THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 20, pp. 15159–15169, May 18, 2007

A Putative Src Homology 3 Domain Binding Motif but Not the C-terminal Dystrophin WW Domain Binding Motif Is Required for Dystroglycan Function in Cellular Polarity in *Drosophila*[*] [S]

Received for publication, September 12, 2006, and in revised form, February 20, 2007 Published, JBC Papers in Press, March 12, 2007, DOI 10.1074/jbc.M608800200

Andriy S. Yatsenko[‡§1], Elizabeth E. Gray[‡1], Halyna R. Shcherbata[‡], Larissa B. Patterson[‡], Vanita D. Sood[‡], Mariya M. Kucherenko[‡§], David Baker[‡], and Hannele Ruohola-Baker[‡2]

From the [‡]Department of Biochemistry, University of Washington, Seattle, Washington 98195 and [§]Ivan Franko National University, Hrushevskogo Str. 4, Lviv 79005, Ukraine

The conserved dystroglycan-dystrophin (Dg·Dys) complex connects the extracellular matrix to the cytoskeleton. In humans as well as *Drosophila*, perturbation of this complex results in muscular dystrophies and brain malformations and in some cases cellular polarity defects. However, the regulation of the Dg·Dys complex is poorly understood in any cell type. We now find that in loss-of-function and overexpression studies more than half (34 residues) of the Dg proline-rich conserved C-terminal regions can be truncated without significantly compromising its function in regulating cellular polarity in *Drosophila*. Notably, the truncation eliminates the WW domain binding motif at the very C terminus of the protein thought to mediate interactions with dystrophin, suggesting that a second, internal WW binding motif can also mediate this interaction. We confirm this hypothesis by using a sensitive fluorescence polarization assay to show that both WW domain binding sites of Dg bind to Dys in humans (K_d = 7.6 and 81 μM, respectively) and *Drosophila* (K_d = 16 and 46 μM, respectively). In contrast to the large deletion mentioned above, a single proline to an alanine point mutation within a predicted Src homology 3 domain (SH3) binding site abolishes Dg function in cellular polarity. This suggests that an SH3-containing protein, which has yet to be identified, functionally interacts with Dg.

The dystroglycan-dystrophin complex contains multiple proteins, including the actin-binding protein dystrophin, the transmembrane protein dystroglycan, and a variety of extracellular proteins, including laminin, agrin, and perlecan (1). The Dg[3] protein is a crucial player in this complex acting as an anchor between the actin cytoskeleton and the extracellular matrix. Dg binds Dys at its proline-rich C-terminal end and laminin at its highly glycosylated N-terminal end (2) (Fig. 1*A*).

When the interactions between components of the Dg·Dys complex are disrupted, the muscle degenerative disease muscular dystrophy (MD) results (3–5). In mouse models, loss of Dg in muscle cells causes mild muscular dystrophy phenotypes (6). Furthermore, several human forms of MD, such as Fukuyama MD, result from mutations in the enzymes that glycosylate Dg. In addition to its role in maintaining the structural integrity of muscle cell membranes, Dg is also required in the brain. When it is knocked out in the mouse brain, disrupted neural migration and disorganized cortical layers are observed (7, 8). This is consistent with the fact that brain malformations as well as learning and memory difficulties are often observed in MD patients (9–12). Dg is not only important in the pathogenesis of MD and the associated brain malformations, but it also has an important role in cell adhesions and anchoring the cell to the extracellular matrix. Loss of Dg protein has been associated with the progression of various epithelial cancers (2, 13). Specifically, Dg is down-regulated in breast and prostate cancers (14, 15).

