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Drosophila Pelle phosphorylates Dichaete protein and influences its subcellular distribution in developing oocytes

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ABSTRACT The *Drosophila Dichaete* gene encodes a member of the Sox family of high mobility group (HMG) domain proteins that have crucial gene regulatory functions in diverse developmental processes. The subcellular localization and transcriptional regulatory activities of Sox proteins can be regulated by several post-translational modifications. To identify genes that functionally interact with *Dichaete*, we undertook a genetic modifier screen based on a *Dichaete* gain-of-function phenotype in the adult eye. Mutations in several genes, including *decapentaplegic*, *engrailed* and *pelle*, behaved as dominant modifiers of this eye phenotype. Further analysis of *pelle* mutants revealed that loss of *pelle* function results in alterations in the distinctive cytoplasmic distribution of Dichaete protein within the developing oocyte, as well as defects in the elaboration of individual egg chambers. The death domain-containing region of the Pelle protein kinase was found to associate with both Dichaete and mouse Sox2 proteins, and Pelle can phosphorylate Dichaete protein *in vitro*. Overall, these findings reveal that maternal functions of *pelle* are essential for proper localization of Dichaete protein in the oocyte and normal egg chamber formation. Dichaete appears to be a novel phosphorylation substrate for Pelle and may function in a Pelle-dependent signaling pathway during oogenesis.

KEY WORDS: *Dichaete, Pelle, oogenesis, phosphorylation*

Introduction

Sox HMG domain proteins act as transcription factors and chromatin architectural proteins and have crucial gene regulatory functions in many developmental processes (reviewed in Guth and Wegner, 2008; Kiefer, 2007). There exist over 20 mammalian Sox genes and several are associated with several human genetic disorders, including sex reversal, campomelic dysplasia, and Waardenburg-Hirschprung disease (reviewed in Chew and Gallo, 2009) and cancer (reviewed in Dong *et al.* 2004). Thus, illuminating the activities of Sox proteins and how they are regulated is of great significance. Interestingly, several Sox genes act early in cell differentiation pathways and many are highly expressed in progenitor or precursor cell types (Chew and Gallo, 2009); Sox2 has critical functions in regulating pluripotency (reviewed in Zhao and Dailey, 2008). Invertebrate Sox genes also mediate important developmental functions (reviewed in Phochanukul and Russell 2009) and the *Drosophila* Dichaete Sox gene is essential for embryonic segmentation and nervous system formation (Nambu and Nambu, 1996; Russell *et al.* 1996). Dichaete exhibits strong sequence similarity to Sox2 and has similar DNA binding and bending activities (Ma *et al.* 1998). Consistent with functions as a regulator of gene transcription, in most cells Dichaete protein exhibits nuclear localization.

Dichaete also has maternal functions that are important for normal oogenesis and Dichaete protein is expressed transiently and specifically in the developing oocyte (Mukherjee *et al.* 2006). Interestingly, Dichaete protein exhibits strictly cytoplasmic distri-

Abbreviations used in this paper: HMG, high mobility group; NLS, nuclear localization signal.

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bution within the oocyte cytoplasm; it is localized in a crescent at the posterior edge of the oocyte nucleus from region II of the germarium through stage 6 oocytes. Dichaete mutant egg chambers exhibit dorsal/ventral pattern disruptions and Dichaete was shown to have sequence specific RNA-binding properties. Dichaete binds to the 3'-UTR of *gurken* mRNA and influences distribution of *gurken* mRNA and protein in the oocyte. Establishment of the anterior/posterior and dorsal/ventral axes during *Drosophila* oogenesis relies on highly regulated and asymmetric localization of specific mRNAs and proteins (reviewed in Kugler and Lasko 2009). Thus the unique distribution pattern of Dichaete suggests that specific signaling processes may regulate Dichaete localization in the oocyte. Significantly, the mechanisms that underlie this unique localization in the oocyte have not yet been established

In a genetic modifier screen to identify genes that influence Dichaete function, we identified several cell signaling pathway genes, including *decapentaplegic*, *engrailed*, and *pelle*. Mutations in each of these genes exhibited dominant modification of a gain-of-function Dichaete adult eye phenotype. We focused further attention on *pelle*, which encodes a conserved serine/threonine protein kinase that also contains a death domain and acts as a downstream transducer in the Toll signaling pathway (Shelton and Wasserman, 1993). Significantly, the Pelle death domain mediates direct association with other proteins, such as Tube (Xiao *et al.* 1999), and Pelle kinase activity influences the stability and subcellular localization of members of the Dorsal/Nf-kB transcription factor family (Shen and Manley, 1998; Towb *et al.* 1998; Edwards *et al.* 1997). In addition to it's critical maternal functions in dorsal/ventral patterning of the embryo Pelle also has important zygotic functions in hematopoesis, muscle development, axon guidance and innate immune response (Mindorff *et al.* 2007; Halfon and Keshishian, 1998; Qiu *et al.* 1998; Lemaitre *et al.* 1996). Identifying additional Pelle interacting or substrate proteins is thus relevant for a wide array of developmental and physiological processes. In this study, we characterize functional interactions between Pelle and Dichaete. We show that maternal Pelle functions are required for the normal distribution of Dichaete protein in the developing oocyte, and that *pelle* mutant ovaries exhibit defects in the elaboration of individual egg chambers. The death domain-containing region of Pelle strongly associates with Dichaete as well as the mouse Sox2 protein. In addition, *in vitro* kinase assays indicated that Pelle can phosphorylate Dichaete. Taken together, our results suggest that phosphorylation of Dichaete by Pelle may be important for normal oogenesis.

