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# Spatially dependent activation of the patterning protease, Easter

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**Abstract The dorsoventral axis of the *Drosophila* embryo is established by the activating cleavage of a signaling ligand by a serine protease, Easter, only on the ventral side of the embryo. Easter is the final protease in a serine protease cascade in which initial reaction steps appear not to be ventrally restricted, but where Easter activity is promoted ventrally through the action of a spatial cue at an unknown step in the pathway. Here, biochemical studies demonstrate that this spatial control occurs at or above the level of Easter zymogen activation, rather than through direct promotion of Easter's catalytic activity against the signaling ligand.**


*Keywords:* *Drosophila*; Easter; Serine protease; Zymogen activation; *pipe*

## 1. Introduction

Patterning of the embryonic body plan relies on spatially restricted signaling by morphogens, and much effort has been focused on determining the mechanisms by which morphogen gradients are formed and interpreted [1,2]. One well-studied model is the establishment of the dorsoventral axis of the early *Drosophila* embryo, in which an NGF-like ligand, Spätzle, is generated in a ventrally restricted domain within the extra-embryonic perivitelline fluid, then diffuses in a gradient that is interpreted by the transmembrane receptor, Toll, and ultimately results in the formation of a corresponding gradient of a transcription factor, Dorsal, in the nuclei of the syncytial embryo (reviewed by [3]). The active form of Spätzle is generated by proteolytic cleavage of a precursor by the serine protease, Easter [4–8]. Easter is the last enzyme in a serine proteolytic cascade comprising either three or four enzymes [8–10], whose output in the form of activated Spätzle is strongly promoted on the ventral side of the embryo through the indirect action of *pipe*, which specifies the ventral domain during oogenesis [6,11].

Easter appears to be at an important control point in the signaling pathway and plays a major role in shaping the gradient of active Spätzle (reviewed by [3]). In addition to feedback inhibition by the N-terminal fragment of the cleaved Spätzle product that shapes the slope of the Spätzle gradient [12], Easter's proteolytic activity is also inhibited by a globally expressed inhibitor, serpin27A, which irreversibly binds to the active site of Easter [13–16]. Loss of serpin27A results in strong ventralization of the embryo [14,15], due to greater catalytic activity of Easter and diffusion of either active Easter or the cleaved Spätzle ligand throughout the perivitelline fluid. In contrast to other serine proteases in the pathway, there are several dominant gain-of-function alleles of Easter that result in ventralization or lateralization of the embryo. These mutations map to the catalytic domain of Easter [17], and result in impairment of interactions with serpin27A while retaining at least partial catalytic activity [14,18]. This altered regulation results in changes in the timing and/or distribution of the proteolytic activity of these dominant Easter enzymes that appear to be critical in shaping the Dorsal gradient [18].

The role of *pipe* in regulating Easter and the proteolytic cascade has remained unclear. While it was predicted that spatial control would occur at the top of this cascade and possibly at each subsequent step as occurs in human blood clotting [19], instead it was found that Gastrulation Defective (GD), acting two steps upstream of Easter in the cascade, was processed in the absence of *pipe* function to what likely corresponds to an active form in embryo extracts [9]. The importance of this GD processing is suggested by the finding that it did not occur in the absence of Nudel, an upstream protease whose role in the pathway could be either direct or indirect [8,9,20,21]. A role for *pipe* in blocking serpin27A activity on the ventral side of the embryo has been ruled out by genetic experiments showing that in the absence of both activities the embryo is ventralized, as occurs if only serpin27A is absent, rather than dorsalized, as would be expected if *pipe*'s role were to antagonize serpin27A [11,15]. The available evidence is consistent with a role for *pipe* in acting upstream of Easter activation in the cascade, but does not rule out the alternate possibility that the ventral domain generated by *pipe* activity bears a cofactor essential for promoting the interaction of a globally activated Easter with its substrate, Spätzle. To distinguish between these two possibilities, I have directly examined Easter processing in *pipe*-mutant embryos with comparison to wild-type embryos and embryos mutant for known upstream and downstream components in the pathway.

