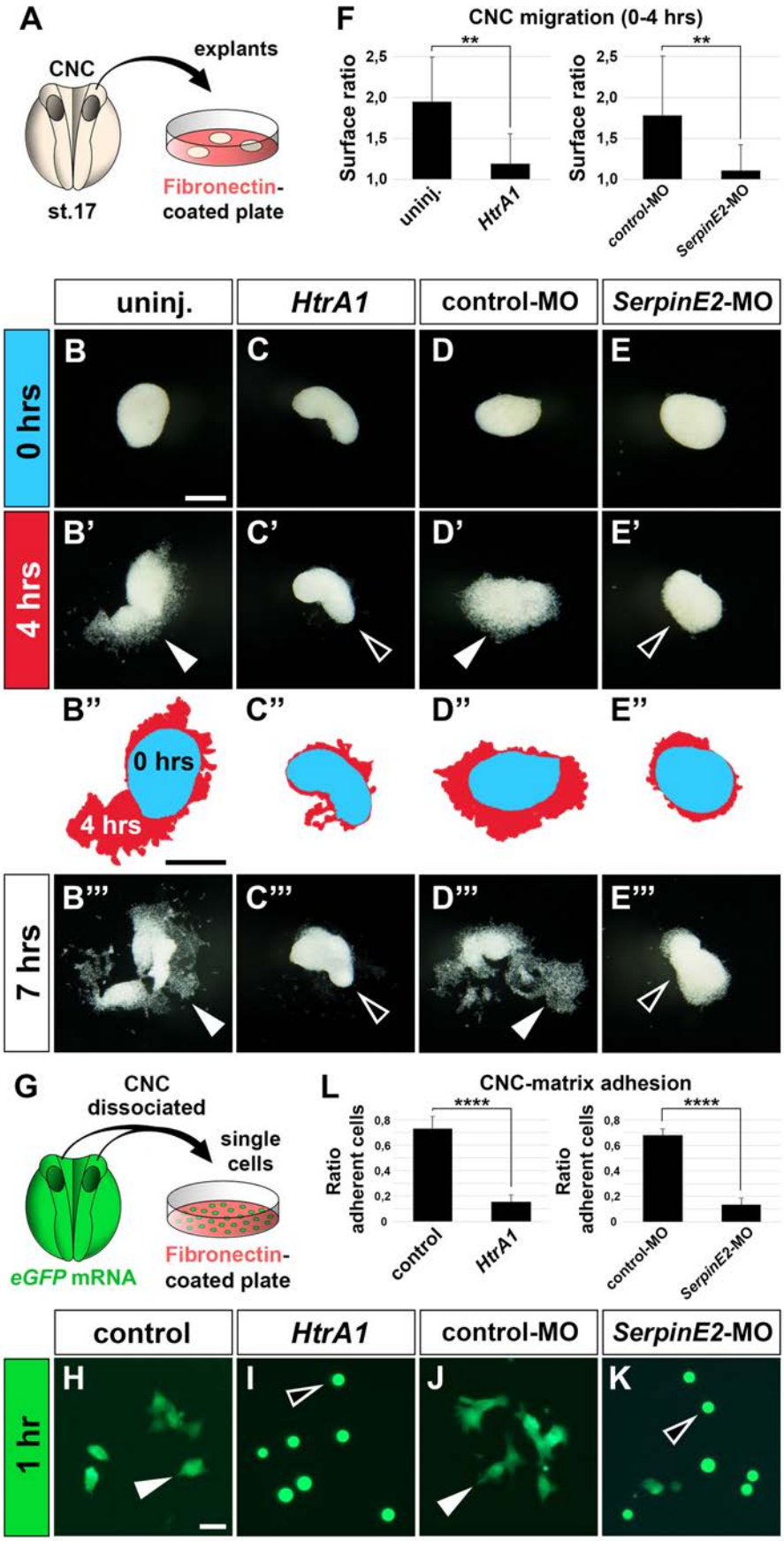


**Figure 5 – Pera et al.**

**Figure 5. HtrA1 overexpression of and SerpinE2 knockdown inhibit cranial neural crest cell migration and adhesion to fibronectin *in vitro***

**(A)** Scheme of migration experiment. The cranial neural crest was explanted from uninjected or injected embryos at stage 17 and cultured on a fibronectin-covered plastic plate.