Results

A genetic modifier screen identifies enhancers of Dichaete *function*

A genetic modifier screen was performed to identify genes that influence *Dichaete* function. P[GMR-*gal4*] was used to target ectopic expression of *Dichaete* in the developing eye imaginal disc via P[UAS-*Dichaete*]. Flies bearing these two P elements exhibit a dosage-sensitive loss and disorganization of ommatidia and mechanosensory bristles in the adult eye (Mukherjee *et al.* 2000). P[GMR-*gal4*]/CyO; P[UAS-*Dichaete*]/TM3 flies were crossed to a series of 169 autosomal deficiencies that span most of the second and third chromosomes and the progeny were scored for alterations in the adult eye phenotype. The screen identified 11 regions on the second chromosome (21D1-2;22B2-

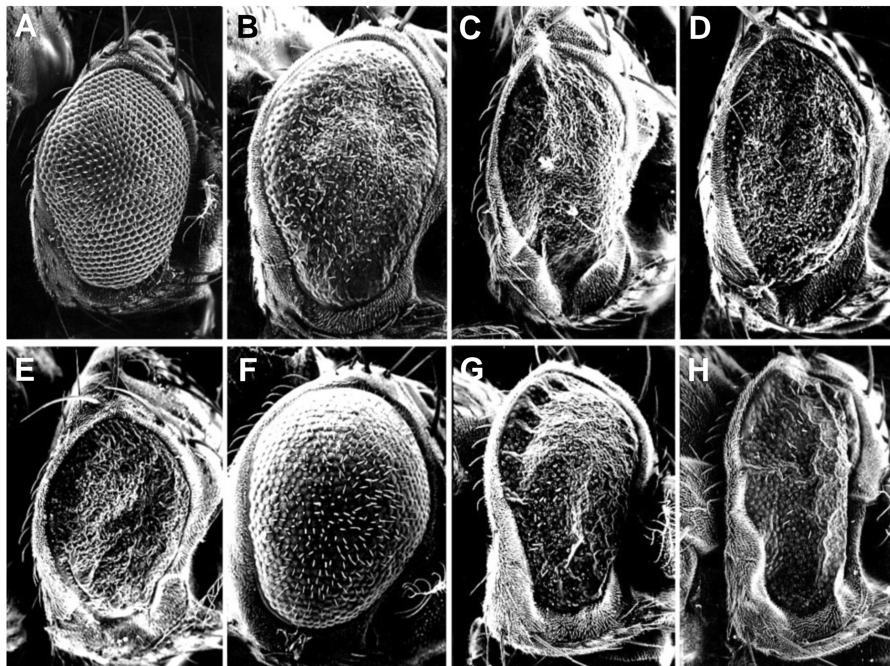

Fig. 1. Dominant modification of a *Dichaete* gain-of-function eye phenotype by chromosomal deficiencies and specific gene mutations. (A) *The head capsule of a P[GMR-*gal4*]/+ fly. Note the ordered array of ommatidia and mechanosensory bristles in the compound eye.* **(B)** *The head capsule of a P[GMR-*gal4*]/+; P[UAS-*Dichaete*]/+ fly. Note the loss and disorganization of the ommatidia and mechanosensory bristles.* **(C)** *Heterozygosity for Df(2L)C144 (23A1-2; 23C3-5) enhances theÄ*Dichaete *gain-of-function eye phenotype. Note the more reduced and collapsed eye with severe loss of mechanosensory bristles in a P[GMR-*gal4*]/Df(2L)C144; P[UAS-*Dichaete*]/+ fly.* **(D)** *Heterozygosity for Df(2R)*en*-A (47D3; 48B2-5) which removes* engrailed, *enhances the* Dichaete *gain-of-function eye phenotype. Note the more severe loss of mechanosensory bristles in a P[GMR-*gal4*]/Df(2R)*en*-A; P[UAS-*Dichaete*]/+ fly.* **(E)** *Heterozygosity for Df(2L)H20 (36A8-9; 36E1-2) enhances the* Dichaete *gain-of-function eye phenotype. Note the more reduced size of the eye and greater disruption of ommatidial organization in a P[GMR-*gal4*]/Df(2L)H20; P[UAS-*Dichaete*]/+ fly.* **(F)** *Heterozygosity for Df(3R)P14 (90C2-D1; 91A1-2) which removes* glass, *suppresses the* Dichaete *gain-of-function eye phenotype. Note the rescue of eye size and ommatidial organization in a P[GMR-*gal4*]/+; P[UAS-*Dichaete*]/Df(3R)P14 fly.* **(G)** *Heterozygosity for Df(3R)R38.3 (97E3-11; 98A), which removes* pelle, *enhances the* Dichaete *gain-of-function eye phenotype. Note the collapsed eye with severe loss of mechanosensory bristles in a P[GMR-*gal4*]/+; P[UAS-*Dichaete*]/Df(3R)R38.3 fly.* **(H)** *Heterozygosity for Df(3R)IR16 (97F1-2; 98A), which removes* pelle, *enhances the* Dichaete *gain-of-function eye phenotype. Note the more reduced and collapsed eye with severe loss in mechanosensory bristles in a P[GMR-*gal4*]/+; P[UAS-*Dichaete*]/Df(3R)IR16 fly.* **(A-H)** *Scanning electron micrographs of 2-3 day old adult flies at 200X magnification.*

