Overexpression of the isolated protease domain of the Nudel protein does not affect Dorsal – Ventral polarity determination in *Drosophila*

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SUMMARY

Most of living organisms begin life from one-cell egg. Generally, an egg begins developments after it gets fertilized. From very early stages, however, depending on the locations in an embryo, each embryonic cell undergoes specific patterns of differentiation. Therefore, the determination and recognition of spatial coordination within an embryo are important before any other developmental events can occur. Due to the lack of zygotic genome, many embryos initially use maternal materials to generate the axes of head-to-tail (Anterior-Posterior, AP) and back-to-belly (Dorsal-Ventral, DV). Also, the environmental surroundings, such as the presence of an egg shell structure, can also influence the axes. In *Drosophila*, the existence of follicle cells surrounding the egg during its formation, which provide nutrition and genetic materials to an egg, generates asymmetrical egg shape. With the ability to distinguish specific region in the egg, researchers found the localization of maternal genetic materials at particular sites within the egg, and showed them to be responsible for the determination of the axes. Those maternal genes were identified to work as switches that will turn on and off zygotic genes we need to be expressed at a specific region or at the specific timing during development. Although the individual functions of maternal genes have been studies in artificial conditions, the combinatorial activities of those materials in living cells are still not fully understood. Mutations affecting the Nudel protein, one of the maternal gene products involved in development, produce an abnormal dorsal-ventral polarity in an embryo or an embryo with aberrant eggshell structure, depending on the position of the mutation within the protein. The unusually large size of protein Nudel is composed of several parts showing different functions. The increasing amount of one portion of protein Nudel, which has a function as an enzyme, does not affect the determination of DV axis or development of eggshell structure. Further research should address the functionality of Nudel protease whether it requires direct activities of Nudel protein or it has the correct construct to express the protease.

INTRODUCTION

Since the studies of Tomas Morgan in genetics using *Drosophila malanoganster* during 1900s, the small fruit flies have been widely used as a model organism due to the wealthy genetic information. The early development of *Drosophila* embryo is controlled by DNA deposited in an egg its mother. Several maternal mRNAs, produced in surrounding cells, transferred into an egg and localized at a certain site to specify Anterior/Posterior and Dorsal/Ventral sides of the egg. [1] After the egg gets fertilized, the maternal mRNA encodes proteins, acting as enzymes or transcription factors regulating the expression of zygotic genes, in order to develop AP and DV polarities in the embryo. Therefore, mutations in those maternal genes do not affect the mother, but generate mutant phenotype in the progeny during development. The DV axis of *Drosophila* embryo is determined by a group of maternal effect genes collectively known as the dorsal group. [1, 2, 3] Females mutant for dorsal group genes produce dorsalized embryos, which only have dorsal structures along the DV axis. A key member of the dorsal group protein is Toll, a receptor that is distributed all around the embryonic outer membrane. The molecule binding to Toll receptor is the dorsal group protein Spätzle, which is secreted from the embryos into the

perivitellin space between the embryonic membrane and the inner layer of the eggshell. Spätzle is secreted as an unprocessed form then is cleaved to form the active Toll ligand. The dorsal group genes, *gastrulation defective* (*gd*), *snake* and *easter*, produce proteases which are also secreted as inactive forms into the perivitelline space. Those proteases are responsible for Spätzle activation mechanism through sequential cleavages of one another. Gastrulation Defective cleaves and activates Snake and Snake cleaves and activates Easter. Once activated, Easter cleaves Spätzle into the active form. It is believed that cleavage of Spätzle by Easter only occurs in the perivitelline space on the ventral side of the embryo. Although the mechanism for spatial limitation of Spätzle to the ventral side is unknown, it is believed that the cleaved form of Spätzle binding to Toll receptor initiates the differential development along DV axis. Another dorsal group gene, *nudel*, also encodes a secreted protease. Nudel is a large protein (2616 amino acids), composed of several functionally different portions including a protease in the middle of the protein. Unlike the other proteases, Nudel is expressed by the somatic follicle cells that surround the oocyte during oogenesis, constructing the eggshell and providing yolk to the developing egg. Currently the role of protein Nudel in DV axis formation is unclear. Female files lacking nudel mRNA produced embryos that arrest during early stages of development, therefore no DV patterning can be distinguished. Those embryos have fragile or collapsed eggshells. [4] A similar set of so-called Class I alleles cause the same defects. Another set of mutations, Class II, generate dorsalized embryo, a phenotype of mutations affecting genes involved in Toll activation pathway. [5] The Class II mutations have been shown to have mutations in the Nudel protease domain. The Nudel protease showed enzymatic activity of capable of cleaving GD protease in cultured cells, although it is not clear if this happens in the embryo. [6] This and the requirement for Nudel for the formation of processed GD *in vivo* led to a suggestion that Nudel protease activity plays direct role in DV patterning via cleavage of GD. On the other hand, the existence of the Class I alleles and the fact that Class II alleles have slightly abnormal egg shells show the effect of Nudel protein also acting in the formation of eggshell. This raises the possibility that Nudel acts indirectly in DV patterning by regulating the formation of the eggshell, whose structure might be necessary for GD activation. Thus experiments were designed to distinguish between these two possibilities. If Nudel protease activates GD directly, expression of an activated form of the protease in embryo should cause constitute activation of GD and produce ventralized embryos. However, if Nudel influence on eggshell is necessary for GD activation, expression of Nudel protease should not ventralize embryos. I expressed Nudel protease domain in either germlines or follicle cells but showed that it had no effect. Possible explanations will be discussed.