**(B-E'''')** Time lapse of cell migration in CNC explants after culturing for 0, 4 or 7 hours. Note collective cell migration (filled arrowheads) in uninjected controls and explants injected with control-MO, whereas *HtrA1* mRNA and *SerpinE2*-MO block migration (open arrowheads). In B''-E'', the surface areas of explants at 0 hours (blue) and 4 hours (red) were determined by ImageJ and superimposed. Scale bar, 0.2 mm

**(F)** Quantification of initial CNC migration. Indicated is the surface ratio of explants 4 hours versus 0 hours after plating. 12 explants were analyzed per sample; two independent experiments.

**(G)** Scheme of adhesion experiment. Upon injection of *eGFP* mRNA, CNC eplants were dissociated in $Ca^{2+}$- and $Mg^{2+}$-free medium, and single cells were cultured on a fibronectin plate.

**(H-K)** Single eGFP-labelled CNC cells after 1 hour culture. Note adhering cells with extended cytoplasmic processes (filled arrowheads) in control sample and after co-injection with control-MO, whereas *HtrA1* mRNA and *SerpinE2*-MO prevent adhesion causing injected cells to acquire a round phenotype (open arrowheads). Scale bar, 0.02 mm.

**(L)** Quantification of CNC adhesion. Indicated is the ratio of adherent cells relative to the control. Analysis of n>1600 cells from at least 6 explants per sample; two independent experiments.

CNC, cranial neural crest; GFP, green fluorescent protein. Embryos were injected with 100 pg mRNAs and 40 ng MOs.