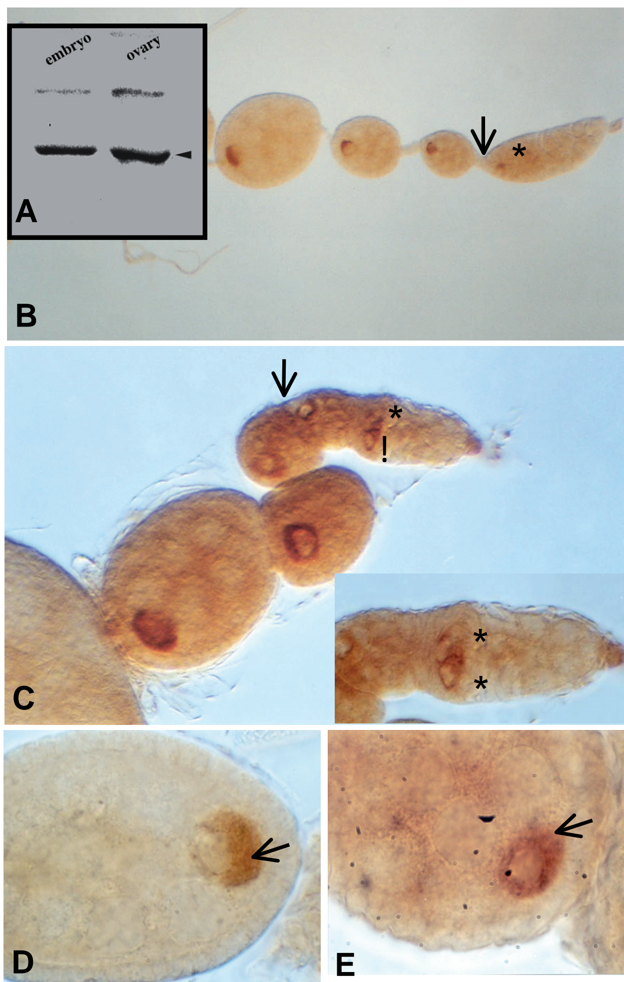

Fig. 2. *pelle* is expressed ovaries and is required for proper distribution of Dichaete protein in the oocyte as well as egg chamber separation. (A) *Pelle protein expression was detected in 0-3 hour wild type embryos and dissected adult female ovaries (arrowhead) via Western blot analysis.* **(B-E)** *Anti-Dichaete immunostaining was performed on ovaries dissected from wild type (B,C) and* pelle *mutant (D,E) females.* **(B)** *In wild type ovarioles, the germarium (arrow) is separated from the stage 1 oocyte by somatic stalk cells. Note the presence of a single cell expressing Dichaete in the germarium (*) and stage 1 oocyte.* **(C)** *In an ovariole from a* pelle ^{rm8}*/Df(3R)38.3.mutant female the germarium (arrow) and stage 1 oocyte are fused, and the stalk is absent. Note also that Dichaete protein is detected around two distinct nuclei in the germarium (*). Inset showing a higher magnification.* **(D)** *In a stage 5 wild type oocyte Dichaete protein is specifically localized around the posterior of the oocyte nucleus (arrow).* **(E)** *In a stage 5 oocyte derived from a* pelle ^{rm8}*/ Df(3R)38.3 mutant female, Dichaete protein is not restricted to the posterior cytoplasm and is dispersed around the entire oocyte nucleus (arrow; also see panel B).*

3, 22F;23D1-2, 24C2;25D3-4, 35E1-2;36A, 36C2-4;37B9, 38A06-B01;40A, 41D;42B1-3, 42C1;43E, 45D9;46F1-2, 47D3;48C6-8, and 54EF;55B9-C1) and 2 regions on the third chromosome (63C1;63D3, 90F1-4;91A1-2 and 97A;98A5) that behaved as genetic enhancers of the *Dichaete*-induced eye phenotype. These enhancers all resulted in more severe disorganization of eye tissue (Fig. 1 A-E,G,H). Three suppressors were also identified that all removed regions containing the *glass* gene (Fig. 2F) and presumably result in reduced Glass-dependent *gal4* and *Dichaete* expression.