In vitro studies have suggested that the interaction between Dg and Dys is mediated by the most C-terminal WW domain binding motif, PP*X*Y, on Dg and the Dys WW and EF-hand domains (16–18). *In vitro* experiments have also shown that when the tyrosine of the PP*X*Y motif is phosphorylated, the binding between Dg and Dys is abolished (19, 20). This suggests a potential mechanism to regulate the Dg and Dys interaction, in which signaling proteins containing SH2 or SH3 domains may bind to Dg in a tyrosine phosphorylation-dependent manner. In the search for a potential regulator, recent studies have revealed several proteins that interact with Dg. Both the Grb2 (growth factor receptor-bound protein 2) adaptor protein, as well as MEK1, and ERK of the Ras-Raf mitogen-activated protein kinase (MAPK) cascade have been shown to interact, *in vitro* and *in vivo*, with the C terminus of Dg (21, 22). However, Dg appears to be only an anchor for MEK1 and ERK rather than a substrate (22), and Dg might not have a direct involvement in this signaling pathway. Independently, recent work has revealed that laminin and dystroglycan-dependent phosphoryl-

* This work was supported by NASA Grant (to E. E. G.), by an American Heart Association fellowship (to H. R. S.), Civilian Research and Development Foundation (to A. S. Y., H. R. S., and H. R.-B.), and by grants from the National Institutes of Health (to D. B., and H. R.-B.), and MDA (to H. R.-B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Results, Experimental Procedures, and Figs. S1 and S2.

[1] Both authors contributed equally to the publication.

[2] To whom correspondence should be addressed: Dept. of Biochemistry, University of Washington, HSB J-591, Box 357350 Seattle, WA 98195. Tel.: 206-543-8468; E-mail: hannele@u.washington.edu.

[3] The abbreviations used are: Dg, dystoglycan; Dys, dystrophin; MD, muscular dystrophy; SH3, Src homology 3; SH2, Src homology 2; MEK1, dual threonine/tyrosine kinase; ERK, extracellular signal-regulated kinase; DBR, dystroglycan binding region; bs, binding site; FL, full-length; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein.

ation of syntrophin affects the Grb2-SOS-Rac1-c-Jun N-terminal kinase (JNK) pathway and ultimately results in the phosphorylation of c-Jun on Ser-65 (23). Thus, although studies suggest a clear role for Dg in signaling, the regulation of Dg by signaling and the specific regions of the Dg C terminus involved in this process are unknown.

To shed light on the regulation of Dg and its role in signaling, we have analyzed the binding motifs that are required for the function of the Dg·Dys complex in cellular polarity in *Drosophila*. The proline-rich C terminus of Dg has several potential protein-binding motifs, which suggests that it may be involved in regulating the complex and potentially may have a signaling role. Proline-rich sequences have been shown to be the targets of several protein interaction domains involved in signal transduction. For example, SH3 domains have been shown to bind core P*XX*P motifs (where P is proline and *X* indicates any amino acid). *Drosophila* Dg contains two putative SH3-binding sites, consisting of the core P*XX*P motif. Proline-rich sequences also serve as targets for binding by WW domains (24). In particular, the class I WW domain ligand, PP*X*Y (where Y is tyrosine), appears twice in the C-terminal region of Dg. The more C-terminal PP*X*Y motif has been established as a binding site for the WW domain of dystrophin in humans (17, 18) and in *Drosophila* by *in vitro* binding studies (25). The role of a putative second, more N-terminal WW domain binding site or the potential SH3 domain binding sites are not yet understood.

Drosophila is an excellent system to study the Dg·Dys complex (25–29). In particular, Dg is required for cellular polarity in the oocyte and epithelial cells in *Drosophila* as well as in mouse mammary epithelial cells (26, 30). Furthermore, *Drosophila* is a model for the MD disease phenotype, as reduction of dystrophin and dystroglycan in the muscles leads to progressive muscle degeneration and loss of muscle function (25). In this study, we test which regions of the Dg C terminus are essential for Dg function in cellular polarity *in vivo*. Specifically, we show by a single amino acid substitution that a putative SH3 domain binding site is critical for Dg function in both loss-of-function and overexpression studies. However, the most C-terminal WW domain binding site previously shown to be essential for dystrophin binding is dispensable for cellular polarity in *Drosophila*.