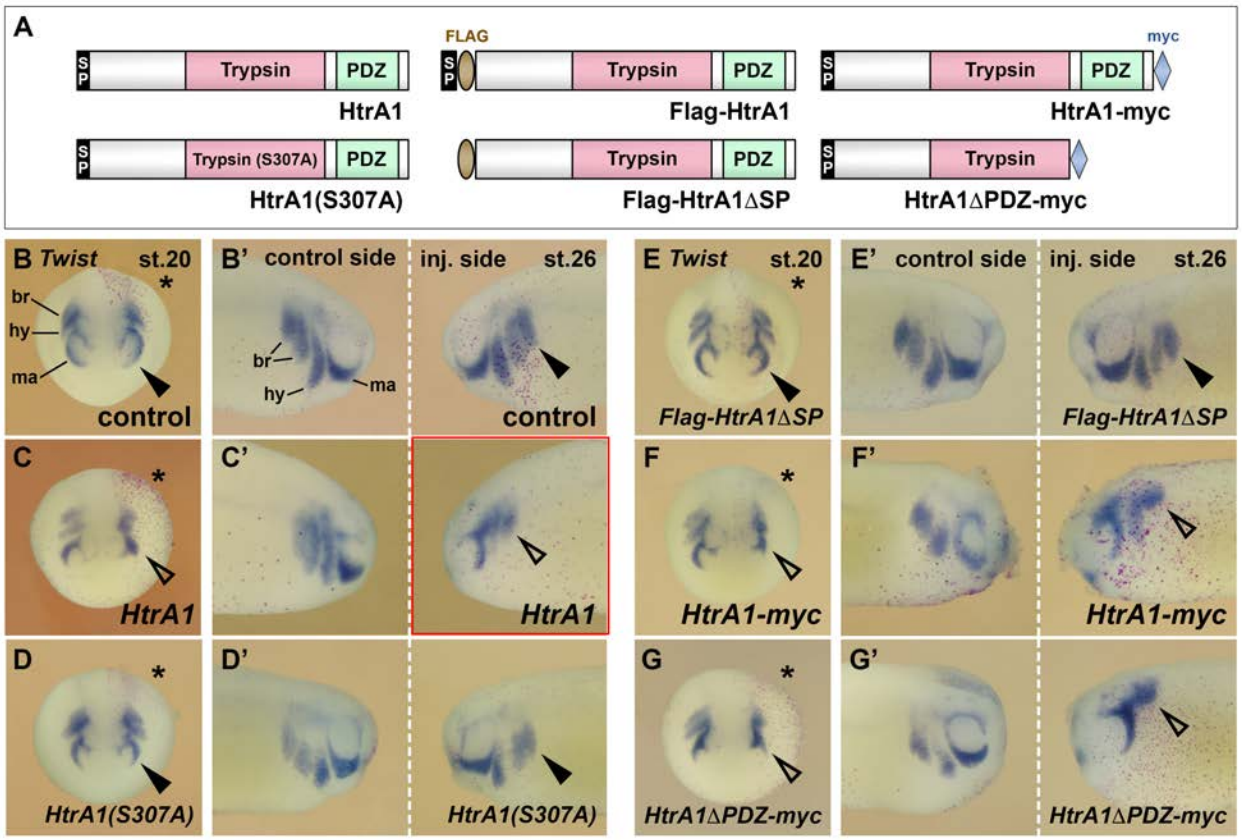


**Figure 6. HtrA1 inhibits neural crest migration as an extracellular protease**
Embryos were injected into a single dorsal animal blastomere at the 8-cell stage. A star labels the injected side. *Twist* expression demarcates the NC in embryos at stage 20 (B-G; anterior view) and stage 26 (B'-G'; lateral view).
**(A)** Overview of wild type (top) and mutant (bottom) HtrA1 protein constructs.
**(B-E')** *HtrA1* mRNA, but neither *Flag-HtrA1ΔSP* nor *HtrA1(S307A)* mRNAs, reduces EMT and migration of NC cells on the injected side (arrowheads). Note that the diffusible HtrA1 protein reduces NC cell migration to a lower extent also on the non-injected side.
**(F-G')** Both *HtrA1-myc* and *HtrA1ΔPDC-myc* mRNAs reduce NC EMT and migration.
br, branchial segment; hy, hyoid segment; ma, mandibular segment. Injected mRNA doses per embryos are 65 pg. For quantification of NC migration defects, see Figure 6-figure supplement 1A,B.