MATERIALS AND METHODS

Fly Stocks

The wile-type sock was Oregon R. For genetic mapping studies of transgenic insertions, I used the following stocks: w/w (Oregon R), w/w; dl cn sca/CyO, w/w, ru st snk[229] e ca/TM3. Males carrying transgenic insertions were first crossed to the w/w virgin females. If all female progeny were W^+, the transgene was on the X chromosome. If not, males carrying transgene were crossed to w/w;CyO or w/w;TM3 virgin females. Among the progeny, flies carrying both the transgene and balancer crossed to w/w virgin females. The pattern of segregation of the transgene and the balancer allowed me to detect which chromosome the transgene insertion was one. pUASP vector constructs carrying the Nudel protease domain was microinjected into embryos of y w;

Δ2-3/Δ2-3 to generate transgenic flies. (Genetivision) The enhancer trap stocks carring GAL4-3 and GAL4-VP16 inserts were used to induce expression of pUASP vectors in somatic follicle cells and germline cells, respectively.

Construction of pUASP-NDL PD

A plasmid carrying a transgene links the Easter signal peptide to the protease domain of Nudel, beginning at the amino acid 1145 of full-length Nudel, has been discussed previously. [6] Expression of this construct together with a construct expressing GD leads to processing of GD in cultured cells. We obtained this plasmid (A kind figt of E. LeMosy) and used high PCR to generate a NotI/BglII fragment bearing transgene. It was then subcloned into NotI/BamHI cut pUASP, a *Drosophila* germline specific expression vector.

Preparation of Injection Mixture

pUASP-NDL PD was grown up in E.Coli and purified using the QIAwell 8 ultra plasmid kit. (QIAgen) 6μg of plasmid DNA were mixed with 1μg of DNA Δ2-3, encoding the constitutively active transpoase, was co-precipitated (1/10 volume of 3M NaOAc pH7 + 3X volume of 100% Ethanol) and kept at -20˚C for overnight. Following centrifugation, the precipitated DNA was washed in 70% Ethanol, dried, and resuspended in 20μL of H_2O. We then sent it to Genetivision for injection.

Examination of embryonic phenotypes

Adult females of specific genotypes were placed in egg lay blocks and allowed to lay eggs. Gastrulation patterns and embryonic morphology of progeny were examined under hydrocarbon oil.

RESULTS

Mapping of transgenes

y w; Δ2-3 flies injection with pUASP-NDL PD were first croseed to w/w flies and transgenic flies with colored (i.e. W^+) eyes were isolated and recrossed to w/w flies to generates a stock. The segregation patterns outlined in Figure 1 would be seen if the transgene on the X, on the second or on the third chromosome.

1) On X

$$P_0 : \frac{w \quad p[W^+]}{Y};\frac{+}{+};\frac{+}{+}\times\frac{w}{w};\frac{+}{+};\frac{+}{+}$$

$$F1: \frac{w \quad p[W^+]}{w};\frac{+}{+};\frac{+}{+} or \frac{w}{Y};\frac{+}{+};\frac{+}{+}$$

2) On 2nd Chromosome

$$P_0 : \frac{w}{Y};\frac{p[W^+]}{+};\frac{+}{+}\times\frac{w}{w};\frac{+}{CyO};\frac{+}{+}$$

$$F1: \frac{w}{Y};\frac{p[W^+]}{CyO};\frac{+}{+}\times\frac{w}{w};\frac{+}{+};\frac{+}{+}$$

$$F2: \frac{w}{w/Y};\frac{p[W^+]}{+};\frac{+}{+} or \frac{w}{w/Y};\frac{+}{CyO};\frac{+}{+}$$

3) On 3rd Chromosome

$$P_0 : \frac{w}{Y};\frac{+}{+};\frac{p[W^+]}{+} \times \frac{w}{w};\frac{+}{+};\frac{+}{TM3\ Sb}$$

$$F1 : \frac{w}{Y};\frac{+}{+};\frac{p[W^+]}{TM3\ sb} \times \frac{w}{w};\frac{+}{+};\frac{+}{+}$$