EXPERIMENTAL PROCEDURES

Fly Stocks—Drosophila melanogaster stocks were raised on standard cornmeal/yeast/agar medium at 25 °C. For overproduction of pUASp-Dg in the germ line, we used the following: *NGT40*; *P*(*w*+:*nanosGal4*:*VP-16*)*Ab-2* (31, 32) and *Mat-α4-Tub* > *Gal4-VP16/CyO* (33). For overproduction of pUASp-Dg in embryos we used *Daughterless-Gal4* (34). For overproduction of pUASp-Dg in the follicle cells, we used *hsFlp; act* < *FRT-CD2-FRT* < *Gal4; UAS-GFP* (35). For generation of dystroglycan clones, we used *FRT42D-Dg*^{323}*/CyO* (Dg^{323} is a dystroglycan loss-of-function mutant with a 3155-bp deletion between bp 32,345 and 35,669 of DS03910) (26) and *hsFLP; FRT42D Ubi-GFP/CyO*. For overproduction of pUASp-Dg in a *dystroglycan* mutant background, we used *FRT42DDg*^{323}*/CyO; P*(*w*+ *nos-Gal4*:*VP16*)*A4-2 III*, and *hsFLP; FRT42D Ubi-GFP/CyO; pUASp-Dg/TM3* or *p*UASp-Dg */FM7; FRT42DDg*^{323}*/CyO,* and *hsFLP; FRT42D Ubi-GFP/CyO; P*(*w*+ *nos-Gal4*:*VP16*)*A4-2 III* (*pUASp-Dg* refers to all *dystroglycan* constructs: FL, C1, C2, 4P, DC2, Pro → Ala, ALLP, AATA).

Generation of pUASp-Dg Transgenic Animals—Full-length and truncated dystroglycan PCR products that can be expressed in the germ line were synthesized from the template LD11619 using the forward primer GGGGTACCAACATGAGATTCCAGTGGTTCT in conjunction with one of the following reverse primers: FL, CTCTAGATTATGGCGACACATATGGCGGT; C1, GCTCTAGATTACTTCTCGTCCTTGAGTATGAC; C2, GCTCTAGATTAATATGGCGGTGGCTTCTCGTCCTTGAGTATAGAC; 4P, GCTCTAGATTATGGCGACACAGGTGGCGGT; DC2, GCTCTAGATTAGTCCACGTCGTTGTCAC (Invitrogen) and cloned into pUASp, a vector that allows efficient germ line expression (36).

To generate a construct with mutated SH3-binding sites (pUASp-2XSH3 knock-out, Pro → Ala), the QuickChange® XL site-directed mutagenesis kit (Stratagene) was used to introduce proline to alanine substitutions in the SH3bsI (PATP → AATA). LD11619 was used as a template with forward primer CGTGGCAAGTCGGCAGCCACGGCCTCTACCGCAAACC and reverse primer GGTTTGCGGTAGGAGGCCGTGGCTGCCGACTTGCCACG, generating the intermediate plasmid pBS-Dg AATA. pBS-Dg AATA then served as a template for PCR with the forward primer GGACGAGAAGCCGGCGCTGCTGCCACCATCCTACAATACC and the reverse primer GGTATTGTAGGATGGTGGCAGCAGCGCCGGCTTCTCGTCC, designed to substitute the first proline of the SH3bsII with an alanine (PLLP → ALLP), thus generating pBS-2XSH3 knockout. This served for the template for standard PCR performed with the forward primer GGGGTACCAACATGACATTCCAGTGGTTCT and reverse primer GCTCTAGATTATGGCGACACATATGGCGGT.

PCR products were digested with KpnI and XbaI and cloned into the pUASp vector (36). The constructs were injected into embryos to obtain at least two independent stable transformant lines.

