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

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ABSTRACT

Here we present an extracellular proteolytic mechanism involving the serine protease HtrA1 and its inhibitor SerpinE2 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 skeleton 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. 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) (Szabó and Mayor, 2018). The NC is a multipotent embryonic cell population that is specified at the border of the neural plate in vertebrates (Le Douarin and Kalcheim, 1999; Schock et al., 2023). EMT initiates the directed migration stream of NC cells towards their target tissues where they contribute to diverse organ systems including skin pigment 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 migration.

Members of the serpin superfamily contain a carboxyterminal reactive center loop that covalently binds to and inhibits target serine proteases (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 as well as in cancer (Arocas and Bouton, 2015; Monard, 2017). SERPINE2 also functions as an oncogene that is upregulated in aggressive variants of several cancer types and prompts metastasis and/or poor

prognosis (Arocas and Bouton, 2015), supporting a positive function in cell migration and invasion. In contrast, a negative function for SERPINE2 has been reported in prostate cancer (Xu et al., 2010) and glioma (Pagliara et al., 2014), where SERPINE2 limits tumor invasion through blocking urokinase plasminogen activator and matrix metalloprotease-9-mediated extracellular matrix remodeling. Despite being a key factor in tumor dissemination, the molecular mechanism by which SerpinE2 governs cell migration and metastasis is largely unknown.

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 was initially identified as being upregulated in human fibroblasts transformed by oncogenic viruses (Zumbrunn and Trueb, 1996). HtrA1 downregulation in several primary tumors and metastatic foci, which correlates with poor clinical outcome and poor response to chemotherapy, together with the observations that overexpression leads to a decrease in cell proliferation, suggests a probable role as a tumor suppressor protein (Zurawa-Janicka et al., 2017). HtrA1 modulates the extracellular matrix and cell signaling as a secreted protein but was found to also be active in the cytoplasm and nucleus (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.

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. The HtrA1 protease releases FGF ligands by triggering the cleavage of cell surface proteoglycans such as Syndecan-4 (Sdc4), thus stimulating FGF signaling in mesoderm and tail formation (Hou et al., 2007). On the other hand, by binding to and inhibiting HtrA1, SerpinE2 restricts FGF signaling and allows 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 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, Syndecan-4 (Sdc4) mRNA can partly revert the NC migration defects that are induced by HtrA1 overexpression or SerpinE2 knockdown. We conclude that the SerpinE2/HtrA1/Sdc4 pathway regulates collective 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 (Fig. 1A,A'; Hopwood et al., 1989). *SerpinE2* transcripts were found in the deep layer of the neural plate and adjacent NC cells (Fig. 1B-B'). *HtrA1* was transcribed in the superficial layer of the neural plate and in the NC (Fig. 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 Fig. 1D-F and Supplementary Figure S1A-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 (Fig. 1D'-F' and Supplementary Figure S1E-G). In addition, *SerpinE2* and *HtrA1* shared overlapping expression in the brain, eye vesicles and otic placodes (Fig. 1E,F and Supplementary Figure S1F,G). These results showed that *SerpinE2* and *HtrA1* are expressed in pre-migratory NC cells and adjacent tissues (Fig. 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 (Fig. 1H).

SerpinE2 is required for the development of neural crest-derived structures

Classical extirpation experiments carried out in the urodele *Amblystoma* demonstrated that the cranial NC is important for the formation of the head skeleton (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 (arrowheads) and reduced melanocyte pigmentation (arrow) (Fig. 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 (arrowheads) and a decreased number of melanocytes (arrow), whereas a standard control-MO had no phenotypic effect at stage 40 (Fig. 2D,E). The striking similarity of the phenotype obtained after knockdown of SerpinE2 with that of NC extirpation (Fig. 2A-C) suggested that SerpinE2 might be important for NC development.

Alcian Blue staining was used to visualize the cartilagenous head and branchial skeleton in more advanced tadpole embryos (Fig. 2G). *SerpinE2*-MO decreased the size of the mandibular, hyoid and branchial cartilage structures at stage 46 (Fig. 2H). The distance between the branchial arch structures as a measure of the skeleton width in *SerpinE2*-MO-injected embryos was decreased by 22% compared to control-morphant siblings (Supplementary Figure S2A,B), showing that SerpinE2 knockdown significantly impairs craniofacial skeleton formation. The coiling pattern of the gut was not affected in *SerpinE2*-morphant tadpoles (Supplementary Figure S2C,D), ruling out an overall delay of embryonic development. These phenotypes were specific, since co-injection of *SerpinE2*-MO together with a *Flag-SerpinE2* mRNA that is not targeted by the MO, rescued normal development (Fig. 2F,I and Supplementary Figure S2B,E). Since NC cells contribute to dorsal fin, melanocyte and craniofacial skeleton formation (Tucker, 1986;

Sadaghiani and Thiébaud, 1987; Tucker and Slack, 2004), the results suggest that SerpinE2 is required 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 (Fig. 2J). *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 (Fig. 2K-N and Supplementary Figure S2F-M). 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 (Fig. 2O,R). 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 (Fig. 2U). A single dorsal injection of *SerpinE2*-MO caused a delay or failure of these NC cells to undergo EMT and migration in advanced neurula (Fig. 2P,S, arrows) and tailbud embryos (Fig. 2V, arrow). 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 (Fig. 2Q,T,W; see also Fig. 6H-K' below and Supplementary Figure S3A,B). Ventrally injected *SerpinE2*-MO was less efficient in reducing NC migration (Supplementary Figure S3C,D) as expression of the *SerpinE2* is highest in dorsal regions of post-gastrula embryos (Fig.

1B). We conclude that SerpinE2 is dispensable for the initial specification but necessary for the migration of NC cells in *Xenopus* embryos.