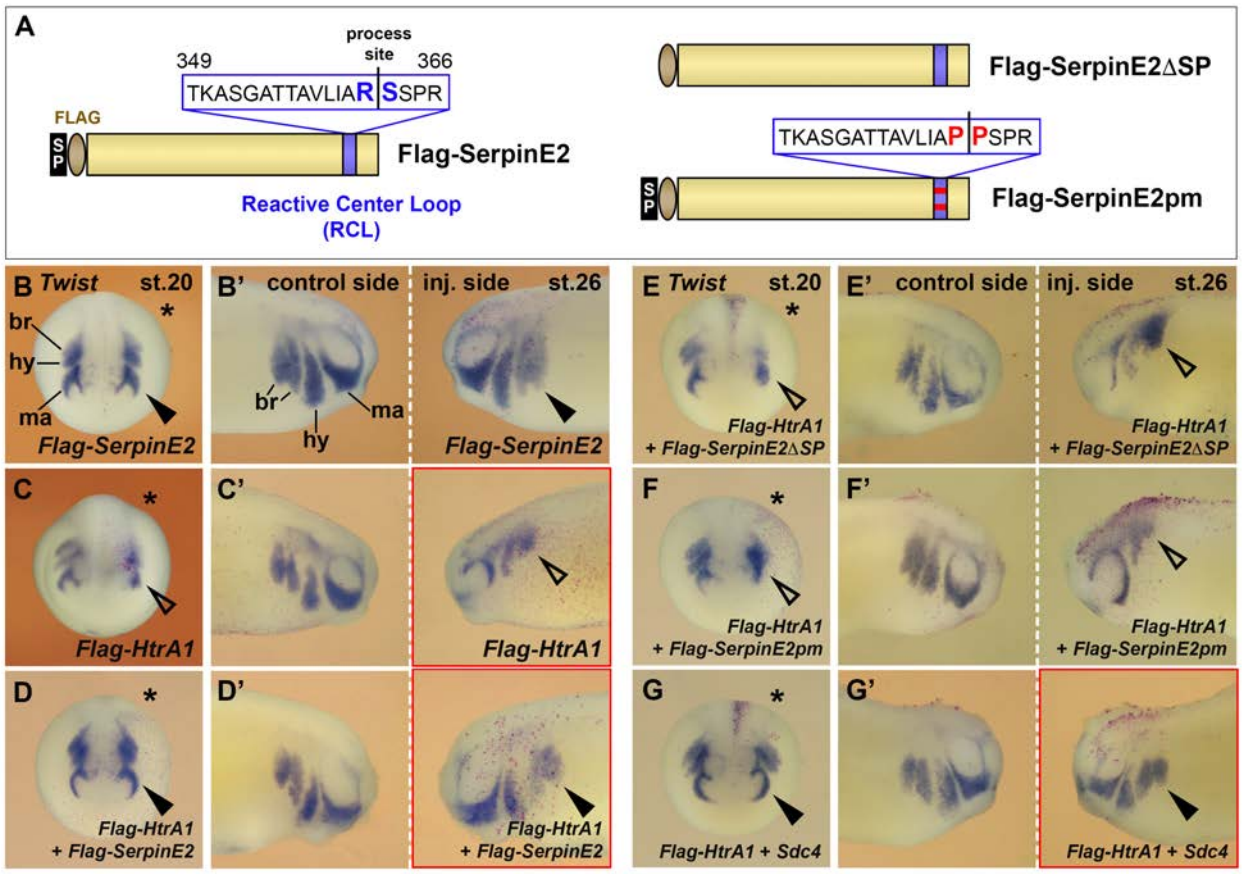


**Figure 7. SerpinE2 and HtrA1 interact with Sdc4 in neural crest cell migration**
mRNAs were injected into one dorsal animal blastomere at the 8-cell stage. Embryos are shown in anterior view (stage 20, injected side labelled with a star, B-G) and lateral view (stage 26, B'-G').
**(A)** Overview of wild type (left) and mutant (right) SerpinE2 protein constructs.
**(B,B')** 4 ng *Flag-SerpinE2* mRNA has no effect on the migration of *Twist*$^{+}$ NC cells (filled arrowheads).
**(C,C)** Flag-HtrA1 inhibits NC cell migration robustly on the injected sides (open arrowheads).
**(D-F')** *SerpinE2* mRNA, but neither *Flag-SerpinE2ΔSP* nor *SerpinE2pm* mRNA, rescues normal EMT and migration of NC cells upon co-injection with *Flag-HtrA1*.
**(G,G')** *Sdc4* mRNA restores normal NC migration in *Flag-HtrA1*-injected embryos.
If not otherwise indicated, injected mRNA doses per embryos are 65 pg (*Flag-HtrA1*), 333 pg (*Flag-SerpinE2* derived constructs) and 450 pg (*Sdc4*). For quantification of NC migration defects, see Figure 7-figure supplement 1A,B.