Overproduction of Dystroglycan in the Germ Line, Follicle Cells, and Embryos—For Dg overproduction in germ line cells, balanced *pUASp-Dg/P*(*w*+:*nanosGal4*:*VP-16*)*Ab-2* or *Mat-α4-Tub* > *Gal4-UP16/CyO* animals were raised in yeasted vials at 25 °C for 3 days, dissected, and analyzed. For Dg overproduction in the follicle cells, *hsFlp; UAS-GFPact* < *FRTCD2FRT* < *Gal4/pUASpDg* animals were heat-shocked at 37 °C for 1 h, raised in yeasted vials at 25 °C for 3 days, dissected, and analyzed. All *pUASp*-Dg constructs used were crossed to these three Gal4 drivers to test for proper overproduction of protein and correct localization of protein to the membrane in the germ line and somatic cells. The following *pUASp-Dg* lines were used for germ line analysis: FL-1, 5; 4P-1, -2, -3, -4; DC2-1, -2; Pro → Ala-1-3; C2-1, 3; C1-1, -2; ALLP-1-3; AATA-1,2,4. For Dg overproduction in the embryo, *pUASp-Dg/Daughterless-Gal4* embryos were collected and left for 20 h at 18 °C to develop to stage 13, stained, and analyzed. The following *pUASp-Dg* lines were used to analyze embryos: FL-1, -2, -4; 4P-1, -3; DC2-1, 3; Pro → Ala-1, -2, -3; C2-1, -5, -10; C1-1, -2. For Dg rescue experiment the following lines were used: FL-1, -2, 3, -5, -6; 4P-1, -3, -4; DC2-2, 3-; Pro → Ala-1–3; C2-1, -3; C1-1, -2; ALLP-1–3; AATA-1,2,4. Importantly, the low level of Dg constructs driven by only one copy of the *nanosGal4*-driver used in the rescue

experiments does not generate significant overexpression phenotypes. Abnormal stage 7–8 clones (severe necrosis, no oocyte, or abnormal Orb staining) were not included in our calculations. For each construct, values for the proportion of ovaries or embryos with mislocalized polarity markers between independent insertion lines were averaged, and average deviations were calculated.

Antibody Staining Procedure—Ovaries were dissected in phosphate-buffered saline (PBS) and fixed while shaking on a nutator for 10 min in PBS containing 5% formaldehyde. Embryos were collected in (0.7% NaCl, 0.3% Triton X-100), dechorionated, and fixed for 20 min in (4% formaldehyde, 0.1 M sodium phosphate buffer, pH 7.2). Embryos were transferred to a 20-ml scintillation vial containing fixture and 100% *n*-heptane 1:1 and fixed for 20 min at room temperature on a shaker. Next, the fixture was removed, and an equal amount of 100% methanol was added. The vial was shaken vigorously to rupture the vitelline membrane. Embryos were rinsed with methanol and dehydrated through an ethanol series and rehydrated prior to antibody staining.

Ovaries and embryos were rinsed with PBT (PBS, 0.2% Triton X-100) four times (15 min each rinse) and blocked in PBTB (PBT, 0.2% bovine serum albumin, 5% normal goat serum) for 1 h at room temperature. The tissue was incubated with primary antibodies overnight at 4 °C and then incubated in secondary antibodies overnight at 4 °C. The next day they were rinsed with PBT for 15 min, stained with DAPI (1 μg/ml in PBT) for 10 min, and rinsed with PBT and mounted onto slides in 70% glycerol, 2% *n*-propyl gallate, 1× PBS. To analyze slides, a two-photon laser-scanning confocal microscope (Leica TCS SP/MP) was used.

The following primary antibodies were used at the following designated dilutions: rabbit anti-dystroglycan (1:3000 (26)), mouse anti-Orb and anti-Crb (1:20; Developmental Studies Hybridoma Bank), and rabbit anti-GFP directly conjugated AF488 (1:1000; Molecular Probes). The following secondary antibodies were used at the designated dilutions: Alexa 568 anti-rabbit and Alexa 568 anti-mouse (1:500; Molecular Probes) and 488 phalloidin (1:50; Molecular Probes).