The HtrA1 protease inhibits the migration and development 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 reduction of dorsal fin tissue (arrowheads) and fewer melanocytes (arrow) in early tadpole embryos (Fig. 3A,B). *HtrA1* overexpression also caused a decrease of head and eye structures in accordance with the previously reported axis posteriorizing activity of this protease (Hou et al., 2007). We further observed that *HtrA1* mRNA injection inhibits craniofacial skeleton development and decreased the cartilaginous skeleton width by 21% in advanced tadpoles (Fig. 3E,F and Supplementary Figure S4A,B,E). We previously reported that wild-type HtrA1 and a mutant *HtrA1(S307A)* derivative, in which the catalytic serine residue in amino acid position 307 is replaced by alanine (Fig. 5A), generates equal amounts of proteins of the expected size but that *HtrA1(S307A)* mRNA fails to block head formation (Hou et al., 2007). Here we show that *HtrA1(S307A)* did not cause any defects in dorsal fin, melanocyte, and craniofacial skeleton formation in mRNA-injected embryos (Fig. 3C, G and Supplementary Figure S4C). This shows that HtrA1 relies on an intact protease domain to regulate the development of these NC derivatives.

A single dorsal injection of *HtrA1* mRNA did not affect the expression of cranial and trunk NC markers but blocked EMT and migration of NC cells (Fig. 3I,J,L-O and Fig. 5B-C'). Quantitative analysis showed that HtrA1 overexpression caused migratory defects at stages 20 and 26 in a concentration-dependent manner (Supplementary Figure S5A,B). *HtrA1(S307A)* mRNA had no effect on NC migration (Fig. 5D,D'). 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 (Supplemental Figure S5C,D). Dorsal injection of an antisense MO sequence against *HtrA1* (*HtrA1*-MO), which efficiently blocks the biosynthesis of endogenous HtrA1 protein in *Xenopus* embryos (Hou et al., 2007), did not affect the induction of the NC marker *Twist* (Fig. 3K) nor alter the migration of NC cells (Fig. 3P). These results indicate that knockdown of HtrA1 appears not to affect NC development and that upregulation of HtrA1 protease activity inhibits the migration but not the specification of NC cells.

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 the extracellular matrix protein fibronectin *in vitro* to investigate collective cell migration in relative isolation, allowing for the identification of extrinsic versus intrinsic mechanisms (Fig. 4A; 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 (Fig. 4B-B'',F). 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 (Fig. 4C-C'',F). 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 (Fig. 4D-E'',F). At 7 hours after plating, distinct segments were seen in uninjected and control-MO-injected explants (Fig. 4B''',D''', arrowheads), resembling the mandibular, hyoid, and branchial streams seen in sibling control embryos at the tailbud stage (Fig. 1D). In contrast, explants injected with either *HtrA1* mRNA or *SerpinE2*-MO failed to display segmentation into distinct streams (Fig. 4C''',E'''). 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 (Fig. 4G). 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 (Fig. 4H, arrowhead). Upon injection of *HtrA1* mRNA, nearly 80% of cells failed to attach to this matrix protein and acquired a round morphology compared to control cells (Fig. 4I,L, arrowhead). Similarly, *SerpinE2*-MO-injected cells also lost adherence and cytoplasmic extensions, whereas the control-MO had no significant effect (Fig. 4J-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 development and cell migration as a secreted protein

HtrA1 contains a cleavable N-terminal signal peptide and shares a carboxyterminal PDZ (Post-synaptic density of 95kD, Discs large, Zona occludens-1) protein-protein interaction domain with other members of the HtrA family (Fig. 5A; Zurawa-Janicka et al., 2017). We previously identified *Xenopus* HtrA1 as a protein secreted into the supernatant of cDNA-transfected and metabolically labelled HEK 293T cells (Hou et al., 2007). On the other hand, human HtrA1 has been shown to associate with cytosolic microtubules in a PDZ domain-dependent manner and to modulate both microtubule stability and cell motility *in vitro* (Chien et al., 2009a,b). We therefore asked if HtrA1 acts in the extracellular space or inside the cell, and whether its PDZ domain is involved in regulating NC development. To this end, we generated a Flag-tagged HtrA1 construct, in which the secretory signal peptide was absent (*Flag-HtrA1ΔSP*; Supplementary Figure S6A) and compared its activity to a signal peptide-containing *Flag-HtrA1* construct (Hou et al., 2007). Western blot analysis of lysates revealed that the two protein constructs are expressed with the expected molecular weights in cDNA-transfected HEK293T cells and mRNA-injected *Xenopus*

embryos; however, while the Flag-HtrA1 protein accumulates at high levels in the supernatant of transfected cells, the truncated Flag-HtrA1ΔSP protein fails to be efficiently secreted into the culture medium (Supplementary Figure S6B-E). We also show that cytosolic Flag-HtrA1ΔSP degrades αTubulin, but not βActin, which validates the proteolytic activity and target specificity of this construct (Supplementary Figure S6B,E,G).

Flag-HtrA1ΔSP did not cause any defects in NC-derived craniofacial, dorsal fin and melanocyte structures (Fig. 3D,H and Supplementary Figure S4D,E) nor did it affect NC migration in mRNA-injected embryos (Fig. 5E,E' and Supplementary Figure S5A,B). We previously described that a myc-tagged HtrA1 construct and a mutant HtrA1ΔPDZ-myc derivative, which has a deletion of the PDZ domain (Fig. 5A), are expressed at the expected size and co-immunoprecipitate at similar levels with Flag-SerpinE2 in embryos (Acosta et al., 2015). Here we show that *HtrA1ΔPDZ-myc* efficiently inhibited EMT and NC migration to a degree comparable to that induced by *HtrA1-myc* control mRNA (Fig. 5F-G' and Supplementary Figure S5A,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 and HtrA1 interact 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 (Fig. 6A). 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? 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 (Fig. 6B,B' and Supplementary Figure S7). 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 (Fig. 6C-D',H,I and Supplementary Figure S7). The newly generated truncated Flag-SerpinE2ΔSP construct lacks a SP, is expressed at the expected protein size and is not efficiently secreted (Supplementary Figure S6A-D). Upon mRNA co-injection, *Flag-SerpinE2ΔSP* failed to rescue the *Flag-HtrA1*-induced NC migration defects (Fig. 6E,E' and Supplementary Figure S7), underscoring that entry into the secretory pathway is important for the function of SerpinE2. We previously showed that *Flag-SerpinE2* and a point mutant *Flag-SerpinE2pm* derivative (Fig. 6A; Onuma et al., 2006), in which two proline residues replace the critical arginine and serine residues (R362P and S363P) at the process site of the RCL, generate proteins in similar amounts and at the expected size but that overexpressed HtrA1 immunopreciptates Flag-SerpinE2pm less efficiently than Flag-SerpinE2 (Acosta et al., 2015). Here we show that *Flag-SerpinE2pm* did not inhibit *Flag-HtrA1* mRNA from blocking NC migration (Fig. 6F,F' and Supplementary Figure S7). We conclude that SerpinE2 functions in the extracellular space and requires an intact RCL for efficient interaction with HtrA1 to modulate NC cell 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 (Figs. 6J,J',L,L' and Supplementary Figure S3A,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 (Fig. 6G,G' and Supplementary Figure S7A,B). Ventral co-injection of *Sdc4* and *Flag-HtrA1* mRNAs was less efficient in rescuing NC cell migration (Supplementary Figure S7C,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. In addition, dorsally injected *Sdc4* mRNA partially restored normal EMT and cell migration in *SerpinE2*-morphant embryos (Fig. 6M,M' and Supplementary Figure S3A,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 cartilages and enteric neurons. (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* (Fig. 6N).