## 2. Materials and methods

### 2.1. Fly stocks and culture

The wild-type strain was Oregon R. Female flies of the following mutant genotypes were generated and crossed to Oregon R males in egg collection cups: $ea^{4}/ea^{5022rx1}$, $snk^{073}/snk^{073}$, $Tl^{9QRX}/Df(3R)ro^{XB3}$,

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*Abbreviation:* GD, Gastrulation Defective

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and *pipe*$^{1}$/*pipe*$^{2}$. For simplicity and by convention, the resulting embryos and their extracts are referred to as *ea*$^{-}$, *snk*$^{-}$, *Tl*$^{-}$ and *pip*$^{-}$, respectively, because they lack critical maternal function in these genes.

*2.2. EA-serpin 27A analysis*

Low-salt extraction of 0–4 h embryos was performed based on the method of Morisato and Anderson [6]. Briefly, dechorionated eggs were homogenized on ice in approximately five volumes of low-salt extraction buffer (20 mM Tris, pH 7.5, 30 mM NaCl, +protease inhibitors), then centrifuged for 5 min at 3000 rpm at 4 °C to remove yolk proteins and debris. Aliquots of the cleared supernatants were transferred to tubes containing an equal volume of Laemmli sample buffer containing 0.1 M DTT + protease inhibitors, immediately frozen on dry ice and stored at −80 °C. Samples were boiled for 5 min then run on 12% SDS–PAGE gels and transferred to nitrocellulose. In all cases, a parallel gel was loaded with an extract made from *ea*$^{-}$ (protein null) ovaries and containing yolk proteins; blotted proteins from this gel were used to preabsorb a 1:1500 dilution of rabbit polyclonal Easter antibody in blocking solution containing 5% condensed milk, 20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton-X 100, in an overnight incubation at 4 °C. This preabsorbed Easter antibody was then transferred directly to the experimental blot that had been stored in blocking solution at 4 °C, and blotting proceeded by standard methods as described [20].

*2.3. mRNA synthesis and injection assay*

The constructs EAS-A and EAR-V,S-A in the S2 cell vector pRMHa-3N [9] were linearized with *Nde*I, which cuts upstream of a T7 promoter sequence engineered in the *easter* 5′ UTR and downstream of the Adh 3′ UTR that is part of the vector sequence [22]. PCR amplification was performed using vector-specific primers including a 3′ primer encoding an artificial 30-base polyA tail downstream of the Adh 3′ UTR to generate PCR-based templates, which in turn were used for T7 mMessage mMachine (Ambion) transcription of 5′-capped, polyadenylated mRNAs. mRNA (1.0 μg/μl in deionized water) was injected into 0.5–1.5 h embryos and the embryos maintained for three additional hours in a humid chamber at 18 °C as described [10,13]. Contents of 18–20 injected, healthy-appearing embryos were harvested using a wide-bore micropipet then transferred to Laemmli sample buffer, flash-frozen for storage, run on 12% gels and blotted with Easter antibody as described above.

## 3. Results and discussion

Misra and colleagues [13] first described Easter processing in vivo by Western blotting of embryo extracts. They found that a processed form corresponding to the 35 kDa Easter catalytic domain could only be detected if the *easter* allele expressed was not capable of catalytic activity, e.g., if the catalytic serine is mutated to alanine (Fig. 1B). Wild-type Easter instead accumulated as an 80 kDa covalent complex with a species they hypothesized to be a serpin, and which was subsequently confirmed to be serpin27A (Fig. 1A; [14,15]). These authors [13] further showed that production of the Easter–serpin27A complex depended on the upstream proteases Nudel, GD, and Snake, but they did not report on whether *pipe* was required, as *pipe* had not yet been defined as a key spatial regulator and spatial control was thought to occur at the top of the protease cascade. The goal of the current study was to apply the methods described by Misra et al. [13] to the specific question of whether *pipe* activity is required for activating cleavage of the Easter zymogen.

