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Research Paper

No requirement for localized Nudel protein expression in Drosophila embryonic axis determination

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Abbreviations: DV, dorsal-ventral; *gd*, *gastrulation defective*; *snk*, *snake*; *ea*, *easter; ndl*, *nudel*; EGFR, epidermal growth factor receptor; GAG, glycosaminoglycan; LDL, low density lipoprotein; FLP, yeast site-specific recombinase from the 2 μ circle; FRT, FLP recombination target sequence; GFP, green fluorescent protein; UAS, upstream activator sequence target for Gal4; *dec-1*, *defective chorion 1*; *y*, *yellow*; *w*, *white*; kD, kilodaltons

Key words: Drosophila, dorsoventral, dorsal-ventral, dorsal group, *pipe*, *nudel*, oogenesis, follicle

Drosophila embryonic dorsal-ventral polarity is defined by a maternally encoded signal transduction pathway. Gastrulation Defective, Snake and Easter comprise a serine protease cascade that operates in the perivitelline space to generate active ligand for the Toll receptor, which resides in the embryonic membrane. Toll is activated only on the ventral side of the embryo. Spatial regulation of this pathway is initiated by the ventrally restricted expression of the sulfotransferase Pipe in the follicular epithelium that surrounds the developing oocyte. Pipe is thought to modify a target molecule that is secreted and localized within the ventral region of the egg and future embryo, where it influences the activity of the pathway such that active Toll ligand is produced only ventrally. A potential substrate for Pipe is encoded by *nudel*, which is expressed throughout the follicle cell layer and encodes a large, multi-functional secreted protein that contains a serine protease domain as well as other structural features characteristic of extracellular matrix proteins. A previous mosaic analysis suggested that the protease domain of Nudel is not a target for Pipe activity as its expression is not required in *pipe*-expressing cells, but failed to rule out such a role for other functional domains of the protein. To investigate this possibility, we carried out a mosaic analysis of additional *nudel* alleles, including some that affect the entire protein. Our analysis demonstrated that proteolytically processed segments of Nudel are secreted into the perivitelline space and stably localized, as would be expected for the target of Pipe, However, we found no requirement for *nudel* to be expressed in ventral, *pipe*-expressing follicle cells, thereby eliminating Nudel as an essential substrate of Pipe sulfotransferase activity.

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Introduction

The determination of dorsal-ventral (DV) polarity in the Drosophila embryo requires the function of 11 maternally expressed genes known collectively as "the dorsal group".[1] Females homozygous for loss-of-function alleles of dorsal group genes produce embryos that are dorsalized. In this pathway, a serine protease cascade acting within the perivitelline space surrounding the egg generates a peptide ligand that activates the Toll receptor only on the ventral side of the embryo. Transmission of the Toll activation signal into the cytoplasm of the embryo ultimately results in the transcription of genes whose spatially regulated expression along the DV axis is responsible for the correct generation of pattern and structure along the DV axis of the embryo.

Four of the genes that act upstream of the *Toll* gene, *gastrulation defective* (*gd*), *snake* (*snk*), *easter* (*ea*) and *nudel* (*ndl*), encode secreted serine proteases.[2-5] Nudel is a very large modular protein with features of extracellular matrix proteins as well as a central domain that is homologous to the catalytic domains of trypsin-type serine proteases.[5] *snk* and *ea* encode trypsin-like serine proteases with secretory signal peptides at their N-termini.[3,4] GD exhibits limited structural similarity to two serine proteases of the mammalian complement system, factors C2 and B.[6] The order of action of the members of the dorsal group protease cascade has been determined through genetic and biochemical analyses.[7-10] GD cleaves and activates Snake, which then processes Easter, leading to its activation. Easter then cleaves Spätzle protein, whose processed form corresponds to the active ligand for Toll. While Nudel protease activity is necessary for GD processing in vivo,[9] it is unclear whether Nudel processes GD directly. The Nudel protease domain has also been shown to be required for modification and maturation of the eggshell,[11] which may represent an indirect avenue through which Nudel regulates the processing of GD.

The dorsal group gene *pipe* plays a crucial role in the mechanism that localizes Toll activation to the ventral side of the embryo.[12] *pipe* is specifically transcribed in the ventral follicle cells of the stage 10 egg chamber. The spatial regulation of *pipe* expression is dependent upon

the establishment of DV polarity in the follicle layer, which is under the control of the EGFR/Torpedo signal transduction pathway[13] *pipe* encodes up to ten distinct protein isoforms. One of these, Pipe-ST2, is essential for the establishment of embryonic DV polarity.[14] Furthermore, misexpression of a *pipe-ST2* cDNA in the follicle cell layer is capable of re-orienting the DV axis of progeny embryos.[12] Thus, spatially restricted transcription of the *pipe* gene integrates ovarian and embryonic DV polarity and represents a key factor in the spatial control of the dorsal group serine protease cascade.

All of the protein isoforms expressed by the *pipe* locus exhibit strong amino acid similarity to the mammalian enzymes heparan sulfate 2-0-sulfotransferase and chondroitin/dermatan sulfate 2-0-sulfotransferase.[12,15,16] These are Golgi resident proteins that mediate the transfer of sulfate to the glycosaminoglycan side (GAG) chains of proteoglycans. Despite the similarity between Pipe and the vertebrate GAG-modifying enzymes, however, heparan sulfate, chondroitin sulfate and dermatan sulfate do not participate in DV patterning and therefore do not represent the targets of Pipe enzymatic action.[14] Nevertheless, it is likely that Pipe-ST2 transfers sulfate to an analogous glycosylated molecule that acts to initiate or enhance the activity of the dorsal group serine protease cascade on the ventral side of the embryo.