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) 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 NC 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 (Fig. 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 (Fig. 1H). Hence, the expression data indicate that opposing activity gradients of SerpinE2 and HtrA1 proteins operate in the NC cell population and influence its collective migratory behavior (Fig. 7B). We propose that only in regions where the SerpinE2 concentration is sufficiently high, HtrA1-mediated repression of cell motility can be relieved allowing NC cell migration to occur. In support of this conclusion, microinjection of *HtrA1* mRNA reduced in a concentration-dependent manner EMT and migration of NC cells; this leads to defects in the formation of craniofacial skeleton structures, dorsal fin tissue, and melanocytes. A protease mutant HtrA1(S307A) construct with a substitution of the catalytic serine against alanine residue had no effect, suggesting that an intact proteolytic trypsin domain is required for HtrA1 to suppress NC migration and formation of NC-derived 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* overexpression and *SerpinE2* knockdown diminished cell migration in isolated NC explants provided evidence that the two proteins act in a NC-autonomous manner. 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) as well as 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 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

The heparan sulfate proteoglycan 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 the Wnt receptor Frizzled7 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 transmembrane 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 Sdc4 in mRNA-injected *Xenopus* embryos (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 showed that SerpinE2 increases the protein stability of Sdc4, that overexpressed SerpinE2 protects Sdc4 from HtrA1-mediated degradation, and that knockdown of SerpinE2 decreases protein levels of Sdc4, suggesting that the endogenous SerpinE2 protease inhibitor is needed to protect the integrity of Sdc4 in the *Xenopus* embryo (Acosta et al., 2015). Here we demonstrated that Sdc4 reduced HtrA1-induced NC migration defects in mRNA-injected embryos in a concentration-dependent manner, indicating that Sdc4 is a relevant target of HtrA1 *in vivo*. The finding that *Sdc4* mRNA also partially rescued EMT and migration of NC cells in *SerpinE2*-MO-injected embryos indicates that SerpinE2 promotes NC migration at least in part via repression of Sdc4-accelerated cleavage by HtrA1. On the other hand, *in vitro* experiments revealed a notable difference in the severity of NC migration defects. While Sdc4 knockdown perturbs the

directionality but not velocity of cell migration (Matthews et al., 2008), both HtrA1 gain-of-function and SerpinE2 loss-of-function reduced cell-matrix adhesion. This as well as the failure of Sdc4 to completely rescue NC migration in HtrA1 overexpressing and SerpinE2-depleted embryos suggests that other components of the extracellular matrix might be targeted by the SerpinE2-HtrA1 axis.

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

Binding of fibronectin to the heparan sulfate 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 cranial and vagal NC migration pathways in the *Xenopus* embryo (Epperlein et al., 1990). This glycoprotein is the only known extracellular matrix component that promotes *Xenopus* NC cell migration as an adhesive substrate (Alfandari et al., 2003). We showed that SerpinE2 knockdown and HtrA1 overexpression inhibited both adhesion and migration of NC cells on 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.

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 (Fig. 7A). The expression data presented here suggest that opposing gradients of SerpinE2 and HtrA1 form in the collective of migrating NC cells (Fig. 7B). Our structure-function analyses and epistatic experiments led us propose a model in which these extracellular proteins regulate directed migration of the NC collective via remodeling of cell-matrix adhesions (Fig. 7C). Since SerpinE2 has a high affinity for heparan sulfate 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 Fig. 7C). 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 collective (right side in Fig. 7C).

SerpinE2, HtrA1 and Syndecan4 in placenta development and cancers

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 cancer. During placenta development, 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 EVTs and spiral arteries (Chern et al. 2011). Silencing of SERPINE2 inhibits the migration and invasion of human EVT cells *in vitro*. In placental tissue, HTRA1 expression is highest in less motile villous trophoblasts and lowest in EVTs (Ajayi et al., 2008). Overexpression of HTRA1 attenuates the migration and invasion of a human EVT cell line. SDC4 is expressed in EVT cells, and SDC4 silencing impairs the migration and invasion of a human EVT cell line (Jeyarajah et al., 2019). Interestingly, the expression levels of both HTRA1 and

SDC4 are upregulated in the placenta of patients with pre-eclampsia (Ajayi et al., 2008; Jeyarajah et al., 2019).

Cell migration is a hallmark of tumor malignancy and essential for metastasis formation (Hanahan and Weinberg, 2011). Breast cancer (BC) is the most prevalent cancer in women worldwide and remains incurable once metastatic. SERPINE2 is upregulated in malignant BC and high expression levels correlate with poor prognosis in patients (Tang et al., 2019). SERPINE2 promotes EMT, migration and invasion of human BC cells *in vitro* and metastasis in xenografted mice *in vivo*. On the other hand, HTRA1 is downregulated in BC; low expression levels associate with poor survival, and HTRA1 inhibits EMT, migration and invasion of human BC cell lines (Wang et al., 2012; Lehner et al., 2013). SDC4 is abundantly expressed in BC, where high expression levels correlate with worse prognosis in patients with an estrogen receptor-negative BC; SDC4 also promotes migration and invasion of human BC cells in culture (Onyeisi et al., 2021) and metastasis in mice (Leblanc et al. 2018). High expression of SERPINE2 correlates with malignancy and promotes cell migration and invasion also in melanoma (Wu, 2016), esophageal squamous cell carcinoma (Zhang et al., 2020), gastric cancer (Wang et al., 2015), and endometrial cancer (Shen et al., 2017). HTRA1 expression is low in tumors and the protease reduces migration and/or invasion in these cancer types (Baldi et al., 2002; Xia et al., 2013; Zhao et al., 2015; Mullany et al., 2011). SDC4 expression is upregulated in aggressive tumors and favors cell migration and invasion in melanoma (O'Connell et al., 2009). Together, the SerpinE2-HtrA1-Sdc4 axis as suggested here might not be confined to NC cell migration in the *Xenopus* embryo but can also regulate human trophoblast cell migration in the placenta and metastasis in several cancers.