Examination of embryo extracts by Western blotting with a rabbit polyclonal anti-Easter antibody reacting with the Easter catalytic domain identified the 80 kDa Easter–serpin27A complex in wild-type extracts, which was absent in *easter*-null abstracts (Fig. 2). The Easter–serpin27A complex could not be

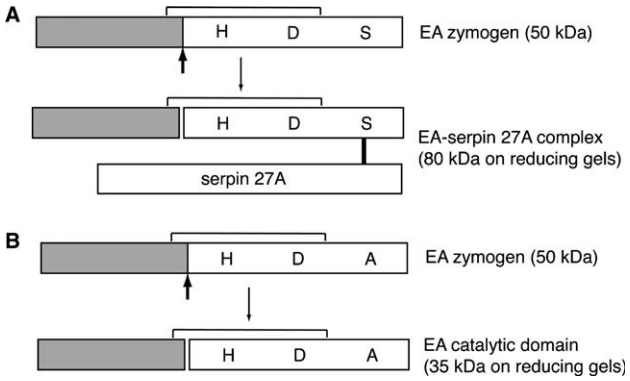


Fig. 1. Diagrams of Easter processing. (A) Processing of the wild-type Easter zymogen at the zymogen cleavage site in embryos results in rapid formation of a covalent complex between the catalytic serine of Easter and serpin27A that is not sensitive to reducing agents, in contrast to the disulfide bond linking the pro-domain (gray) and the catalytic domain (with catalytic triad residues indicated). (B) Processing of an Easter zymogen in which the catalytic serine is mutated to alanine results in production of a stable but inactive catalytic domain fragment, which cannot form a covalent complex with serpin27A. Sizes of fragments recognized by the Easter antibody are indicated at right.

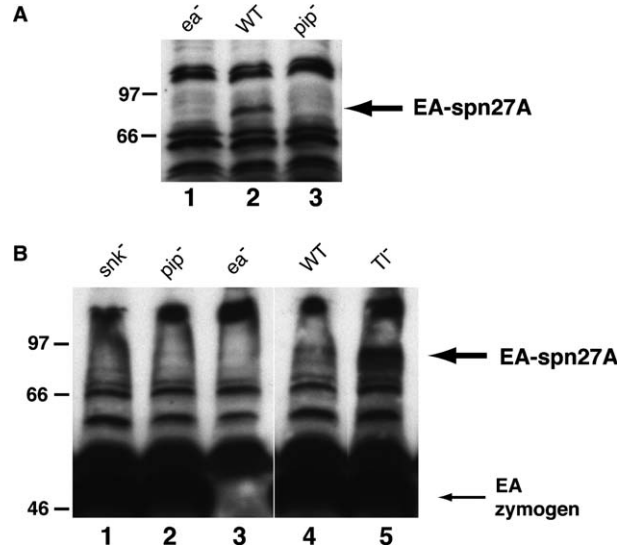


Fig. 2. Easter–serpin27A complex in embryo extracts. Low-salt extracts from wild-type (WT), *ea*$^{4}$/*ea*$^{5022rx1}$ (easter protein null, *ea*$^{-}$), *pipe*$^{1}$/*pipe*$^{2}$ (*pip*$^{-}$), *snk*$^{073}$/*snk*$^{073}$ (*snk*$^{-}$), and *Tl*$^{9QRX}$/*Df(3R)ro*$^{XB3}$ (*Tl*$^{-}$) embryos were separated on SDS–PAGE gels and blotted with preabsorbed Easter antibody. The EA–serpin27A complex was readily detected in WT, though showed variability in its electrophoretic behavior and resolution (cf. panel A, lane 2 and panel B, lane 4), and was significantly increased in *Tl*$^{-}$ extracts (panel B, lane 5). No easter–serpin27A complex could be detected in *pip*$^{-}$, *snk*$^{-}$, and *ea*$^{-}$ extracts.

detected in *snake*$^{-}$ extracts or in multiple independent *pipe*$^{-}$ extracts, but was increased in *Toll*$^{-}$ extracts as previously reported ([13]; Fig. 2). These results strongly suggest that *pipe* is behaving, like *snake*, as an upstream gene required for normal processing of Easter.

For a complementary test of the requirement for *pipe* in Easter processing, independent of the formation of the Easter–serpin27A complex, I examined the processing of Easter containing a catalytic serine mutation (EAS-A). This mutant