The product of the dorsal group gene *ndl* represents a potential candidate to be the molecule that carries the carbohydrate moiety that is the substrate of the Pipe-ST2 sulfotransferase. Both the spatial and temporal patterns of Nudel protein expression overlap with those of *pipe*,[5,12] which is an essential requirement for the expression of the substrate of the Pipe sulfotransferase. In contrast to *pipe*, the expression of *ndl* is not ventrally restricted. However, the ability of ectopically expressed *pipe* to ventralize progeny embryos is consistent with a target that is expressed uniformly in the follicle cell layer. We have proposed that *pipe* sulfates a target molecule that is then secreted and localized in the ventral region of the egg, where it influences the activity of the serine protease cascade and/or the activation of the Toll receptor.[12] According to this model, mosaic follicles, in which the target molecule is absent from the ventral *pipe*-expressing follicle cells, would generate progeny embryos with localized DV defects that are correlated to the position of the mutant clone. In earlier work, Nilson and Schüpbach[17] investigated whether follicles containing ventral follicle cell clones mutant for *ndl* would produce embryos with DV defects. Using a marking method that allowed the location of the clone in the follicle cell layer to be identified based on chorion defects in the embryo, Nilson and Schüpbach[17] showed that egg chambers containing large, ventrally located clones homozygous for ndl^{3} produce embryos with normal DV polarity. This result was interpreted to mean that Nudel does not serve as the target of Pipe. ndl^{3} is a missense mutation in the protease domain of the protein that does not affect the expression or secretion of the Nudel protein.[18] In addition to the protease domain, a number of other structural motifs are present in the Nudel amino acid sequence.[5] This includes 11 repeats of a peptide motif found in the Low Density Lipoprotein Receptor (LDL type A repeats),[19] 3 amino acid motifs conforming to the canonical target site for GAG addition,[20] 23 potential sites for N-glycosylation[18] and two serine/threonine-rich stretches of amino acids that might represent target sites for O-linked glycosylation. Nudel protein has also been demonstrated to undergo complex proteolytic processing during oogenesis and embryogenesis.[18,21] The modular structure of Nudel raised the possibility that a functional domain distinct from the protease domain might be acting as the substrate for Pipe.

The finding that *ndl* alleles fall into two discrete maternal effect phenotypic classes[22] supports the possibility that determinants of Nudel other than the protease domain also provide essential functions. Class II alleles lead to the production of embryos with a dorsalized phenotype, while Class I alleles additionally cause early embryonic arrest and/or the production of collapsed eggs. Although most Class I/Class II transheterozygotes produce dorsalized embryos, some heterozygous combinations of Class I with Class II alleles are capable of complementing one another, leading to the production of hatching larvae with normal DV polarity.[18] This interallelic complementation provides strong evidence that different Nudel domains provide distinct and separable functions. The dorsalizing effect of the Class II alleles results from mutations causing amino acid substitutions within the central serine protease domain of the protein. In contrast, none of the Class I alleles have been found to carry mutations within the protease domain. While the large size of the protein has precluded the identification of the specific lesions associated with most of the Class I alleles, analysis of Nudel protein produced by these alleles has demonstrated that they are associated with: (1) Reduced levels or complete absence of the protein, (2) Truncation or deletion of portions of the protein, or (3) Altered processing, secretion or stability of the protein.[18]

The observations outlined above indicate that, in addition to providing a proteolytic function essential for embryonic DV axis formation, the Nudel protein contributes other functions necessary for correct oogenesis and embryogenesis. The previous mosaic analysis reported by Nilson and Schüpbach[17] employed a Class II allele in which only the protease function of Nudel is disrupted. If another domain of Nudel were required to be expressed in ventral follicle cells in order for it to undergo an essential modification mediated by Pipe-ST2, this would not have been detected in those experiments. Since the Nudel protein is secreted and processed into several fragments, it is possible that some of the secreted fragments may be diffusible within the perivitelline space and capable of acting at a distance from their site of synthesis, while others might be limited in their diffusion and act nearby their site of synthesis. According to this model, the protease-containing fragment would be freely diffusible, while a Pipe-ST2-modified fragment would remain restricted nearby its site of synthesis and provide its sulfation-dependent function in that location. This model would require a functional version of the Nudel domain targeted by Pipe-ST2 to be expressed in ventral follicle cells that also express *pipe*. In contrast, follicle cells anywhere along the DV axis of the egg chamber could provide functional Nudel protease activity.

To address this question, we examined the spatial requirement for Nudel expression in the follicle cell layer using mutations that affect non-protease functions of Nudel. These studies support the conclusions of Nilson and Schüpbach[17] that Nudel protein is not required to be specifically expressed in the ventral, *pipe*-expressing follicle cells and thus confirm that Nudel is not the essential sulfated target of Pipe. Interestingly, examination of the distribution of Nudel protein in mutant-bearing follicles indicates that N-terminal and C-terminal determinants of Nudel, presumably present on distinct processed fragments, are localized near their site of synthesis, a