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 and pre-eclampsia, together with their implication in metastasis of numerous tumor types, the SerpinE2-HtrA1-Sdc4 axis constitutes a potential therapeutic target for the treatment of a common pregnancy disorder and various aggressive cancers.

Materials and Methods

Xenopus laevis embryos

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 fixed in MEMFA and processed by whole-mount *in situ* hybridization as described (Pera et al., 2015).

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 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) on Fibronectin-coated plastic dishes as described (Gouignard et al., 2016).

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

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).

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 Machine™, Invitrogen).

Statistical analysis

Results are shown as mean ±SEM. Statistical analyses were performed using two-tailored t-test for two-sided comparisons where statistical significance was defined as **p< 0,01 and ****p<0,0001.

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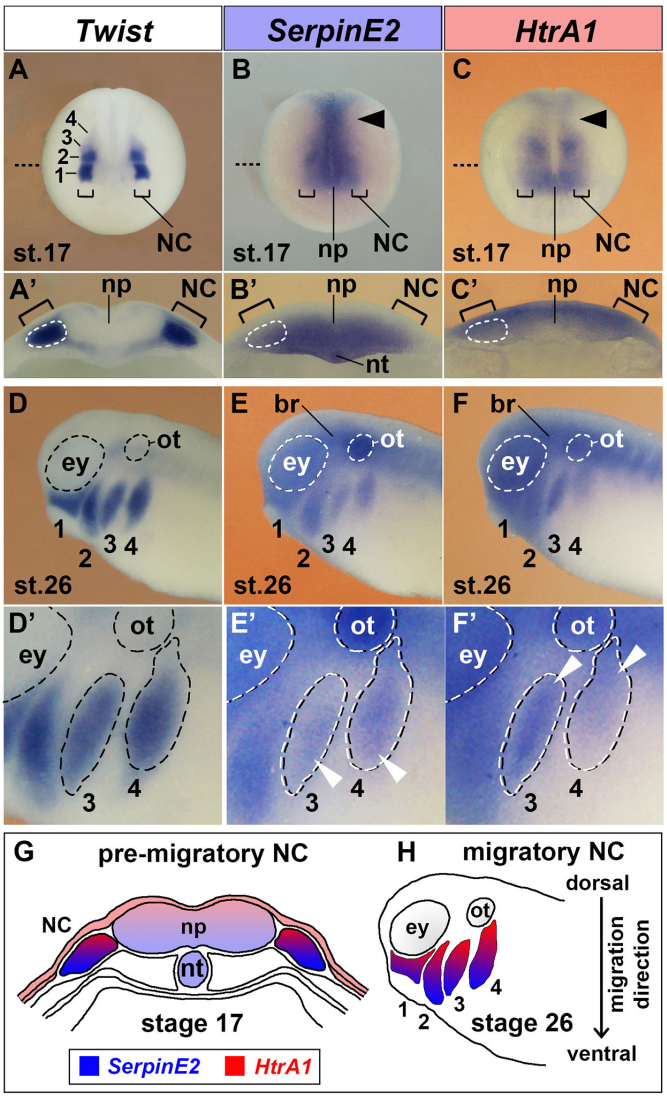
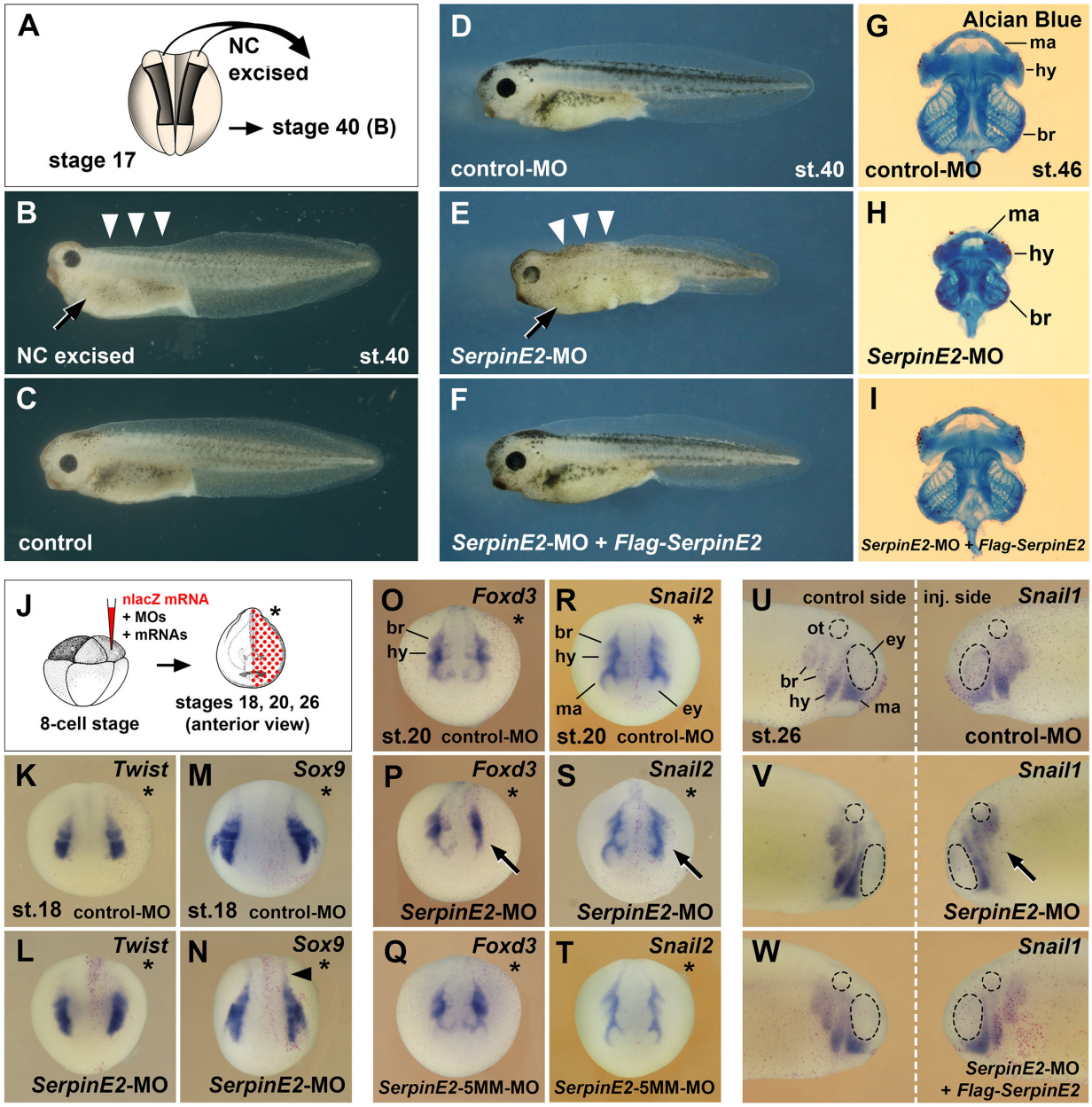
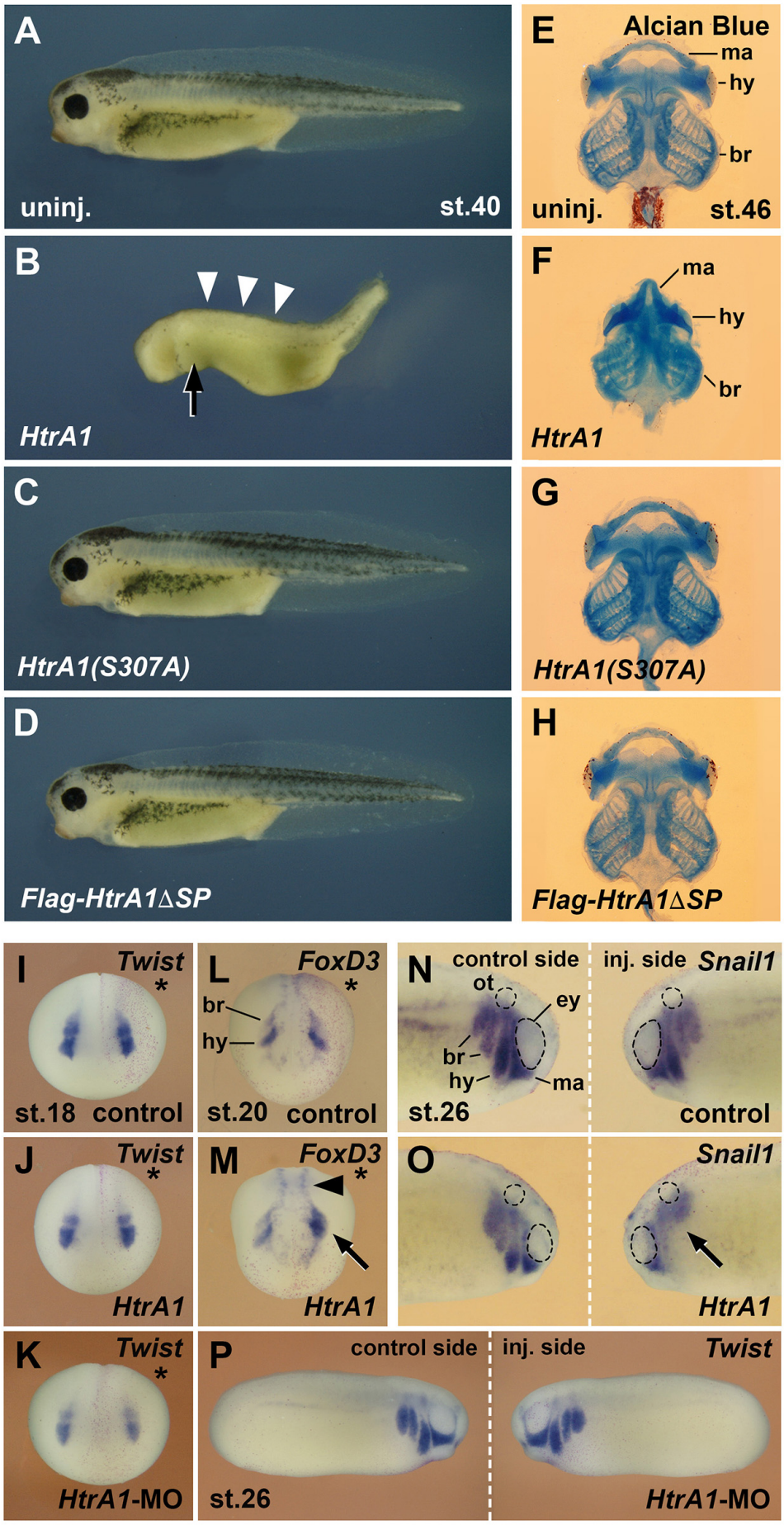


Figure 1. SerpinE2 and HtrA1 are expressed in the neural crest
Xenopus embryos were analyzed by whole-mount *in situ* hybridization.
(A-C) Anterior view of embryos at stage 17. The brackets point to pre-migratory NC cells on each side of the neural plate. The numbers label the *Twist*-expressing cranial 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.