To identify the specific modifier genes, single gene mutations that mapped within the relevant deficiency intervals were crossed to P[GMR-*gal4*]/CyO; P[UAS-*Dichaete*]/TM3 flies. This analysis revealed that mutations in *decapentaplegic* (*dpp*^{S1} and *dpp*$^{d\text{-}ho}$ alleles), *engrailed* (*en*^{1} allele), and *pelle* (Df(3R)*R38.3* allele) mimicked the effects of the corresponding deficiencies at 22F, 48A, and 97F, respectively (Fig. 2 G,H). *dpp* encodes a TGF-β-related protein that plays an essential role in morphogenetic furrow formation and retinal patterning during eye development (Cordero *et al.* 2007). Based on the ability of ectopic Dichaete to repress *dpp* expression in the eye disc (Mukherjee *et al.* 2000), removal of one copy of *dpp* could exacerbate the P[GMR-*gal4*]/ P[UAS-*Dichaete*] eye phenotype by further reducing levels of *dpp* expression. The *en* gene encodes a conserved homeodomain protein whose precise role in eye development is unclear. Loss of *Dichaete* does disrupt *en* expression in the embryo and larval CNS (Mukherjee *et al.* 2000; Nambu and Nambu, 1996; Russell *et al.* 1996) suggesting ectopic *Dichaete* could influence *en* expression in the eye imaginal disc. Alternately, En could repress the activities of Dichaete in the eye disc; direct interactions between other homeodomain and Sox proteins have been reported (Wissmüller *et al.* 2006). The ability of *pelle* mutants to modify the *Dichaete* gain-of-function eye phenotype suggests that *pelle* may normally function in eye development. *pelle* mutants do exhibit mis-targeting of photoreceptor R1-5 axons to the medulla (Mindorff *et al.* 2007), suggesting that *pelle* may attenuate the effects of ectopic Dichaete expression in the developing eye disc. As there is only limited knowledge of the signaling pathways that influence Sox protein activities, we chose to further investigate functional interactions between Dichaete and Pelle.

Pelle is required for proper distribution of Dichaete protein in the oocyte and egg chamber separation

Maternal *pelle* function is required for embryonic dorsal/ventral pattern formation and Western blot analyses confirmed that Pelle protein is expressed in early embryos as well as adult female ovaries (Fig. 2A). *Dichaete* is also expressed during oogenesis (Mukherjee *et al.* 2006), and strikingly, in early stage egg chambers Dichaete protein is specifically restricted to the cytoplasm around the posterior margin of the oocyte nucleus (Fig. 2B). We thus examined whether *pelle* might influence Dichaete expression or distribution by performing anti-Dichaete immunostaining on ovaries from *pelle* ^{385}/Df(3R)*38.3* and *pelle*^{rm8}/Df(3R)*38.3* mutant females. This analysis indicated that Dichaete protein is still expressed and present in the cytoplasm of *pelle* mutant oocytes. However, in 12.8% (6 out of 47) of stage 5-6 *pelle* ^{385}/ Df(3R)*38.3* and 20.5% (9 out of 44) of stage 5-6 *pelle*^{rm8}/Df(3R)*38.3* mutant oocytes, Dichaete protein exhibited an abnormal diffuse distribution and was not restricted to the posterior cytoplasm (Fig. 2C). In addition, *pelle* mutant ovaries also exhibited defects in the elaboration of individual egg chambers. Unlike wild type ovarioles, 12.8% (6 out of 47) of the *pelle*^{385}/Df(3R)*38.3* and 6.8% (3 out of 44) of the *pelle*^{rm8}/Df(3R)*38.3* ovarioles contained two or more fused egg chambers (Fig. 2 D,E). In addition, the stalk cells between adjacent egg chambers were often lacking and some mutant egg chambers contained multiple sites of Dichaete ex-