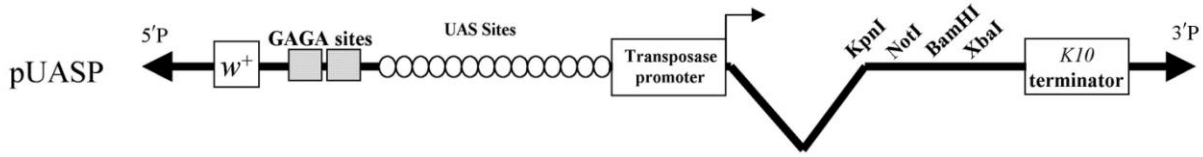
$$F2 : \frac{w}{w/Y};\frac{+}{+};\frac{p[W^+]}{+} or \frac{w}{w/Y};\frac{+}{+};\frac{+}{TM3\ Sb}$$

[Figure 1] Crosses for mapping and balancing P element insertions

Balancer chromosomes carry multiple inversions to inhibit recombination and reassortment of linked markers. They also carry a dominant marker to follow their segregation as well as a recessive lethal maker to inhibit the balancer from becoming homozygous. Phenotypic markers in those chromosomes can be used for finding the P element insertion location in mapping crosses. pUASP vector carries the W^+ (colored eye) marker within it. Transgenic flies with P element insertion on X chromosome would only produce W^+ females and w (white eye) males from the first cross with W^- due to sex linkage. For insertions on 2nd and 3rd chromosomes, balancer chromosomes of CyO (Curly wings) and TM3 (short bristle because of stubble) can be used respectively. For transgenes shown not to be on the X, the F1 generation of transgenic flies, having both the P insertion and balancer marker, would be crossed with w wild type (Oregon R) to examine the segregation of W^+ with respect to the balancer. A chromosome carrying the P element insertion would be expected to segregate among from the balancer from that chromosome. Among twelve transgenic fly lines carrying pUASP-NDL PD, candidate NDL PD6 was shown to have a p element insertion on X chromosome. This insertion was used in subsequent studies.

Overexpression of the Nudel Protease Domain

GAL4/UAS binary system has been widely used for studies in *Drosophila*. GAL4 encodes an activator of transcription from the years *Saccharomyces cerevisiae*. [7] GAL4 initiates the transcription by binding to Upstream Activating Sequences (UAS). In the GAL4/UAS binary system, fusion of the gene of interest with UAS sequences enables its transcription by GAL4 expression in specific tissues. The absence of GAL4 in transgenic flies having only the UAS fusion gene keeps the gene of interest in a silent state. Expression is only seen in tissues expressing GAL4. We used this system to express the Nudel protease domain in germline or somatic cells of the egg and examine its effects. Among several possible GAL4 based expression vectors, we used pUASP because it can be expressed in both germline and somatic cells. (Figure 2) Most UAS-controlling expression vectors can not be expressed in the germline. To express NDL PD in somatic follicle cells, where the original full-length Nudel is normally expressed, GAL4-3 enhancer trap were used. For expression in the germline, a transgenic insertion of GAL4-VP16 under the control of the Nanos promoter (Nanos-GAL4-VP16) was used.



[Figure 2] pUASP element construct

The male progeny having w pUASP-NDL PD6 were crossed with either virgin female of 1) w/w;GAL4-3/TM3, Sb or 2) w/w;ndl093 Nanos-GAL4-VP16/Nanos-GAL4-VP16. (Figure 3)

1)
$$P_0 : \frac{w\ pUASP-NDL\,PD6}{Y};\frac{+}{+};\frac{+}{+}\times\frac{w}{w};\frac{+}{+};\frac{GAL4-3}{TM3\ Sb}$$
$$F1: \frac{w\ pUASP-NDL\,PD6}{w/Y};\frac{+}{+};\frac{GAL4-3}{+}$$

2)
$$P_0 : \frac{w\ pUASP-NDL\,PD6}{Y};\frac{+}{+};\frac{+}{+}\times\frac{w}{w};\frac{+}{+};\frac{Nanos-GAL4-VP16}{Nanos-GAL4-VP16}$$
$$F1: \frac{w\ pUASP-NDL\,PD6}{w/Y};\frac{+}{+};\frac{Nanos-GAL4-VP16}{+}$$

[Figure 3] NDL-PD overexpression using GAL4 enhancer

In the F1 generations, female flies having colored eyes (p element) and long bristle (GAL4) as makers were selected. All of them would have both pUASP-NDL PD and the GAL4 driver. If Nudel protease regulates DV polarity determination through Toll activation pathway by initiating GD cleavage, we would expect overexpression of the NDL PD domain to induce ventralization of embryo, since it would lead to the accumulation of cleaved GD. This would be expected to lead to higher than normal levels of cleaved Snake, Easter, and Spätzle, causing the expansion of Toll activation to produce ventralized embryo. The progeny of both types of F1 females appeared normal and showed no defects in development, suggesting that Nudel protease overexpression in either somatic follicle cells or the germline cells was insufficient to increase the levels of activated GD in the embryo.