Pera et al. – Figure 2

Figure 2. Knockdown of SerpinE2 causes defects in neural crest-derived dorsal fin, melanocyte, and craniofacial skeleton structures 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 (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.
(E) *SerpinE2*-MO causes a reduction of head tissue, dorsal fin structures (arrowheads) and melanocytes (arrow).
(F) Co-injection of *SerpinE2*-MO and 2 ng non-targeted *Flag-SerpinE2* mRNA restores a normal phenotype.
(G-I) Ventral view of cartilaginous skeleton extracted from embryos at stage 46 after Alcian Blue staining. Note that SerpinE2 knockdown specifically reduces mandibular, hyoid and branchial structures.
(J) 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.
(K-T) Anterior view of neurula embryos. Neither control-MO nor *SerpinE2*-MO affect *Twist* and *Sox9* expression in NC cells in the head and trunk (arrowhead) at stage 18 (K-N). *SerpinE2*-MO inhibits the EMT of *Foxd3*$^+$ and *Snail2*$^+$ NC cells (arrows) at stage 20, whereas the control-MO and *SerpinE2*-5MM-MO have no effect (O-T).
(U-W) Lateral view of stage 26 embryos. *SerpinE2*-MO, but not control-MO, leads to defective migration of *Snail1*$^+$ NC cells (arrow) on the injected side. 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 (K-W). Indicated phenotypes were shown in B, 10/11; D, 89/90; E, 64/83; F, 122/132; G, 83/88; H, 73/77; I, 77/87; K, 29/29; L, 24/26; M, 7/7; N, 10/11; O, 9/9; P, 7/7; Q, 7/9; R, 7/8; S, 8/9; T, 9/11; U, 10/10; V, 13/15; W, 9/9.