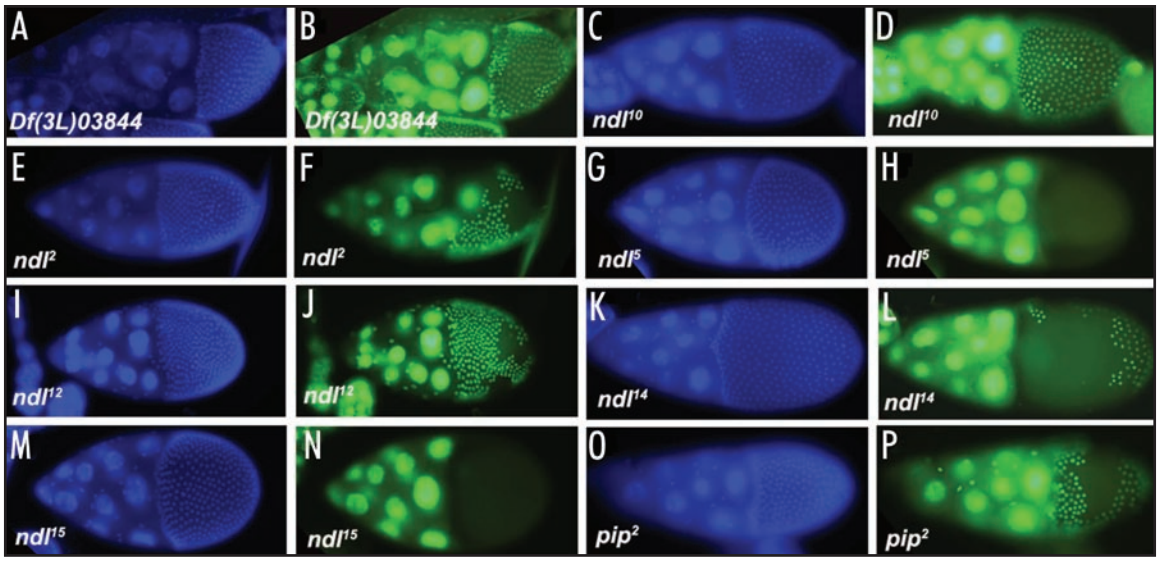

Figure 1. Large clones of cells homozygous for Class I *ndl* alleles can be generated in the follicle cell layer. Follicle cell clones homozygous for the following mutations were generated by e22c-Gal4/UAS-FLP-mediated site-specific recombination in the follicle cell layer: (A and B) *Df(3L)03844*; (C and D) ndl^{10}; (E and F) ndl^{2}; (G and H) ndl^{5}; (I and J) ndl^{12}; (K and L) ndl^{14}; (M and N) ndl^{15}; (O and P) $pipe^{2}$. Staining with DAPI (A, C, E, G, I, K, M and O) shows the position of nurse cell and follicle cell nuclei. The absence of GFP-associated fluorescence from regions of the follicle layer in panels (F, H, J, L, N and P) indicates the position of homozygous mutant cells. Note that mutant follicle clones of cells homozygous for *Df(3L)03844* and ndl^{10} could not be generated, while cells mutant for the class II *ndl* allele, ndl^{2}, for the Class I alleles ndl^{5}, ndl^{12}, ndl^{14} and ndl^{15}, and for the *pipe* allele, pip^{2}, were readily obtained.

property that may contribute to their roles in forming the eggshell or in maintaining its integrity.

Results

Large follicle cell clones homozygous for most class I *ndl* alleles exhibit normal viability. We first examined the consequences of generating mutant clones homozygous for several Class I *nudel* alleles to determine whether a ventral requirement for Nudel could be uncovered when expression or function of the full-length protein was perturbed. We used the following *ndl* Class I alleles in this analysis, each of which was recombined onto an FRT2A(79D)-bearing chromosome to enable the generation of mutant mosaic clones:

Df(3L)03844 carries a deletion of the *ndl* gene as well as at least two nearby genes, *anon-65Ba* and *anon-65Bb*.[5] ndl^{10} is a putative null allele that fails to express detectable levels of mRNA or protein.[5,18,22] Consistent with this, antibodies directed against regions of the Nudel protein fail to detect any expression of protein produced by ndl^{10}.[18] ndl^{14} is a Class I allele carrying a point mutation (G3400A) that introduces a premature stop codon (W1044stop) which results in a protein that is truncated prior to the protease domain.[18] The molecular lesions responsible for the phenotypes associated with the alleles ndl^{5}, ndl^{12} and ndl^{15} have not been identified. The ndl^{5} and ndl^{12} alleles produce proteins that fail to exhibit normal processing and are not secreted into the perivitelline space between the follicle cells and the oocyte.[18] ndl^{15} produces a full-length translation product of apparently normal size that fails to undergo the normal proteolytic processing observed for wild-type protein. Finally, the Class II allele ndl^{2}, used as a control in these studies, carries a missense mutation (H1355L) within the protease catalytic domain of the protein.

No follicle cells homozygously mutant for *Df(3L)03844* or for ndl^{10} were obtained in experiments in which the e22c-Gal4 enhancer trap insertion was used to direct the expression of the FLP recombinase (Fig. 1A and B). The absence of homozygous *Df(3L)03844*|*Df(3L)03844* mutant follicle cells is presumably attributable to a requirement for the expression of either or both of *anon-65Ba* and *anon-65Bb* to support the viability of follicle cells. Our failure to obtain follicle cell clones homozygous for ndl^{10} (Fig. 1C and D) is unlikely to be due to a requirement for Nudel protein for follicle cell viability as ndl^{10}|*Df(3L)03844* adult flies exhibit normal viability despite a complete absence of Nudel protein. A more likely explanation for the inviability of homozygous ndl^{10} mutant cells is that the mutation is associated with a chromosomal aberration, either a deletion or an inversion that also perturbs the expression of another essential gene. Supporting this possibility is the fact that our attempts to separate the ndl^{10} allele from its associated lethality by recombination have been unsuccessful.

In contrast to the results for *Df (3L) 03844* and ndl^{10}, large follicle cell clones homozygous for the FRT2A (79D)-bearing chromosomes carrying the Class II allele ndl^{2}, and the class I alleles ndl^{5}, ndl^{12}, ndl^{14} and ndl^{15} were readily produced via e22c-Gal4-mediated expression of the FLP recombinase (Fig. 1E–N). In some egg chambers all follicle cells appeared to be mutant (Fig. 1H and N). These observations indicate that the various Class I *ndl* alleles did not confer any defect in viability or growth that, when present in the same follicle with wild-type cells, would preclude the examination of potential phenotypes associated with localized mutant clones. Additionally, these observations demonstrated that the recombinant chromosomes carrying the various *ndl* alleles that had been generated did not carry any other independent mutations that when homozygous, would interfere with the production of large clones whose *ndl*-dependent effects could be examined.