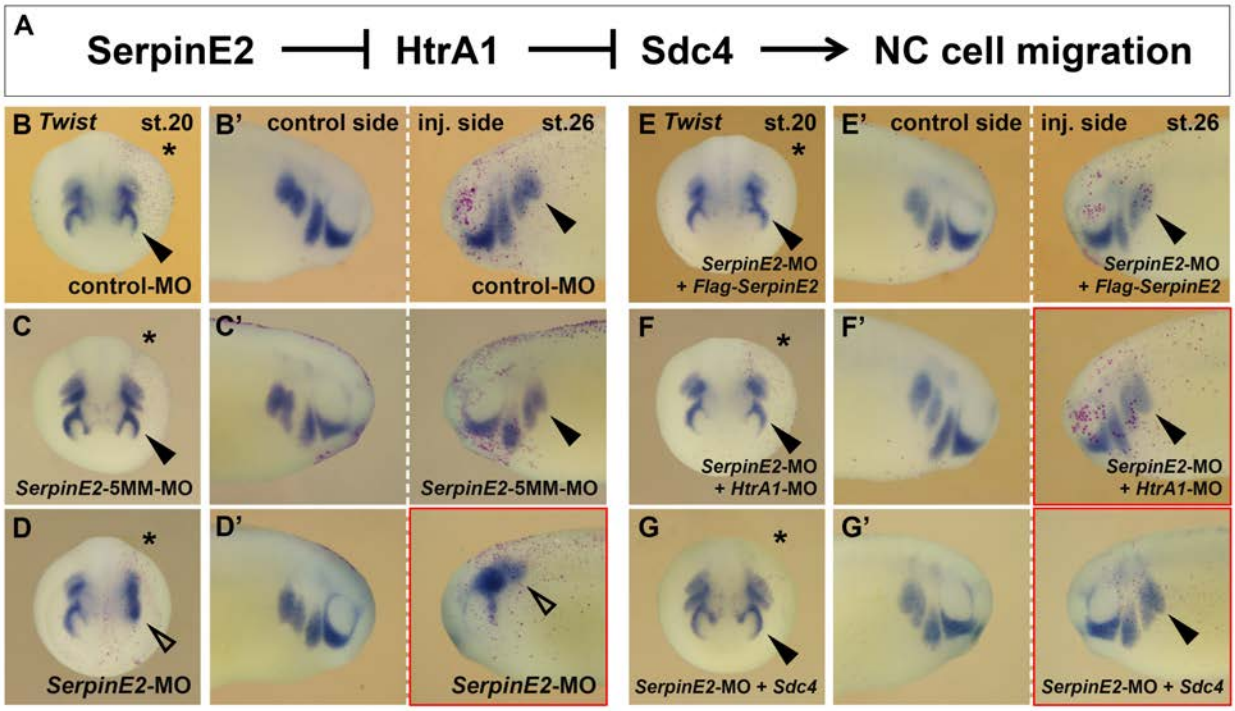


**Figure 8. SerpinE2 functions in neural crest cell migration in an HtrA1- and Sdc4-dependent manner**

mRNAs and morpholino oligonucleotides (MOs, 10 ng) were injected into one dorsal animal blastomere at the 8-cell stage. Embryos are shown in anterior view (stage 20, injected side labelled with a star, B-G) and lateral view (stage 26, B'-G').

**(A)** Proposed mechanism for the regulation of NC migration by SerpinE2, HtrA1 and Syndecan-4.

**(B-D')** *SerpinE2*-MO, blocks EMT and migration of *Twist*$^{+}$ NC cells (arrow) on the injected side, while control-MO and *SerpinE2*-5MM-MO have no effect.

**(E-G')** *Flag-SerpinE2* mRNA, *HtrA1*-MO, and *Sdc4* mRNA restore normal NC migration in *SerpinE2*-morphant embryos.

Injected mRNA doses per embryos are 333 pg (*Flag-SerpinE2*) and 450 pg (*Sdc4*). For quantification of NC migration defects, see Figure 8-figure supplement 1A,B.