Plasmid Construction for in Vitro Analysis and Protein Expression—The WW-EF hand region (DBR) of *Drosophila* dystrophin was amplified from the template LD11292 using PCR with forward primer GGAATTCCATATGACCATTGGACCACTGCCC and reverse primer CCGCTCGAGTTACTGGTGCTTGGCCGCCTC and cloned between the NdeI and XhoI restriction sites of the His tag expression vector pET-15b (Novagen). *Drosophila* DBR protein was expressed in *Escherichia coli* strain BL21(DE3) after induction by 1 mM isopropyl 1-thio-β-D-galactopyranoside in standard LB medium (Qbiogene/Bio 101, Inc.). Cell pellets were collected, resuspended in Binding Buffer solution (150 mM MOPS, 150 mM NaCl, 5 mM imidazole), and lysed by a French press. Protein was purified using nickel-nitrilotriacetic acid (Qiagen) affinity chromatography. Protein was concentrated using an Amicon ultracentrifugal device (Millipore), and imidazole was removed by dialysis. Purified DBR protein was stored in 50 mM MOPS, pH 6.5, 150 mM NaCl, 400 mM Na_2SO_4, 10 mM dithiothreitol.

The Drk (Dreadlock)-FL gene of *Drosophila* was amplified via PCR from the template LD12029 with forward primer CCGCTCGAGATGGAAGCGATTGCCAAACACG and reverse primer CGCGGATCCTTATGAATGATATGGCGTCACAT and then cloned into the His tag expression vector pET-15b (Novagen) using the XhoI and BamHI restriction sites. *Drosophila* Drk protein was expressed in *E. coli* strain BL21(DE3) after induction by 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. Cell pellets were collected and lysed by a French press. Protein was purified using nickel-nitrilotriacetic acid (Qiagen) affinity chromatography. Protein was concentrated using an Amicon ultracentrifugal device (Millipore) and imidazole removed by dialysis. Purified Drk protein was stored in 20 mM Tris, pH 7.9, 150 mM NaCl, 150 mM Na_2SO_4.

The DBR of human dystrophin (18) was expressed as a glutathione *S*-transferase fusion protein and purified by glutathione affinity chromatography. Five hundred units of thrombin (Amersham Biosciences) were loaded onto the glutathione column with DBR bound, and the column was sealed and incubated overnight at 4 °C to cleave the glutathione *S*-transferase from the DBR. The DBR was washed off the column and concentrated, and the buffer was exchanged during concentration to the same storage buffer used for the *Drosophila* DBR.

Fluorescence Polarization Experiments—Synthesized dystroglycan peptides (Fig. 5*C*) were N-terminally tagged with tetramethylrhodamine by Invitrogen Evoquest Services (sequences DmWWbsI, GKSPATPSYRKPPPYVSP; HmWWbsI, KNMPTYRSPPPYVPP; DmWWbsII, PVI-LKDEKPPLLPPSYNT; HmWWbsII, PL-ILQEEKAPLPPPEYSN). Six additional tetramethylrhodamine-labeled peptides were ordered from Genemed Synthesis Inc. (DmWWbsI-pY, GKSPATPYRKPPPpY-VSP; DmWWbsII-pY, PVILKDEKPPLL-PPSpYNT; HmWWbsI-pY, KNMPTYRSPPPpYVPP; DmWWbsI-W, GKSPATPYRKWPPYVSP; DmWWbsII-G, PVI-LKDEKPPLLLPPSGNT; and DmSH3bsII-2A, PVILKDEKPALLPPSYNT). All peptides were over 95% pure based upon high pressure liquid chromatography and mass spectrometry analysis. Fluorescence polarization experiments were performed at 25 °C using a Wallac 1420 Victor3 fluorescence plate reader (PerkinElmer Life Sciences). Dystroglycan peptide (200 nM) was incubated with increasing concentrations of dystrophin protein in storage buffer to a final volume of 250 μl. Anisotropy values were measured at an excitation wavelength of 531 nm and an emission wavelength of 595 nm. Dissociation constants (K_d) were determined by plotting millianisotropy *versus* the concentration of Dys and fitting the data to the equilibrium binding Equation 1,