Secreted carboxy- and amino-terminal epitopes of the Nudel protein remain localized within the egg chamber perivitelline space nearby their site of synthesis. Following sulfation, the enzymatic

target of Pipe-ST2 is expected to be secreted from follicle cells and become stabilized, either in association with the oocyte membrane, or with the forming vitelline membrane. It is essential that the modified molecule not diffuse far from its site of synthesis, following secretion. This is necessary in order for the polarizing effects of Pipe-ST2 action to be maintained throughout subsequent oogenesis and during early embryogenesis. To test whether Nudel protein exhibits this property, we generated follicles carrying large *ndl* mutant clones, and examined the distribution of the wild-type Nudel protein with respect to the position of the wild-type follicle cells from which the protein had been secreted.

During oogenesis, the 350 kD full-length Nudel protein is first processed to form a 210 kD amino terminal fragment and a 250 kD carboxy-terminal fragment, which contains the protease domain.[18] Later during oogenesis, the 210 kD fragment is further processed to form a 170 kD fragment. During early embryogenesis, the carboxy terminal fragment is processed further, first generating a 38 kD protease domain-containing fragment and a 110–130 kD carboxy terminal fragment. Later during oogenesis the 38 kD fragment is processed to form the mature 33 kD protease fragment and the 110–130 kD fragment is processed to form a 50–60 kD fragment. The processing events outlined above were elucidated using a set of three Nudel-specific antisera, one specific for epitopes in the amino terminal half of the protein (amino acids 548–657), another specific for epitopes in the carboxy terminal half of the protein (amino acids 2518 to 2607), and a third specific for the protease domain (amino acids 1135–1379).[21] We carried out whole mount immunostaining of egg chambers using the antisera targeting the amino and carboxy terminal regions of the protein in order to examine the fates of these regions of the protein following synthesis in the follicle cells.

We examined egg chambers in which e22c-Gal4 coupled with UAS-FLP was used to generate mosaic egg chambers with large clones of follicle cells mutant for the *ndl* alleles ndl^{2}, ndl^{5}, ndl^{12} and ndl^{14}. The ndl^{2} Class II allele is not expected to alter the expression, secretion, processing or stability of Nudel protein during oogenesis. Consistent with this, egg chambers carrying large mutant clones homozygous for ndl^{2}, nevertheless showed a uniform distribution of Nudel protein all around the perivitelline space of stage 10 egg chambers (Fig. 2C and F). The molecular lesions associated with the ndl^{5} and ndl^{12} alleles have not been identified. However, both alleles are reported to lead to a failure in the secretion of Nudel protein.[18] If Nudel protein becomes stably localized following its secretion, then in egg chambers carrying clones of cells mutant for these two alleles, we would expect to observe Nudel protein localized to the perivitelline space adjacent to wild-type cells, while no protein would be observed in the perivitelline space adjacent to the mutant cells. Although this result was observed for both of these alleles, it was especially evident in the case of ndl^{12}, presumably due to instability of the mutant protein retained in the cells (Fig. 2H, I, K and L). Although secreted Nudel protein was evident adjacent to wild-type cells in follicles carrying ndl^{5} mutant cells, assessment of the presence or absence of secreted protein near the mutant cells was obscured by the bright staining of mutant Nudel protein retained within the mutant cells (data not shown). The ndl^{14} allele carries a premature stop codon and is truncated following amino acid 1043. Thus, cells homozygous for ndl^{14} should not express any protein detectable by the carboxy terminus-specific antisera. Consistent with this, in egg chambers carrying follicle cells mutant for ndl^{14}, C-terminal Nudel staining was observed in the perivitelline space only adjacent to wild-type cells (Fig. 2Q and R). Interestingly, the same result was observed using the antisera specific for the amino terminal fragment (Fig. 2N and O). This suggests either that the first 1043 amino acids of Nudel are unstable, and rapidly degraded prior to, or following its secretion or alternatively, that the primary transcript encoding the truncated protein undergoes nonsense-mediated decay.[32] Supporting an involvement of nonsense-mediated decay, in their initial characterization of the *ndl* locus, Hong and Hashimoto[5] designate ndl^{14} as a null allele due to the fact that they were not able to detect any *ndl* mRNA in mutant females.

Taken together, these observations indicate that following secretion, Nudel protein is maintained near the cells from which it is secreted. Presumably, the protein interacts with determinants on either the oocyte surface, the apical surface of the follicle cells, or with components of the forming vitelline membrane, thus restricting its movement. Thus, Nudel protein satisfies one of the requirements of the putative target of Pipe-ST2. It has the capacity to stably maintain the localization, within the egg, of any modification added to it during expression in the follicle cell layer.

Expression of functional Nudel protein in ventral follicle cells is not required for the production of embryos with normal dorsal-ventral polarity. Pipe-ST2 is localized to the Golgi apparatus of a ventral subset of follicle cells in the stage 9/10 egg chamber. As described above, because its presumed glycoprotein target must come into contact with Pipe-ST2 in that same compartment, the gene encoding the target of Pipe must also be expressed in the ventral follicle cells. It follows, therefore, that if a motif present in the Nudel protein represents a required target of Pipe, then the *pipe*-expressing cells must also express a functional form of that Nudel domain for normal embryonic DV polarity to result. Correspondingly, if the *pipe*-expressing cells instead express a mutant form of that Nudel domain, those follicles would be expected to yield embryos that exhibit perturbations in DV pattern formation. To test this possibility we generated egg chambers containing clones of follicle cells mutant for the Class I *ndl* alleles, ndl^{5}, ndl^{12} and ndl^{14}. In order to unambiguously determine whether or not the presence of ventral mutant clones affecting Nudel protein led to alteration of the DV phenotype, we used the marking system of Nilson and Schüpbach.[17] In this approach follicle cells homozygous for mutant alleles of the gene of interest (in this case *ndl*), also fail to express a wild-type copy of the *dec-1* gene, leading to a detectable alteration in the structure of the chorion produced by these follicle cells, thus enabling the position of the mutant clone in the follicular epithelium to be determined. Chorion produced by *dec-1* mutant cells appears transparent compared to chorion produced by wild-type cells.