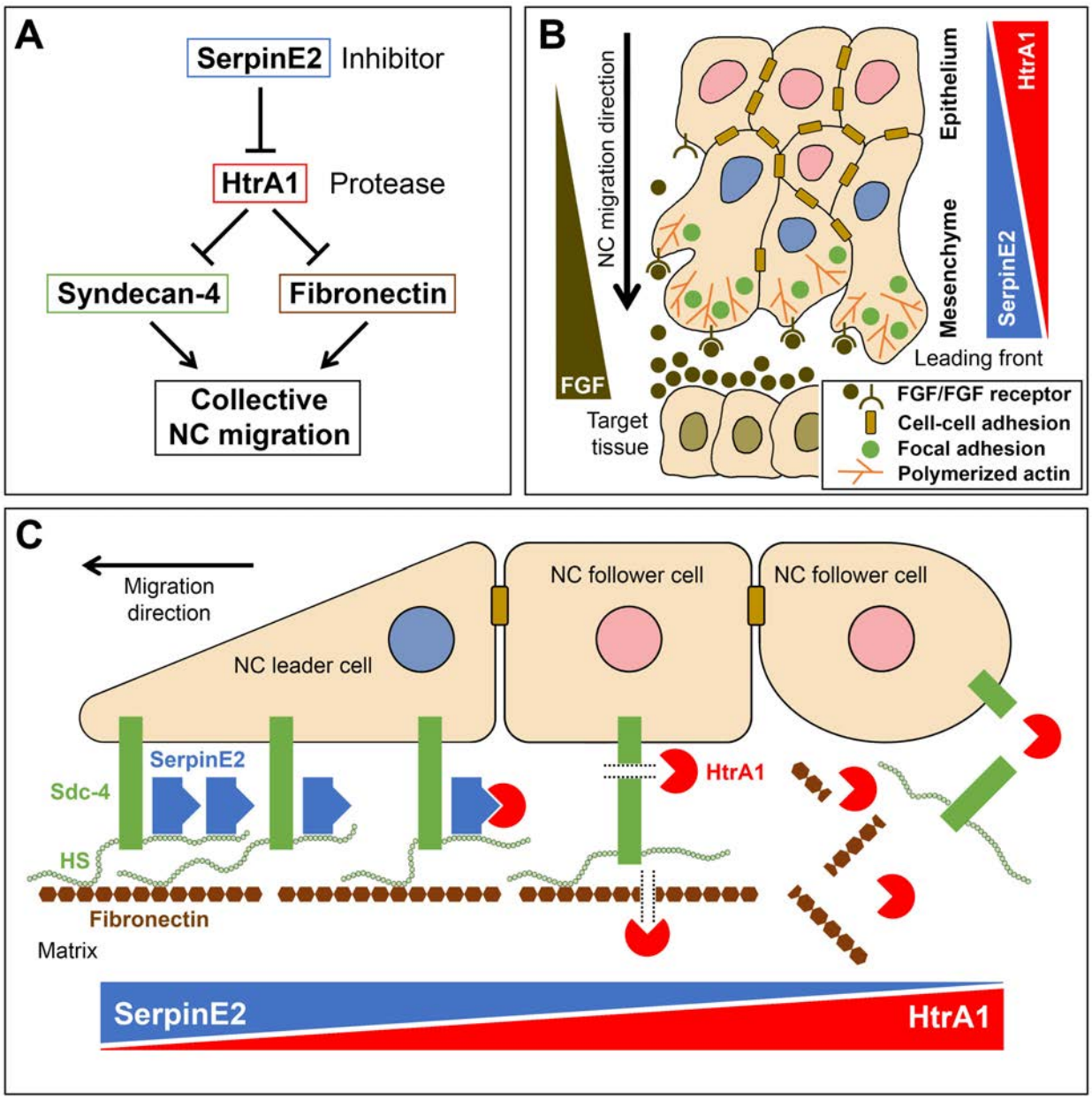


**Figure 9. Model for a proteolytic pathway of SerpinE2 and HtrA1 that regulates collective neural crest migration**

**(A)** SerpinE2 stimulates collective NC migration by a double-inhibitory mechanism involving the secreted serine protease HtrA1 and its proteolytic substrates Syndecan-4 and Fibronectin.

**(B)** Opposing gradients of SerpinE2 and HtrA1 activities regulate the directed migration in a collective of NC cells. The SerpinE2/HtrA1 pair contributes to the formation of a chemoattractant gradient that guides the NC stream towards a source of FGF signals in the target tissue. High SerpinE2 and low HtrA1 levels coincide with abundant focal adhesion sites and polymerized actin that drive mesenchymal migration at the leading edge.

**(C)** SerpinE2 anchored to the heparan sulfate chains of the transmembrane proteoglycan Syndecan-4 protects the integrity of focal adhesions at the leading front and allows collective cell migration to occur (left side). Unbound HtrA1 triggers the proteolytic cleavage of Syndecan-4 and degrades the matrix protein Fibronectin (middle), causing loss of cell-matrix adhesion at the rear end of the NC cell collective (right side).

FGF, fibroblast growth factor; HS, heparan sulfate; NC, neural crest; Sdc4, Syndecan-4.

## Supplementary Figures

**Figure 1-figure supplement 1. *Twist*, *SerpinE2* and *HtrA1* gene expression in sections of tailbud stage embryos after whole-mount *in situ* hybridization**
**(A)** Schematic of embryo at stage 26. Labelled in violet are the *Twist*-positive NC cells in the mandibular arch (1), hyoid arch (2), anterior branchial arch (3), and posterior branchial arch (4). Indicated are the level of horizontal sections in panels B-D (strippled line) and the level of transversal sections in in panels E-G (continuous line).
**(B-D)** Horizontal hemisections viewed from dorsal.
**(E-G)** Transversal sections. The strippeled line labels the *Twist*$^+$ NC. Note the arrowheads indicating the ventral *SerpinE2* and the dorsal *HtrA1* expression domains in the NC of the hyoid arch.
ey, eye; hb, hindbrain; ot, otic placode.

**Figure 2-figure supplement 1. SerpinE2 depletion does not affect the specification of neural crest cells**
**(A-H)** Anterior view of embryos at stage 18 after whole-mount *in situ* hybridization. Note that neither control-MO (A,C,E,G) nor *SerpinE2*-MO (B,D,F,H) affect the expression of the neural crest cell markers *cMyc*, *FoxD3*, *Snail1*, and *Snail2*. The arrow demarcates the trunk neural crest. Embryos were injected into a single animal blastomere at the 8-cell stage with 10 ng morpholino oligonucleotide (MO) and 100 pg *nlacZ* mRNA as lineage tracer (red nuclei). Indicated phenotypes were shown in A, 5/5; B, 9/9; C, 8/8; D, 9/9; E, 6/6; F, 6/7; G, 8/8; H, 5/6.

**Figure 4-figure supplement 1. Overexpression of HtrA1 decreases cartilaginous elements and craniofacial structures.**
Embryos were injected into four animal blastomeres at the 8-cell stage with a total of 100 pg mRNA.
**(A-D)** Ventral view of embryos at stage 46 after Alcian Blue staining. Note that *HtrA1* mRNA decreases the skeleton width (double arrow at the level of the ceratobranchial cartilage structures) but does not affect gut coiling (arrowhead). *HtrA1(S307A)* and *HtrA1ΔSP* mRNAs have no effects.
**(E)** Quantification of the skeleton width. The number of analyzed specimen is indicated above each column.
int, intestine. Indicated phenotypes were shown in A, 73/74; B, 58/62; C, 73/77; D, 67/78 embryos. At least two experiments were done.