Pera et al. – Figure 3

Figure 3. HtrA1 protease inhibits 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.

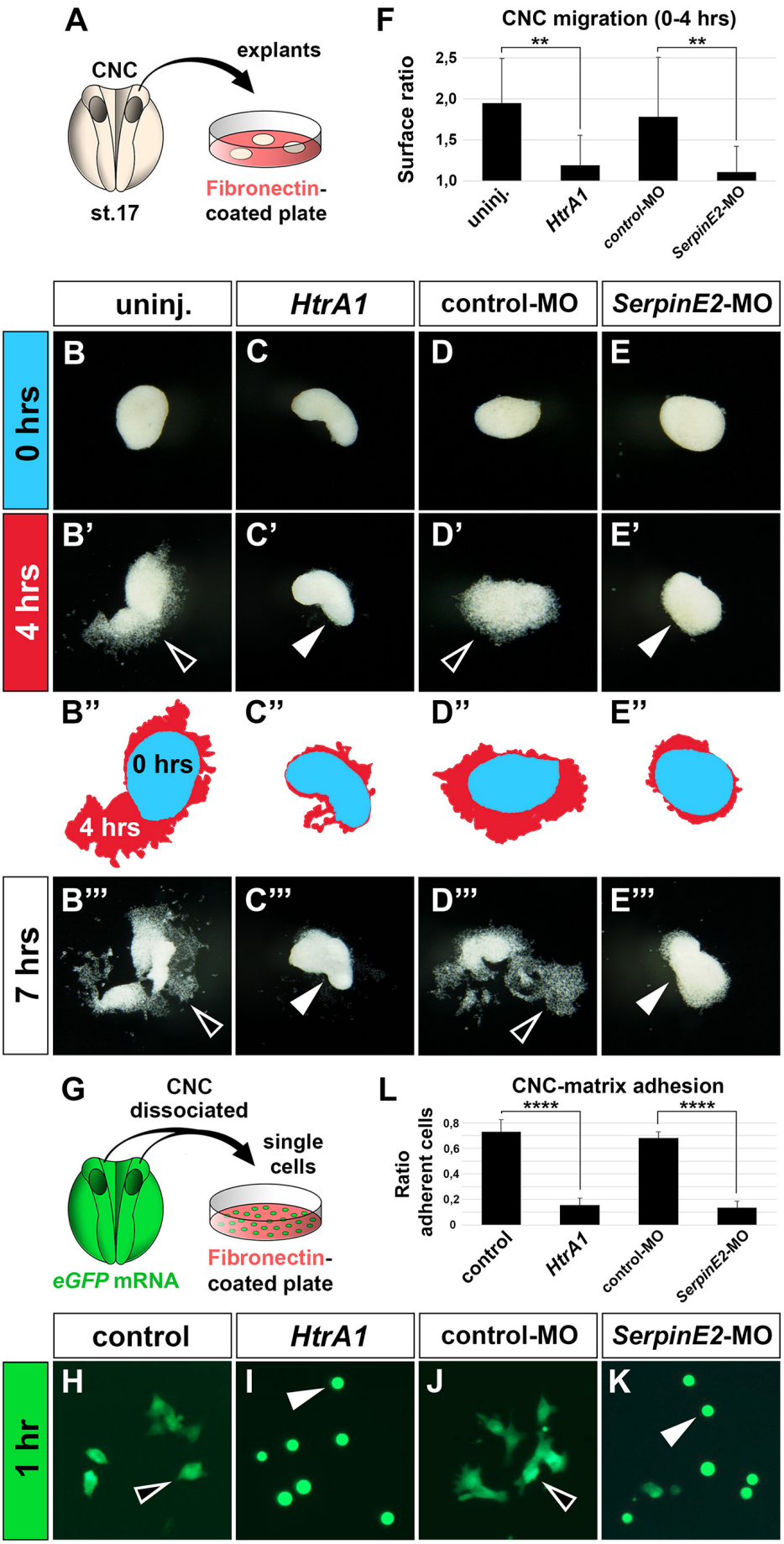
(E-H) Ventral view of isolated skulls at stage 46. Note that *HtrA1* mRNA, but not *HtrA1(S307A)* and *Flag-HtrA1ΔSP* mRNAs, diminishes cranial cartilage structures.

(I-K) 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.

(L,M) Anterior view of embryos at stage 20. 65 pg *HtrA1* mRNA reduces the EMT of *Foxd3*$^{+}$ cranial NC cells (arrow) but does not affect the specification of trunk NC cells (arrowhead).

(N-P) Lateral view of embryos at stage 26. The migration of NC cells is reduced by 65 pg *HtrA1* mRNA (arrow) 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, 57/58; F, 17/21; G, 72/77; H, 67/78; I, 31/31; J, 51/53; K, 59/60; L, 28/31; M, 23/26; N, 9/10; O, 21/21; P, 58/79 embryos.



Pera et al. – Figure 4

Figure 4. Overexpression of HtrA1 and knockdown of SerpinE2 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 (open arrowheads) in uninjected controls and explants injected with control-MO, whereas *HtrA1* mRNA and *SerpinE2*-MO block migration (filled arrowheads). In B''-E'', the surface areas of explants at 0 hours (blue) and 4 hours (red) were determined by ImageJ and superimposed.

(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.

(G) Scheme of adhesion experiment. Upon injection of *eGFP* mRNA, CNC explants 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 (open 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 (filled arrowheads).

(L) Quantification of CNC adhesion. Indicated is the ratio of adherent cells relative to the control. 6 explants were analyzed per sample.