Using the strategy outlined above, we identified eggs derived from egg chambers with large ventral clones of follicle cells lacking *dec-1* activity, and therefore mutant for *ndl*, for all alleles tested. We did not observe an association between the presence of a ventral mutant clone and embryonic dorsalization for any of the three Class I *ndl* alleles tested (Fig. 3E–G) (similar results have been obtained by Dr. L. Nilson, in mosaic studies of the ndl^{14} allele, personal communication). As a negative control, we also generated eggs from *dec-1* marked clones mutant for the Class II allele ndl^{2}. As previously demonstrated using the Class II allele ndl^{3},[17] ventrally positioned follicle cell clones

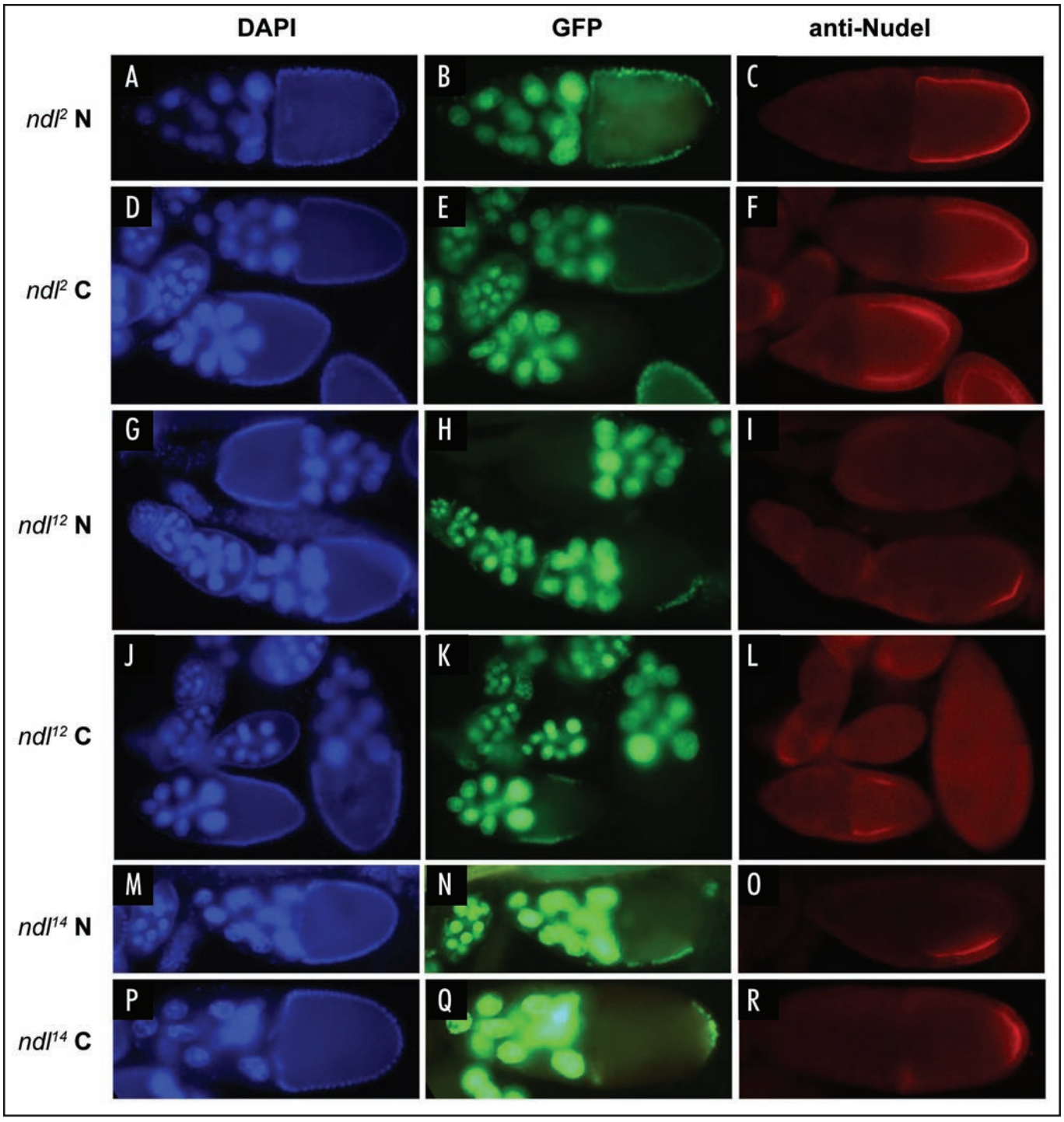

Figure 2. N- and C-terminal segments of the Nudel protein maintain their localization in the perivitelline space following secretion from follicle cells. e22c-Gal4/UAS-FLP-mediated site-specific recombination was used to generate follicle cell clones homozygous for the *ndl* alleles ndl^{2} (A–F), ndl^{12} (G–L), and ndl^{14} (M-R). Clone-bearing follicles were visualized with DAPI to illuminate nuclei (A, D, G, J, M and P), with GFP to identify wild-type, fluorescent follicle cells (B, E, H, K, N and Q), and with antibodies specific for determinants within the N-terminal (C, I and O), and C-terminal (F, L and R) regions of Nudel protein. Nudel protein, detected by both antibodies was present throughout the perivitelline space of egg chambers containing ndl^{2} mutant clones (C and F), consistent with the fact that the ndl^{2} allele is a missense mutations that does not affect the level of Nudel protein produced. In contrast, in the cases of follicles carrying cells mutant for ndl^{12} and ndl^{14}, which lead to dramatic decreases in the amount of Nudel protein produced and secreted, epitopes detected using both the N- and C-terminal specific antisera were only detected in the perivitelline space adjacent to the position of wild-type cells (Compare panels H and I, K and L, N and O, and Q and R), indicating that Nudel protein does not diffuse away from its site of secretion within the perivitelline space. (N and C) refer to stainings using antibodies specific to the amino- and carboxy-terminal fragments of Nudel.