$$\frac{W\mathrm{Dg}}{\mathrm{Dg}} = \frac{\mathrm{Dg} + W + K_d - \sqrt{\mathrm{Dg}^2 + K_d{}^2\,W^2 - 2 \times \mathrm{Dg} \times W + 2 \times W \times K_d + 2 \times \mathrm{Dg} \times K_d}}{2 \times \mathrm{Dg}} \qquad \text{(Eq. 1)}$$

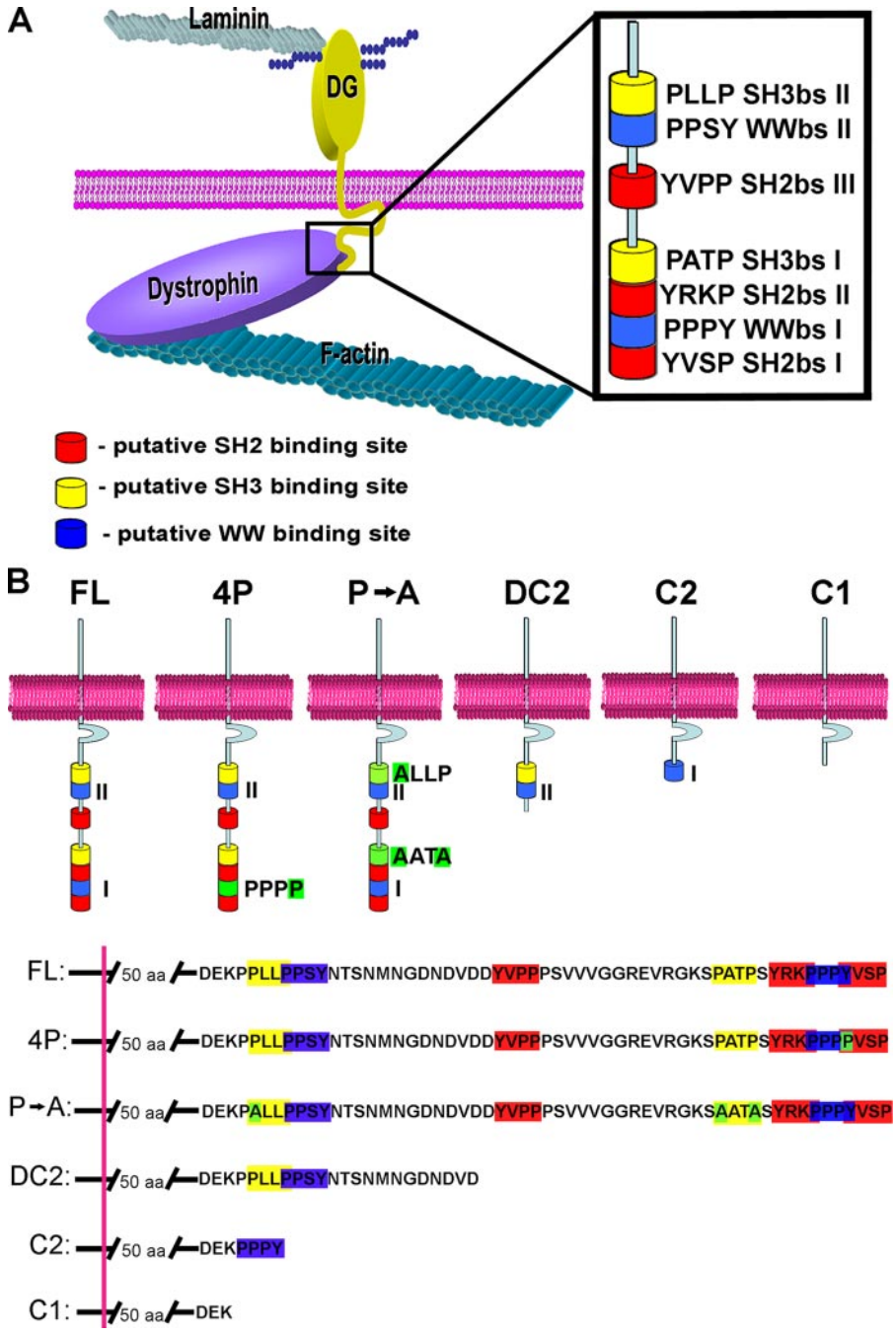


FIGURE 1. **The *Drosophila* dystroglycan-dystrophin complex.** *A,* Dg·Dys complex consists of the actin-binding protein dystrophin, the transmembrane protein Dg, and extracellular proteins, including laminin. Dg is a crucial player in this complex acting as a connector between the actin cytoskeleton and extracellular matrix. Dg binds Dys at its proline-rich C-terminal end, which contains several well conserved putative binding sites for signaling proteins. *B,* schematic drawing of pUASp-Dg constructs with various changes at the Dg C terminus (*FL*, full length; *4P,* Tyr to Pro substitution in the WWbsI; *P → A,* Pro to Ala substitutions in the SH3bsI and SH3bsII; *DC2, C2*, and *C1* = stepwise deletions of the proline-rich C terminus).

where Dg is the total concentration of dystroglycan peptide; *W* is the total concentration of dystrophin protein; *W*Dg is the concentration of dystroglycan-dystrophin complex, and K_d is the apparent dissociation constant for the complex. The assays using phosphorylated peptides were performed with (1:100; phosphatase inhibitor cocktail 2, Sigma) and without phosphatase inhibitors resulting into the same K_d values. This suggests that no obvious effects of contaminant phosphatases were observed in this assay.

RESULTS