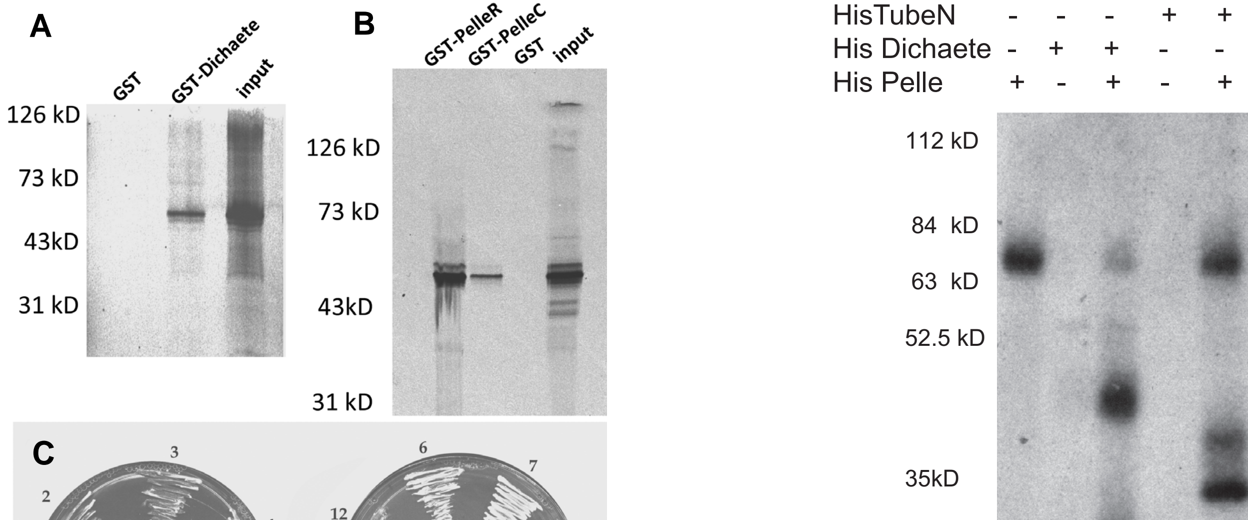

Fig. 3 (Left). Dichaete associates with the death domain-containing region of Pelle. (A,B) *GST-pulldown assays using Dichaete and Pelle proteins.* **(A)** *Binding of a GST-Dichaete fusion protein to ^{35}S-labeled full length Pelle generated via* in vitro *translation. ^{35}S -labeled Pelle protein migrates at ~55 kD (input). Pelle protein specifically associated with GST-Dichaete and not with GST alone.* **(B)** *Binding of GST-Pelle proteins to ^{35}S-labeled full length Dichaete generated via* in vitro *translation. ^{35}S-labeled full length Dichaete migrates at ~45 kD (input). Dichaete protein associated strongly with GST-Pelle R and more weakly with GST-Pelle C proteins. Dichaete did not associate with GST alone.* **(C)** *Yeast 2-hybrid assays using Dichaete, Sox2, and Pelle bait and prey constructs. Full length Dichaete and mouse Sox2 associate with full length Pelle (2,3,7) as well as the Pelle NH_2 region (4,8-10). Dichaete and Sox2 only weakly associate with the Pelle COOH region (5, 11,12). 1. A positive control of p53 bait and pB42AD prey vectors permitted strong growth of transformed yeast cells on Leu- selective medium. 2. A combination of full length (FL) Dichaete bait and FL Pelle prey permitted strong growth of yeast cells, indicating interaction. 3. A combination of FL Pelle bait and FL Dichaete prey permitted strong growth of yeast cells, indicating interaction. 4. A combination of the Pelle NH_2 region (amino acids 1-209) bait and FL Dichaete prey permitted strong growth, indicating interaction. 5. A combination of the Pelle COOH region (210-501) bait and FL Dichaete prey permitted only weak growth, indicating minimal interaction. 6. A positive control of a p53 bait and pB42AD prey vectors permitted strong growth of transformed yeast cells on Leu- selective medium. 7. A combination of FL Sox2 bait and FL Pelle prey permitted strong growth, indicating interaction. 8. A combination of FL Sox2 bait and Pelle NH_2 prey (1-209) permitted strong growth, indicating interaction. 9. A combination of FL Sox2 bait and Pelle NH_2 prey (26-209) permitted strong growth, indicating interaction. 10. A combination of FL Sox2 bait and Pelle NH_2 prey (1-209) permitted strong growth, indicating interaction. 11. A combination of FL Sox2 bait and Pelle COOH region (142-501) permitted only weak growth, indicating minimal interaction. 12. A combination of FL Sox2 bait and Pelle COOH region (210-501) permitted only weak growth, indicating minimal interaction.*

Fig. 4 (Right). Pelle phosphorylates Dichaete protein. *Autoradiogram from an* in vitro *kinase assay using purified 6XHis-Pelle, 6XHis-Dichaete, and 6XHis-TubeN proteins. Note that Pelle exhibits autophosphorylation activity as evidenced by a prominent ^{32}P-labeled band at ~65 kD [see Shen and Manley, 2002]. Pelle also phosphorylates the NH_2 region of Tube (note bands at ~35 kD) and Dichaete (note band at ~48 kD). No labeling of Dichaete or TubeN proteins were detected in the absence of Pelle protein.*

pression. Thus, maternal *pelle* functions are important both for proper distribution of Dichaete protein in the oocyte and normal elaboration of individual egg chambers.