**Figure 4-figure supplement 2. Knockdown of SerpinE2 reduces craniofacial skeleton formation.**
Morpholino oligonucleotides (MOs) were injected into *Xenopus* embryos at the 8-cell stage.
**(A-C)** Ventral view of tadpoles at stage 46 after Alcian Blue staining. Note that *SerpinE2*-MO decreases the branchial skeleton width (double arrow) but does not affect gut coiling (arrowhead). The control-MO and a combination of *SerpinE2*-MO and *Flag-SerpinE2* mRNA have no effects.
**(D)** Quantification of the skeleton width. The number of analyzed specimen is indicated above each column. Embryos were injected into four animal blastomeres with a total of 40 ng MO.
cb, ceratobranchial; ch, ceratohyal; et, ethmoid-trabecular; int, intestine; mc, Meckel's cartilage; pq, palatoquadrate. Indicated phenotypes were shown in A, 76/88; B, 72/74; C, 78/85. At least two experiments were done. At least three experiments were done.

**Figure 6-figure supplement 1. *HtrA1* mRNA inhibits neural crest migration in a concentration-dependent manner.**
If not otherwise indicated, embryos were injected with 65 pg *HtrA1*-derived mRNAs into a single dorsal animal blastomere at the 8-cell stage. Quantification of EMT and migration of *Twist*$^+$ NC cells in embryos at stages 20 and 26. Defects were assessed based on a comparison of injected embryos and non-injected control siblings. Normal phenotypes are distinguished from phenotypes with either mild or strong defects in NC cell migration. A mild defect is defined as a reduction by more than 25% in at least one NC segment. A strong defect is a reduction by more than 50% in all NC segments. The number of analyzed embryos per sample is indicated above the columns. At least two experiments were done.
**(A,B)** Following dorsal injection, *HtrA1* mRNA blocks EMT and cell migration of NC cells in a dose-dependent manner. Note that *HtrA1-myc* and *HtrA1ΔPDZ-myc* mRNAs cause migration defects with a similar strength, whereas HtrA1(S307A) and *Flag-HtrA1ΔSP* mRNAs have only little effects.
**(C,D)** Ventral injection of *HtrA1* mRNA cause relatively mild NC migration defects.

**Figure 6-figure supplement 2. Cytoplasmic HtrA1 causes reduction of αTubulin protein levels in mammalian cells and *Xenopus* embryos.**
**(A)** Schematic representation of the protein constructs. Indicated are antibody binding sites. RCL, reactive center loop; SP, signal peptide.
**(B,C)** Western blot analysis of cell lysate and culture supernatants. HEK293T cells were transiently transfected with pCS2 vector constructs containing Flag epitope-tagged cDNAs encoding HtrA1, HtrA1ΔSP, SerpinE2, and SerpinE2ΔSP. Note that Flag-HtrA1 and Flag-SerpinE2 have higher molecular weights in the supernatant (54 and 48 kD) than in the cell lysate (52 and 46 kD), indicating that these proteins are glycosylated in the secretory pathway. Flag-HtrA1ΔSP and Flag-SerpinE2ΔSP are less abundant in the supernatant, and their molecular weights (49 and 43 kD) the same as in the cell lysate, suggesting that these signal peptide-deficient proteins are not secreted and their low levels in the supernatant likely due to cell lysis. Empty vector was used as negative control. Ponceau Red staining of membrane shows equal amounts of protein in the lysate samples.
**(D,E)** Western blot analysis of *Xenopus* embryos at stage 10.5. Embryos were injected into the animal pole at the 4-8 cell stage. Injected mRNA doses per embryo were 320 pg (*HtrA1*-derived) and 4 ng (*SerpinE2*-derived). Coomassie blue staining shows equal protein loading in the gels.
**(F,G)** Quantification of αTubulin protein levels relative to βActin in HEK293T cells (F) and relative to Ponceau Red staining in *Xenopus* embryos at stage 10.5 (G). Experiments were done at least twice.