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

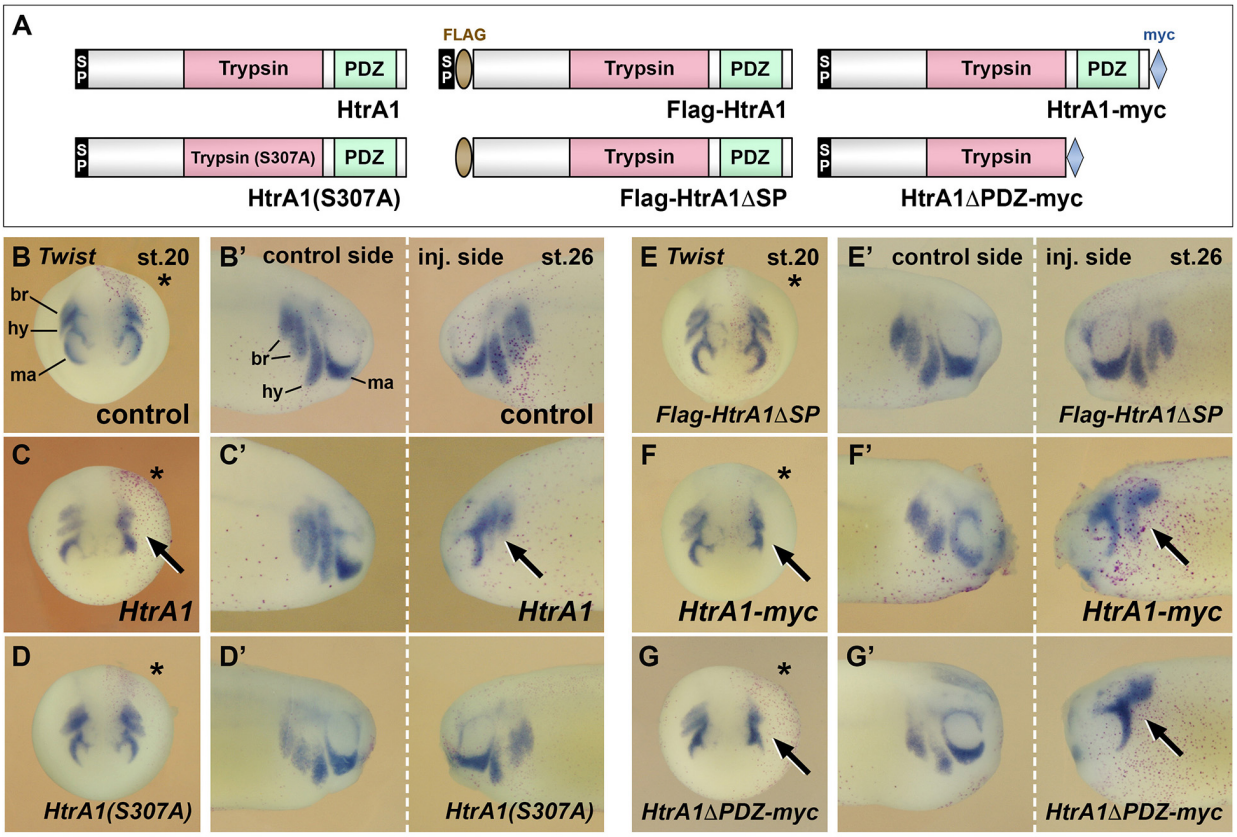
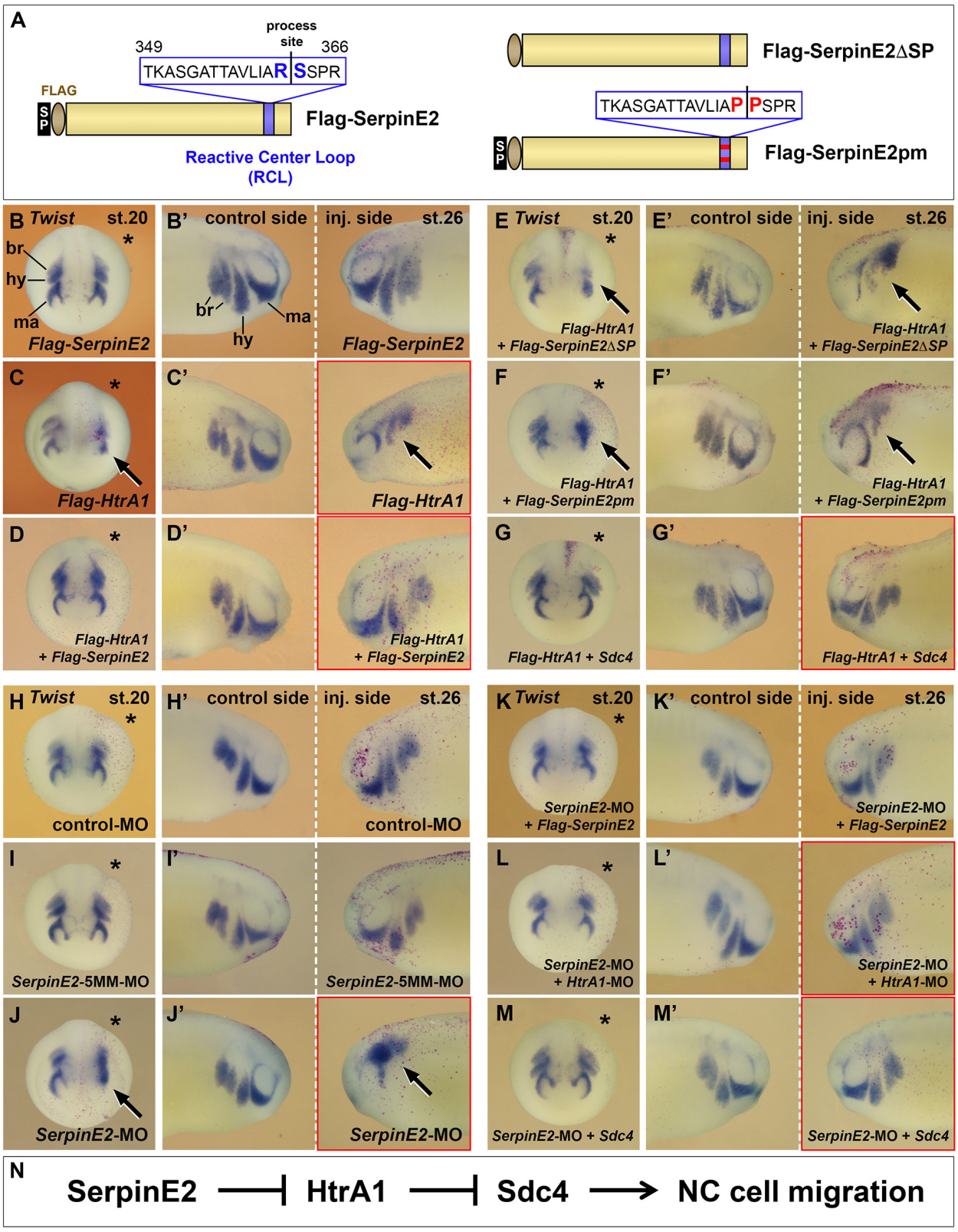