Pelle directly associates with and phorphorylates Dichaete

To determine whether the Pelle and Dichaete proteins may directly interact, GST-pulldown and yeast 2-hybrid assays were performed. ^{35}S-labeled full length Pelle protein specifically associated with GST-Dichaete, but not GST alone (Fig. 3A). In addition, ^{35}S-labeled full length Dichatete associated strongly with GST-Pelle R (amino acids 1 to 209 of Pelle) which includes the death domain and weakly with GST-Pelle C (amino acids 209 to 501 of Pelle) which includes the kinase catalytic domain (Fig. 3B). Yeast 2-hybrid assays also indicated association between full length Dichaete and Pelle proteins (Fig. 3C) and further revealed that Dichaete strongly associated with the Pelle NH_2 region (1-209) and only weakly associated with the Pelle COOH region (amino acids 210-501). These data indicate that Dichaete and Pelle proteins can directly associate and that these interactions are predominantly mediated via the death domain-containing NH_2 region of Pelle. As the HMG domain of Dichaete is 88% identical to that of Sox2, additional yeast 2-hybrid assays were carried out to show that full length Pelle can also associate with Sox2 (Fig. 3C). Similar to Dichaete, Sox2 exhibited strong interaction with the Pelle NH_2 region (amino acids 1-209 or 26-209) and a weak interaction with the Pelle COOH region (amino acids 142-501 or 210-501). This result suggests that the HMG domain of Dichaete and Sox2 is important for association with Pelle.

The ability of Dichaete and Pelle proteins to directly associate suggested that Dichaete might be a phosphorylation substrate for Pelle. To test this hypothesis, *in vitro* kinase assays were performed using full length 6XHis-Pelle and 6xHis-Dichaete proteins.

As previously demonstrated (Shen and Manley, 2002; Shen and Manley, 1998), incubation of 6xHis-Pelle in the presence of γ-^{32}P-ATP results in autophosphorylation, and 6xHis-Pelle also phosphorylates the NH_2 region of *Drosophila* Tube (Fig. 4). Co-incubation of 6xHis-Pelle and 6xHis-Dichaete resulted in the presence of a prominent ^{32}P-labeled band corresponding to 6xHis-Dichaete (Fig. 4). These data thus identify Dichaete as a novel target for the Pelle kinase.

Discussion

Regulation of Sox protein functions by post-translational modifications

Sox proteins can undergo several types of post-translational modification that influence their subcellular localization and transcriptional activation properties (reviewed in Wegner, 2005). Consistent with their functions as transcription regulators, Sox proteins are typically detected in cell nuclei and the HMG domains of several Sox proteins, including Dichaete, contain a bipartite and a basic cluster nuclear localization signal (NLS) (reviewed in Smith and Koopman, 2004). The absence of Dichaete protein in the oocyte nucleus suggests that these NLSs are masked, either via a distinct Dichaete protein conformation, binding to another molecule, or a post-translational modification. Several Sox proteins have been shown to undergo phosphorylation, including Sry, Sox9, and Sox3 (Huang *et al.* 2000; Desclozeaux *et al.* 1998; Stukenberg *et al.*, 1997). While the site of Pelle-mediated Dichaete phosphorylation is not yet established, use of the NetPhos 2.0 Protein Phosphorylation Prediction Server (http://www.cbs.dtu.dk/services/NetPhos/) identified 17 consensus phosphorylation sites in Dichaete (data not shown). Two of the predicted sites reside within the HMG domain; Ser186 (context = LLAESEKRP) received a score of 0.996 (maximum score = 1.0; scores over 0.5 are predictive) and a Tyr209 (context = EHPDYKYRP) received a score of 0.942. Similar potential phosphorylation sites were predicted in the HMG domain of Sox2 (data not shown). The Dichaete basic cluster NLS overlaps the predicted Tyrosine phosphorylation site, suggesting that phosphorylation at this site might influence the activity of this NLS. However, Protein Kinase A-mediated phosphorylation of Serine-211 near the basic cluster NLS of Sox9 did not alter its nuclear localization (Huang *et al.* 2000).

While Pelle kinase does not appear to be responsible for controlling cytoplasmic versus nuclear localization of Dichate in the oocyte, its absence does result in more diffuse localization of Dichaete protein. These findings suggest that Pelle-mediated phosphorylation is important for proper positioning of Dichaete in the oocyte cytoplasm and that distinct post-translational mechanisms regulate it's cytoplasmic versus nuclear distribution. Acetylation of both Sry and Sox2 regulates nuclear localization (Baltus *et al.* 2009) and this acetylation is mediated by the p300 histone acetyl transferase (HAT) at the lysine residue in an ISKRL motif in the central portion of the HMG domain. This motif and the lysine residue are strongly conserved among vertebrate Sox2 proteins and are also present in the Dichaete HMG domain. Perhaps constitutive modification of this site by a *Drosophila* HAT maintains cytoplasmic localization of Dichaete in the oocyte.