**Figure 7-figure supplement 1. *SerpinE2* and *Sdc4* mRNAs partially rescue neural crest migration defects that are induced by HtrA1 overexpression.**
Quantification of EMT and migration of *Twist*$^+$ NC cells in embryos at stages 20 and 26. Defects were assessed based on a comparison of injected embryos and non-injected control siblings. The number of analyzed embryos per sample is indicated above the columns. Note that secreted Flag-SerpinE2, but not the cytosolic Flag-SerpinE2ΔSP and mutant Flag-SerpinE2pm constructs, revert NC migration defects in *Flag-HtrA1* mRNA-injected embryos.

**(A,B)** Dorsal injection of *Sdc4* mRNA rescues *Flag-HtrA1*-induced migration defects in a dose-dependent manner.
**(C,D)** *Sdc4* mRNA is less efficient in rescuing NC migration upon ventral co-injection with *Flag-HtrA1* mRNA.
Embryos were injected into one animal blastomere at the 8-cell stage with mRNA doses of 65 pg (*Flag-HtrA1*) and 200 pg (*SerpinE2*-derived). Indicated *Sdc4* mRNA amounts per embryo are in pg. Experiments were done at least three times.

**Figure 8-figure supplement 1. SerpinE2-MO causes neural crest migration defects which are rescued by *HtrA1*-MO and *Sdc4* mRNA.**
EMT and NC migration defects were quantified in embryos at stages 22 and 26 after whole-mount in situ hybridization with the NC marker *Twist*. Defects were assessed based on a comparison between the injected and non-injected side within the same specimen. Normal phenotypes were distinguished from phenotypes with either mild or strong defects in NC cell migration. A mild defect is defined as a reduction by more than 25% in at least one NC segment. A strong defect is a reduction by more than 50% in all NC segments. The number of analyzed embryos per sample is indicated above the columns.
**(A, B)** Dorsal injection of *SerpinE2*-MO blocks EMT and migration of NC cells, while control-MO and *SerpinE2*-5MM-MO have no or only little effect. *Flag-SerpinE2* mRNA, *HtrA1*-MO, and *Sdc4* mRNA restore normal NC migration in *SerpinE2*-morphant embryos.
**(C,D)** Upon ventral injection, *SerpinE2*-MO mildly blocks EMT and migration of NC cells.
Morpholino oligonucleotides (MOs, 10 ng) and mRNAs were injected into one animal blastomere at the 8-cell stage. Injected mRNA doses per embryos were 333 pg (*Flag-SerpinE2*) and 300 pg (*Sdc4*). Experiments were done at least three times.

**Videos**

**Figure 5-video 1. Collective migration of neural crest cells *in vitro*.** Embryos were injected into the animal pole at the 4-cell stage with 40 ng standard control-MO. The cranial NC was explanted at stage 16/17, plated on fibronectin and imaged 7 hours after culture. Filming was done for 20 minutes. Note that cells at the leader front of the cluster extend lamellipodia and filopodia.

**Figure 5-video 2. Knockdown of SerpinE2 prevents adhesion and migration of neural crest cells.** Embryos were injected into the animal pole at the 4-cell stage with 40 ng *SerpinE2*-MO. The cranial NC was explanted at stage 16/17, plated on fibronectin and imaged 7 hours after culture. Filming was done for 10 minutes. Note that cells acquire a ball-like shape and freely float around.

**Source data files**

**Figure 6-Figure supplement 2-Source data 1. Western blot analysis of HEK293T cell lysate.** HEK293T cells were transfected with pCS2 vector alone as a control and pCS2 constructs containing Flag epitope-tagged cDNAs encoding HtrA1, HtrA1ΔSP, SerpinE2, and SerpinE2ΔSP. Ponceau Red staining validates that in all samples equal amounts of protein were transferred to the membrane. The same membrane was then sequentially incubated with antibodies against Flag epitope, βActin and αTubulin. Note that traces of anti-Flag antibody are seen in the βActin blot,

and traces of βActin antibody in the αTubulin blot due to incomplete membrane stripping between the antibody incubations.

**Figure 6-Figure supplement 2-Source data 2. Western blot analysis of HEK293T cell supernatant.** The same membrane was sequentially incubated with antibodies against Flag epitope, βActin and αTubulin.

**Figure 6-Figure supplement 2-Source data 3. Western blot analysis of *Xenopus* embryos at stage 10.5.** *Xenopus* embryos were uninjected or microinjected with mRNA encoding Flag-tagged HtrA1, HtrA1ΔSP, SerpinE2, and SerpinE2ΔSP. Coomassie-Blue staining of the gel validates that in all samples equal amounts of protein were loaded. Membranes were incubated with anti-Flag antibody (left side) or sequentially with antibodies against HtrA1 and αTubulin (right side).