Figure 5. HtrA1 inhibits neural crest migration as an extracellular protease
Embryos were injected into a single dorsal animal blastomere at the 8-cell stage. A star labels the injected side. *Twist* expression demarcates the NC in embryos at stage 20 (B-G; anterior view) and stage 26 (B'-G'; lateral 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 (arrow). 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 (arrows). br, branchial segment; hy, hyoid segment; ma, mandibular segment. If not otherwise indicated, injected mRNA doses per embryos are 65 pg. For quantification of NC migration defects, see Supplementary Figure S5A,B.



Pera et al. – Figure 6

Figure 6. SerpinE2 functions in neural crest cell migration in an HtrA1- and Sdc4-dependent manner

mRNAs and morpholino oligonucleotides (MOs, 10 ng) and 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-M) and lateral view (stage 26, B'-M').

(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.

(C,C) Flag-HtrA1 inhibits NC cell migration robustly. Arrows label defects on the injected sides.

(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.

(H-J') *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.

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

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

Injected mRNA doses per embryos are 333 pg (*Flag-SerpinE2*) and 450 pg (*Sdc4*). For quantification of NC migration defects, see Supplementary Figures S3A,B and S7A,B.

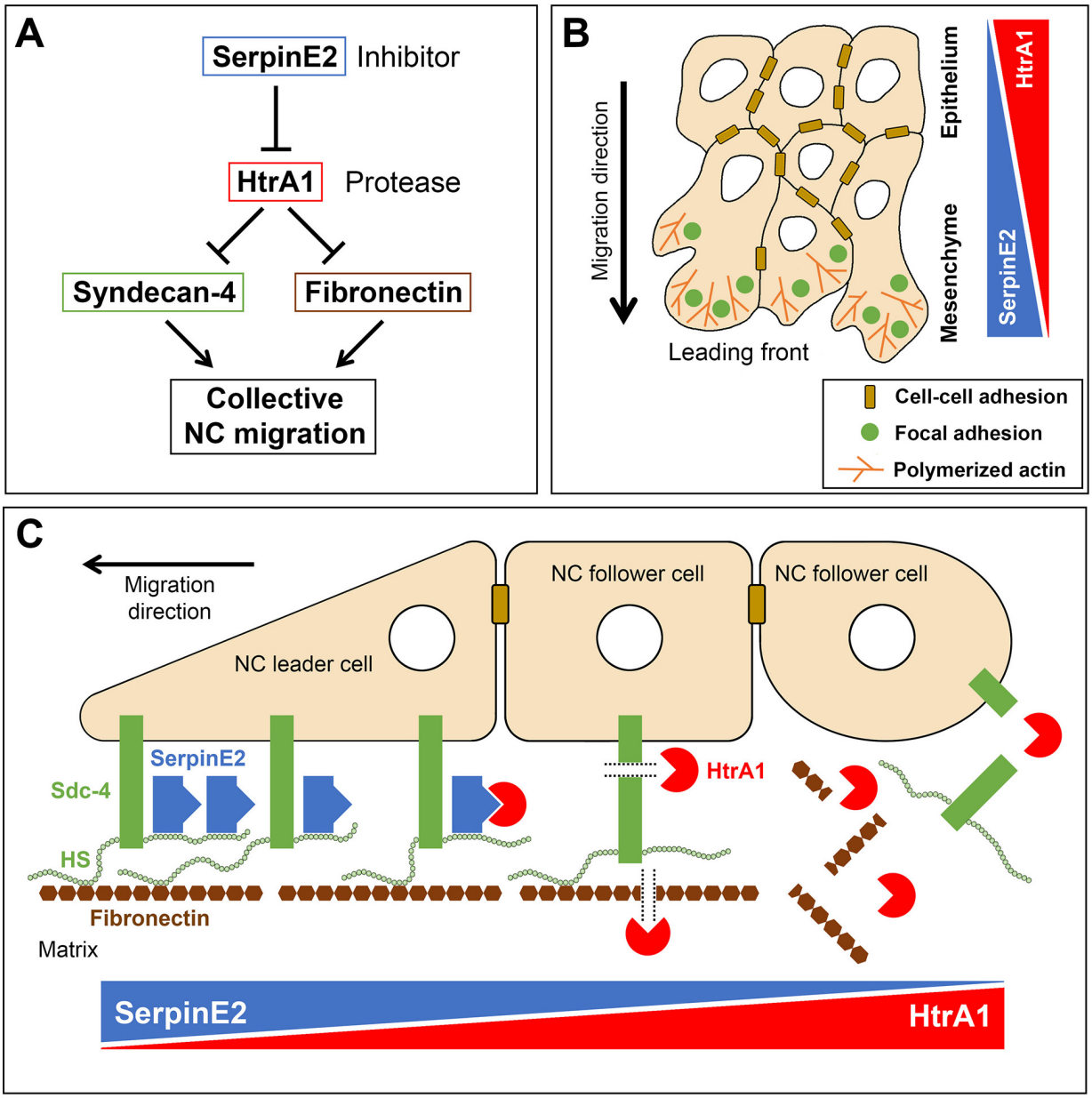


Figure 7. Model for a proteolytic pathway of SerpinE2 and HtrA1 that regulates collective neural crest migration

(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 migration in a collective of NC cells. 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).

HS, heparan sulfate; NC, neural crest; Sdc4, Syndecan-4.