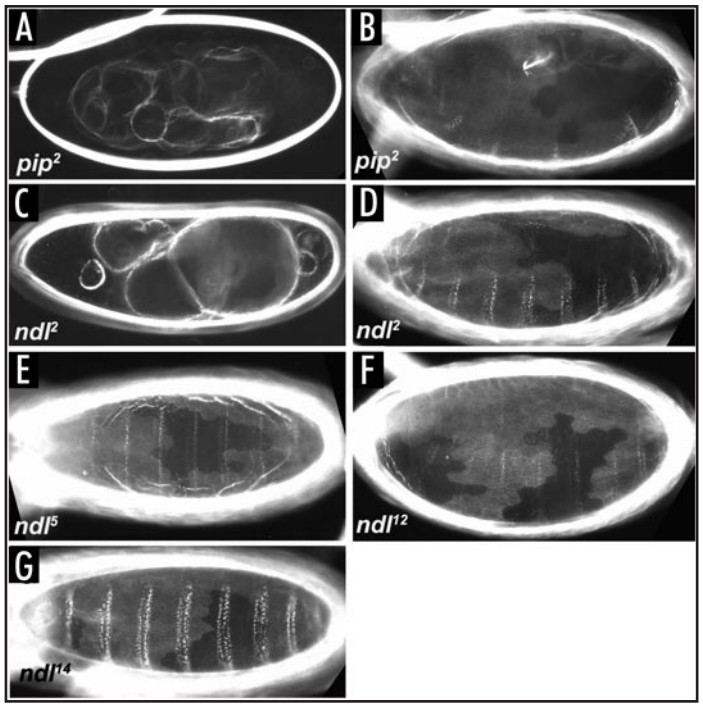

Figure 3. Expression of Nudel protein in ventral follicle cells is not necessary for the establishment of dorsal/ventral polarity in progeny embryos. (A and C) Completely dorsalized (D0) embryo produced by e22c-Gal4/UAS-FLP-mediated generation of *pip*^{2} (A) and *ndl*^{2} (C) homozygous mutant cells, respectively, in the follicle cell layers of the egg chambers that generated these eggs. In (B and D–G), the technique of Nilson and Schüpbach (1998) was used to generate marked mutant follicle cells whose position in the follicle layer could be determined by the transparent appearance of the chorion imprints that they left on the egg, in comparison to the opaque appearance of the chorion imprints made by wild-type cells. (B) A ventral clone of *pip*^{2} homozygous mutant cells led to the production of a partially dorsalized embryo as indicated by the tail-up phenotype and narrowed ventral denticle bands. In contrast, ventral follicle cells homozygous for the class II *ndl* allele, *ndl*^{2} (C and D), and for the Class I *ndl* alleles, *ndl*^{5}, *ndl*^{12} and *ndl*^{14} (E–G) did not perturb DV patterning in the progeny embryos. For each of the mutations tested, the number of dorsalized embryos/total number of eggs exhibiting a ventral *dec-1* clone are as follows: *pipe*^{2} (95/169), *ndl*^{2} (0/51), *ndl*^{5} (0/76), *ndl*^{12} (1/71), *ndl*^{14} (3/58). The small number of embryos scored as dorsalized in the mosaic analyses of *ndl*^{12} and *ndl*^{14} included three embryos exhibiting a tail-up phenotype, a defect arising from a perturbation in gastrulation that is occasionally seen in embryo collections independent of alterations in DV patterning, and one embryo from an egg carrying a very large mutant clone that likely resulted in insufficient Nudel protease activity to support DV axis formation.

mutant for *ndl*^{2} did not perturb DV polarity in progeny embryos (Fig. 3D). In contrast, when we generated *dec-1* mutant clones that were also homozygous for the mutant *pipe* allele, *pipe*^{2}, we did observe perturbations in the DV of most of the embryos derived from egg chambers carrying a ventral clone of mutant follicle cells (Fig. 3B). Dorsalized phenotypes observed included a narrowing of the ventral denticle bands and the display of a "tail-up" phenotype.

In principle, we might have expected to see the production of dorsalized embryos from egg chambers in which most of the follicle cells were homozygous for the mutant *ndl* alleles being tested, due to lack of sufficient quantities of the Nudel protease required for embryonic DV patterning. Indeed, when *e22c-Gal4* was used to drive the expression of GFP minus follicle cells clones as described previously, we did observe the production of dorsalized embryos (Fig. 3C), although in those experiments it was not possible to correlate the size or positioning of the clones within the follicle cell layer. However, we did not observe strongly dorsalized embryos in the experiments employing the *dec-1* marker. Dec-1 protein is necessary for the production of eggshells with normal integrity. It is likely, therefore, that eggs derived from follicles carrying *ndl* clones large enough to significantly decrease the levels of Nudel protease activity were either not fertilized or failed to develop due to the concomitant absence of *dec-1*. However, this caveat does not preclude us from concluding that expression of Nudel protein, and of determinants distinct from the protease domain, is not required in ventral follicle cells in which Pipe is also expressed. Thus, Nudel does not represent an essential enzymatic target of Pipe-ST2-mediated sulfation in ventral follicle cells.

Discussion

The structure, tissue-specific expression, and subcellular localization of the Nudel protein have made Nudel an intriguing candidate to be the sulfated target of Pipe-ST2. The observations reported here unambiguously confirm and extend the conclusions of Nilson and Schüpbach,[17] showing that no determinants within the full-length Nudel protein are required to be expressed in ventral follicle cells of the egg chamber for normal DV polarity to be established in progeny embryos. As such, Nudel cannot represent a unique effector whose modification by the Pipe-ST2 sulfotransferase during oogenesis acts to polarize the embryonic DV axis. These results do not absolutely rule out Nudel as a target of Pipe-ST2-mediated sulfation. The results that we have obtained would also be consistent with a situation in which Nudel acts redundantly with one or more other molecules that are all modified by Pipe and capable of providing the spatial cue that polarizes the dorsal group serine protease cascade. In that situation, expression of Nudel in ventral follicle cells would not be essential as one or more other molecules sulfated by Pipe in ventral follicle cells would be capable of substituting for Nudel. While the most straightforward interpretation of our results is that Nudel is not the target of Pipe, the identification and characterization of the bona fide target or target(s) of Pipe-ST2 will settle this question definitively.