Functions for Dichaete and Pelle in oogenesis

This study revealed functions of *pelle* in oogenesis; *pelle* mRNA expression was detected in dissected ovaries and *pelle* mutants exhibited a partially penetrant defect in egg chamber separation. In addition, multiple sites of Dichaete expression were detected in some *pelle* mutant egg chambers. It is possible that *pelle* is required for the normal function of stalk cells and other follicle cells in the individualization of adjacent egg chambers. This phenotype resembles that seen for germline mutations in the gene encoding a nonmotor kinesin II-associated protein (Pflanz *et al.* 2004). The ectopic sites of Dichate expression could correspond to potential oocyte duplication or failure of developing cysts to fully separate. While the important sites of *pelle* expression during oogenesis are unknown, *pelle* mutants do appear to affect relatively early stages of oogenesis as defects in egg chamber separation and Dichaete expression were observed in the germarium. As yet there is little evidence indicating a role for Sox proteins in the Toll/Interleukin-1 Receptor signaling pathway. However, Pelle can function in distinct pathways (Murdiff *et al.* 2007) and Pelle may influence Dichaete distribution independently of the Toll pathway. Taken together, the data suggest that a non-canonical Pelle pathway may influence Dichaete localization during oogenesis. Given the tightly regulated localization and translation of mRNAs in the *Drosophila* oocyte (reviewed in Kugler and Lasko 2009), the highly restricted positioning of Dichaete protein could be important for post-transcriptional regulatory processes during oogenesis.

Materials and Methods

Drosophila stocks

Generation of P[UAS-*Dichaete*] is described in (Mukherjee *et al.* 2000). P[GMR-*gal4*] and the second and third chromosome deficiency kits were obtained from the Bloomington *Drosophila* Stock Center. Df(3R)R38.3 (97E3-11; 98A) is a P element excision *pelle* null allele (Hecht and Anderson, 1993), Df(3R)IR16 (97F1-2; 98A) is a γ-ray induced null allele (Shelton and Wasserman 1993), and *pll*^{385} and *pll*^{m8} are EMS-induced strong alleles (Hecht and Anderson, 1993).

Genetic modifier screen

P[GMR-*gal4*]/CyO; P[UAS-*Dichaete*]/TM3 females were crossed to males carrying a second or third chromosome deficiency carried over a balancer chromosome. At least 100 non-balancer progeny from each cross were analyzed for adult eye morphology and the phenotypes were compared to P[GMR-*gal4*]/+; P[UAS-*Dichaete*]/+ flies. Modifier phenotypes were confirmed via secondary crosses between P[GMR-*gal4*]/CyO; P[UAS-*Dichaete*]/TM3 males and deficiency chromosome females. All crosses were maintained at 25°C.

Scanning electron microscopy

Two to three day old adult flies were prepared for scanning electron microscopy by fixation with 2% glutaraldehyde in PBS (175 mM NaCl, 1.86 mM NaH_2PO_4 8.41 mM Na_2HPO_4 pH) for 6 hours on ice, followed by dehydration through a graded ethanol series. Samples were critical point dried in a Polaron CPD unit and were sputter coated with palladium/gold. They were examined and photographed using the UMass Central Microscopy Facility (http://www.bio.umass.edu/microscopy/) using a Nikon JEOL 5400 scanning electron microscope.

Immunostaining and immunoblotting

Anti-Dichaete immunostaining of ovarioles was performed using anti-Dichaete serum (Ma *et al.* 1998) at a 1:1000 dilution in PBT (PBS and 0.1% Triton X-100) and a biotin-conjugated anti-rabbit secondary antibody (Vector Laboratories) at a 1:400 dilution. Detection of antibody

labeling was achieved using streptavidin-horseradish peroxidase (Vector Laboratories) and diaminobenzidine/H_2O_2 reactions.

For Western blot analysis, protein extracts were prepared from 100 µl of dechorionated 0-3 hour Canton-S embryos or ~50 ovaries dissected from adult female flies. Tissues were homogenized in 100 µl of lysis buffer provided in the Immunoprecipitation Kit (Boehringer Mannheim) and centrifuged to pellet debris. 20 µl of protein extracts were electrophoresed on an 8% SDS polyacrylamide gel and electro-transferred to nitrocellulose membrane. Anti-Pelle serum was purified by preabsorption to fixed third instar larval salivary glands (lacking associated fat body) for one hour. The membranes were blocked in PBS containing 5% nonfat dry milk and 0.2% Tween 20 for one hour, and then incubated with a 1:1000 dilution of preabsorbed anti-Pelle serum at 4^0C overnight. The blot was washed in PBS + 0.2% Tween 20, incubated in biotin-conjugated anti-rabbit secondary antibody (Vector Laboratories) for one hour at room temperature, and washed several times. Labeled proteins were detected via chemiluminescence using reagents from the ECL kit (Amersham Lifesciences) and autoradiography.