Function of Nudel Protease

If the effect of Nudel protein in determination of DV polarity is indirect, acting through the formation of an eggshell, the overexpression of NDL PD would not necessarily produce phenotypic changes, causing ventralization. However, in that case we might expect the expression of the Nudel protease domain to restore normal DV polarity to the progeny of females carrying Class II mutations. To test this, we expressed NDL PD in the germline or follicle cells of females carrying class II mutations. ndl^{10} is an RNA null allele of Nudle. The allele ndl^{046} and ndl^{093} are Class II alleles with mutations in the protease domain. Homozygous virgin females having w pUASP-NDL PD6 were crossed to W^+/Y; ru st e ca ndl^{10} /TM3, Sb. The females carrying w pUASP-NDL PD6/Y; ndl^{10} ru st e ca/+ were collected and crossed to either males of 1) w/w; ndl^{046} e GAL4-3/TM3, Sb or 2) w/w; ndl^{093} e Nanos-GAL4-VP16/TM3, Sb. Progenies in F2 generation having W^+ eyes (pUASP-NDL PD6), ebony body and long bristle (ndl^- ,GAL4

driver containing) were sorted to test function of Nudel protease domain in mutant background. (Figure 4)

$$P_0 : \frac{w\ pUASP-NDL-PD6}{w\ pUASP-NDL-PD6};\frac{+}{+};\frac{+}{+}\times\frac{W^+}{Y};\frac{+}{+};\frac{ru\ st\ e\ ca\ ndl^{10}}{TM3,Sb}$$

$$F1 : \frac{w\ pUASP-NDL-PD6}{Y};\frac{+}{+};\frac{ru\ st\ e\ ca\ ndl^{10}}{+}\times\frac{w}{w};\frac{+}{+};\left(\frac{ndl^{046}\ e\ GAL4-3}{TM3\ Sb}\right) or \left(\frac{ndl^{093}\ e\ Nanos-GAL4-VP16}{TM3\ Sb}\right)$$

$$F2 : \frac{w\ pUASP-NDL-PD6}{w/Y};\frac{+}{+};\frac{ru\ st\ e\ ca\ ndl^{10}}{ndl^{046}\ e\ GAL4-3\ or\ ndl^{093}\ e\ Nanos-GAL4-VP16}$$

[Figure 4] NDL PD expression in Class II mutant background

Neither expression of NDL PD in the germline or the follicle cells was capable of rescuing the dorsalized phenotype of the progeny of ndl^{046}/ ndl^{10} or ndl^{093}/ndl^{10} females. The result suggested either the pUASP-NDL PD construct was not expressing functional Nudel protease or the expression of the free protease domain alone is insufficient to provide full function. The NDL PD construct can have a genetic problem. In other words, it is possible that Nudel protease might require a certain conformation or a specific timing to be realized from the full length protein to provide normal function.

DISCUSSION

The objective of these experiments was to determine whether the Nudel protease domain was sufficient to cause ventralization of embryo which would support a model in which Nudel directly cleave GD. Since the Class II mutants having point mutations within Nudel protease domain produced dorsalized embryo and cleaved GD was not observed in a Nudel mutant background, it was hypothesized that the function of Nudel protease might be the direct cleavage of GD. Alternatively, egg shell defects associated with both Class I and Class II alleles provided the theoretical basis for possibility of the Nudel protein being responsible for DV polarity determination indirectly, through egg shell formation mechanism. The overexpression of NDL PD experiments did not lead to the production of ventralized embryos. Moreover, expression of the NDL PD did not appear to rescue the phenotype of nudel mutants with defects in protease domain. Tests for pUASP-NDL PD constructs should be preceded. To ensure the insufficient function of isolated Nudel proteases domain in embryos, other transgenic insertions other than pUASP-NDL PD6 should be crossed with Class II mutants. We can also PCR the NDL PD from pUASP-NDL PD6 genomic DNA of transgenic flies to make sure the sequence of NDL PD is correct. Antibodies were previously made but are no longer available to see presence of Nudel protease in embryo. We could make our own antibodies and test expression under the control of GAL4-3 and Nanos-GAL4-VP16. If the expression occurs, we can conclude that the expression of free protease domain is insufficient to encode fully functional protease and requires other conditions. Other larger expression constructs of protease domain could be tried to see whether isolated Nudel protease domain was able to provide its normal function. Ultimately, an

understanding of the role of the Nudel protease will provide important insight about the mechanism controlling the establishment of DV patterning in *Drosophila* embryo.

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