The findings reported here demonstrate that the non-protease domains of Nudel do not influence the signaling pathway that establishes embryonic DV polarity. However, mutations affecting these domains do cause perturbations in egg formation and embryogenesis that occur prior to the time at which signaling of DV polarity to the embryo occurs. Embryos produced by females homozygous or transheterozygous for Class I alleles arrest very early in development,[22] with few embryos progressing to the stage at which DV defects can be observed. Many of the eggs are collapsed and appear unfertilized. Immunohistochemical studies have revealed the presence of Nudel protein in a layer between the vitelline membrane and the oocyte surface.[21] Correspondingly, Nudel protein can be isolated through a biochemical purification protocol that enriches for extracellular matrix components of the ovary, including components of the developing eggshell.[33] Thus, it appears that non-protease domains of the Nudel protein are required for the proper assembly of the vitelline membrane or that they represent structural components of the vitelline membrane. We have carried out an extensive complementation analysis in which all available Class I *ndl* alleles were

crossed individually to each other and all allelic combinations exhibited defects in eggshell integrity or early embryonic development (Balakrishnan D and Stein D, unpublished). Thus, there do not seem to be separable determinants present in the Nudel protein that play independent roles in these processes. Rather, it is likely that multiple Nudel domains provide an integrated role in eggshell construction and subsequent embryogenesis.

Interestingly, although a spatially localized distribution of Nudel along the DV circumference of the egg is not important for its function, we have shown that portions of the protein nevertheless exhibit stable localization in the egg chamber perivitelline space following their secretion and processing. A similar ability to be localized would be expected of the bona fide target(s) of Pipe-ST2. The Torsolike protein, which is involved in the maternal control of terminal patterning in the Drosophila embryo, is expressed in terminal follicle cells.[34,35] Following its secretion, Torsolike is maintained stably at the anterior and posterior ends of the developing oocyte,[36] and is localized within the egg as a component of the vitelline membrane.[37] The determinants within the Torsolike protein that accomplish its stable localization have not yet been identified. However *tsl*^{146}, which converts tyrosine 279 to an asparagine residue, produces a protein that fails to remain localized to the poles of the egg (Stevens L, unpublished). Two other members of the maternal terminal class of genes, *fs(1)polehole* and *fs(1)Nasrat* are required for the maintenance of Torsolike protein at the ends of the oocyte and later, the eggshell.[36,37] Like Nudel protein, the products of *fs(1)polehole* and *fs(1)Nasrat* exhibit structural features of extracellular matrix proteins, and strong loss-of-function alleles of both genes lead to the production of collapsed eggs and to a failure in the cross-linking of vitelline membrane proteins.[36,38] It is not yet known whether the products of *fs(1)polehole* and *fs(1)Nasrat* play direct roles in the maintenance of Torsolike protein or whether their roles in maintaining eggshell structural integrity have an indirect effect on Torsolike localization.

The role of Nudel in DV patterning may be analogous to the roles of Fs(1)polehole and Fs(1)Nasrat in patterning the embryonic termini. As noted above, embryos from egg chambers carrying very large *ndl* clones nevertheless produce embryos with normal polarity, so the target of Pipe-ST2 must be capable of maintaining its localization even in regions of the egg chamber where no Nudel is present in the perivitelline space. Thus, Nudel cannot be acting directly to bind to and maintain localization of the modified target of Pipe. Nevertheless, it remains possible that Nudel's function in the generation of an eggshell with normal integrity may indirectly play a role in maintaining the localization of the bona fide target of Pipe-ST2. The fact that embryos with correct DV polarity and eggshells with apparently normal structural integrity can be produced when relatively large patches of *ndl* mutant follicle cells are present in the follicular epithelium indicates that the roles played by Nudel in eggshell structure and embryonic development are not required uniformly around the developing oocyte. Unfortunately, we have not been able to determine the maximum number of *ndl* mutant follicle cells that can be present in an egg chamber and still allow normal egg development and embryogenesis as the *dec-1* marker itself produces nonviable eggs when large clones are generated.

The studies of *ndl* that have been carried out to date have not illuminated the precise role of the protease domain in the process of embryonic DV patterning. Epistasis analysis indicates that Nudel protease function acts upstream or in parallel with the function of GD[39] and it has been proposed that the Nudel protease acts to process and activate GD protein directly. Consistent with this view, a truncated form of Nudel consisting of the protease domain alone is capable of processing GD when the two proteins are co-expressed in cultured cells.[9] However, activated forms of the Snake and Easter protease, as well as an activated form of the GD protease, are also capable of processing GD in the same assay.[9] These results indicate that the requirements for GD cleavage in co-expression assays are relatively broad, and cast doubt on whether the results from these experiments accurately reflect the situation in vivo. A more convincing demonstration of a requirement for Nudel protease activity in the processing of GD is the observation that the processed form of GD detected in wild-type eggs is not observed in eggs from females carrying protease-defective forms of Nudel.[9] Moreover, while it is relatively clear that the Nudel protease function is essential for the processing of GD in embryos, it is unclear whether the Nudel protease itself is directly involved in the cleavage of GD. An alternative model is that Nudel protease participates in a processing event involved in the formation or maturation of the eggshell, with the correct integrity of the eggshell being a prerequisite for the processing or activation of GD. Consistent with this view, females carrying Class II mutant *ndl* alleles produce eggshells displaying minor defects in eggshell integrity. The vitelline membranes of these eggs are soft, unusually permeable to the dye neutral red, and fail to undergo the covalent cross-linking of vitelline membrane component proteins observed in wild-type eggs.[11] Ultimately, further study should illuminate the precise role of this enigmatic and essential protein in the formation of the eggshell and the establishment of embryonic DV polarity in Drosophila.