GST-pulldown assays

Preparation of GST-Dichaete fusion protein using a pGEX-2T subclone was performed as described in (Ma *et al.* 2000). GST-Pelle R and GST-Pelle C fusion proteins were prepared as described in (Shen and Manley, 1998). ^{35}S-labeled full length Dichaete or Pelle proteins were generated using full length *Dichaete* and *pelle* cDNA clones via *in vitro* transcription/ translation reactions (Promega) and ^{35}S-methionine (Amersham Pharmacia). GST-pulldown assays were performed by incubating 1-4 µg of purified GST fusion protein (GST-Dichaete, GST-Pelle R, or GST-Pelle C) with 20 µl of glutathione-sepharose-4B beads in 100 µl of NETN (20mm Tris-HCl pH 8.0, 100mm NaCl, 1mm EDTA and 0.5% NP40) for 2 hours at 4 °C followed by several washes with NETN. 5 µl of *in vitro* translated ^{35}S-methionine-labelled protein (Dichaete or Pelle) was incubated with 20 µl of glutathione-sepharose-4B beads in 40 µl of NETN for 2 hours at 4 °C. The supernatant containing the ^{35}S-methionine-labelled protein was then transferred to the sepharose-4B beads bound to GST-Dichaete or GST-Pelle and incubated for 2-6 hours at 4 °C. The complexes were washed several times with NETN and bound protein eluted with buffer containing reduced glutathione (Amersham Pharmacia). The eluted samples were briefly boiled and electrophoresed on either an 8% or 10% SDS polyacrylamide gel. The gels were fixed in 1:1 methanol:acetic acid, dried, and autoradiographed.

Yeast 2-hybrid assays

The pEG202 bait (fusion to LexA DNA binding domain) and the pJG4-5 prey (fusion to B42 transcriptional activation domain) vectors (Origene) were used to generate constructs for yeast two hybrid analyses. Full length *pelle* bait and prey constructs were generated via PCR using a full length *pelle* cDNA clone (Shelton and Wasserman, 1993) as template and the following oligonucleotide primers:

5'-GAGGCCGAATTCGGCGTCCAGACCGCCGAAGCC-3'

5'-CTTGGCAAACAATGGCTGGAGCTCCGACCG-3'

The PCR products were digested with EcoRI and XhoI, purified and subcloned into pEG202 and pJG4-5. pEG202 bait constructs expressing Pelle-NH_2 (amino acids 1-209) and Pelle-COOH (amino acids 210-501) were kindly provided by Steve Wasserman (Shelton and Wasserman, 1993). Generation of the Dichaete bait and prey constructs are described in (Ma *et al.* 1998; 2000).

A pEG202 bait construct that expresses full length mouse Sox2 (amino acids 4-319), including the HMG domain, was generated via PCR from a Sox-2 cDNA clone kindly provided by Lisa Dailey. The following primers were utilized:

5'-GGGGGAATTCATGGAGACGGAGCTGAAGCCGCCG-3'

5'-GGGGCTCGAGCATGTGCGACAGGGGCAGTGTGCC-3'

The PCR product was digested with EcoRI and XhoI and subcloned into the pEG202 vector.

Combinations of bait and prey constructs were co-transformed into EGY48 host yeast cells (Matα *trp1 his3 ura3 leu2*::6lexAop-*LEU2*) according to the supplier's instructions (Origene). The transformed cells were plated on YNB/Leu/Ura (-His, -Trp) glucose medium and incubated at 30°C for 3-4 days. The transformants were then re-plated on the YNB/ Ura (-His, -Leu, -Trp) galactose/raffinose medium and incubated at 30°C for 4 days to assay for the presence of colonies and activation of the *Leu2* reporter gene.

In vitro *phosphorylation assay*

Phosphorylation assays were performed essentially as described in (Shen and Manley, 1998). Reactions with various combinations of 6xHis-Pelle, 6xHis-TubeNH_2 (amino acids 1 to 259), and 6xHis-Dichaete were incubated for 30 minutes at 30°C in 40 µl reactions containing kinase buffer (25mM HEPES pH 7.5, 10mM $MgCl_2$, 5 mM $MnCl_2$, 50 mM β-glycerol phosphate, 20 mM ATP and 5 µCi γ-^{32}P-ATP). 50% of each reaction was electrophoresed on an 8% SDS polyacrylamide gel. The gel was subjected to Coomassie blue staining and autoradiography.

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