Materials and Methods

Stocks. All stocks were maintained and crosses carried out employing standard conditions and procedures. The wild-type stock used was Oregon R. Mutant alleles have been described as follows: *pipe*^{1} (FBal0013836) and *pipe*^{2} (FBal0013837)(formerly *pipe*^{386} and *pipe*^{664}) are described in Anderson et al.,[23] *Df(3L)03844* (FBab0024404), which deletes *ndl* as well as two nearby genes, *anon-65Ba* and *anon-65Bb*, is from Hong and Hashimoto.[5] *ndl*^{10} (FBal0051337), *ndl*^{12} (FBal0051335), *ndl*^{14} (FBal0051333) and *ndl*^{15} (FBal0051332) are described in Hong and Hashimoto.[22] *ndl*^{2} (FBal0012922) and *ndl*^{5} (FBal0012925)(formerly designated *ndl*^{093} and *ndl*^{169}) are from Tearle and Nüsslein-Volhard.[24] The two stocks with the genotypes *y*^{1} (FBal0018607) *w dec-1*^{VA28} (FBal0089355) P{dec-1^{+tWa}}3L (FBti0058762) P{FRT(w^{hs})}2A (FBti0002046) and *y*^{1} *w dec-1*^{VA28} P[hsFLP, *ry*$^{+}$] (FBti0002044)/FM6 (FBba0000003); *ru*^{1}(FBal0014832) *h*^{1}(FBal0005318) *th*^{1}(FBal0016769) *st*^{1}(FBal0016127) *cu*^{1}(FBal0002131) *sr*^{1}(FBal0016068) *e*^{s} (FBal0003292) *Pr*^{1}(FBal0013944) *ca*^{1}(FBal0001461)/TM3 (FBba0000047), *Ser*^{1} (FBal0015427) are described in Nilson and Schüpbach.[17]

The stock with the genotype *y*^{1} *w* ; P{en2.4-GAL4}e22c (FBti0002970) P{UAS-FLP1.D}JD1 (FBti0012284)/CyO (FBba0000025); *w*; P{His2Av$^{T:Avic/GFP-S65T}$}62A (FBti0017565) P{FRT(w^{hs})}2A/TM3, *Sb*^{1} (FBal0015145) is from Zhu et al.[25]

Generation of *ndl* mutant follicle cell clones and immunohistochemistry. In order to test whether any of the *ndl* mutant

lesions influence follicle cell viability, clones of mutant follicle cells homozygous for various *ndl* alleles were generated by FLP/FRT-mediated site-specific recombination.[26] Mosaic females carried a *ndl* mutation and FRT2A(79D) element in trans to a chromosome carrying FRT2A(79D) together with a transgene expressing the constitutively expressed *D. melanogaster* variant H2A.F/Z class histone fused to Green Fluorescent Protein (hGFP).[27] To achieve a high frequency of homozygous clones in the follicle cell layer, these females also carried the somatically expressed *e22c-Gal4* enhancer trap insertion[26] together with a UAS-FLP transgene on the second chromosome.[26] Following dissection of ovaries, homozygous mutant follicle cells could be identified by the absence of GFP-associated fluorescence.

In some of these experiments the distribution of wild-type Nudel protein was examined in egg chambers carrying homozygous mutant follicle cell clones. Whole mount immunostaining of fixed egg chambers was carried out as described in Maines et al.,[28] using primary antibodies generated in rabbits that had been targeted against either the amino terminus (amino acids 548–657) or carboxy terminus (amino acids 2518–2607) of Nudel.[21] Primary antibodies were used at a dilution of 1/1000 in conjunction with secondary antibodies (Alexa Fluor 594-coupled goat anti-rabbit antibodies [Molecular Probes/Invitrogen]) used at a dilution of 1/500.

In order to test for a localized requirement for the expression of the *ndl* gene in ventral cells of the follicular epithelium, FLP/FRT-mediated site-specific recombination was again employed to generate follicle cells homozygously mutant for various *ndl* alleles. In this instance, the clones were marked using the system of Nilson and Schüpbach.[17] In this strategy, follicle cells mutant for *ndl*, are also homozygous mutant for the gene encoding *dec-1*, a protein involved in chorion formation.[29] An alteration in the appearance of follicle cell imprints on the chorion enables the determination of the position within the follicle cell layer at which the *ndl* mutant clones have been generated. In these experiments, production of FLP protein was induced in response to heat-shock using the *hsFLP1* insertion,[30] which places FLP under *hsp70* promoter transcriptional control. Virgin adult females of the genotype:

y w dec-1^{VA28}/ y w dec-1^{VA28} P[hsFLP, *ry*$^{+}$]; *ndl* P{FRT(w^{hs})}2A/ P{dec-1^{+tWa}}3L P{FRT(w^{hs})}2A

were subjected to two rounds of heat shock of 1.5-hour duration, separated by several hours. Heat shocked females were placed together with OregonR strain wild-type males in yeasted food vials. After four days, the flies were placed in egg collection containers and collections of eggs were made on days 5 and 6 after heat shock. Collected embryos were allowed to develop for one day, at which time cuticle preparations were made using the protocol of van der Meer,[31] with the exception that the eggs were not de-chorionated prior to mounting in Hoyer's/Lactic acid. For each genotype tested, several thousand eggs were examined to identify ones that contained an embryo that had produced cuticle, allowing its DV phenotype to be assessed. Those eggs were then examined for the presence of a patch of *dec-1*-derived chorionic imprints that crossed the ventral midline, indicating the presence of a ventral mutant clone in the follicle cell layer of the egg chamber in which the egg had developed. Embryos exhibiting either a tail-up or U-shaped phenotype, a twist in the body or a narrowing of ventral denticle bands were scored as being dorsalized.

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