Drosophila Dg is required for proper polarity of different cell types (25, 26). The C terminus of Dg contains several putative WW, SH2, and SH3 domain binding sites where signaling proteins may bind and participate in the regulation of the Dg·Dys complex (Fig. 1*A*) (26). Specifically, *Drosophila* Dg contains two class I WW domain binding sites, WWbsI and WwbsII; three putative SH2-binding sites, SH2bsI, SH2bsII, and SH2bsIII; and two putative SH3-binding sites, SH3bsI and SH3bsII (Fig. 1*A*). To analyze the function of each of these potential binding sites, we generated transgenic animals expressing a variety of Dg constructs, in which some of these binding sites are deleted, and we examined their capacity to affect polarity in overexpression and loss-of-function experiments (Figs. 3 and 4). We first generated the C1 construct, which lacks all C-terminal binding sites, and compared its function to full-length Dg (*C1, FL*; Fig. 1*B*). The most C-terminal PPPY motif (WWbsI) binds dystrophin (16, 18, 25). To test the importance of this interaction *in vivo* in *Drosophila*, we generated C2, which only contains this PPPY motif (Fig. 1*B*). When the tyrosine of the PPPY motif is phosphorylated, Dys binding to the Dg C terminus is reduced (Fig. 5) (19). To test whether tyrosine phosphorylation is an important component in the regulation of the Dg·Dys complex *in vivo*, we generated 4P, in which the PPPY motif (WWbsI) has been altered to PPPP. To analyze the importance of the second WW domain binding site, we generated DC2, which contains the second WW and SH3 domain binding sites (WWbsII and SH3bsII). Finally, to analyze whether the two SH3 domain binding sites are critical, we generated P → A, in which both sites (SH3bsI and SH3bsII) have been disrupted by proline to alanine substitutions (Fig. 1*B*). The Gal4-UAS system for protein expression was utilized to express the Dg constructs in the follicle cells, the germ line cells, and the embryos. To avoid problems because of positional effects, 2–6 independent lines were generated and

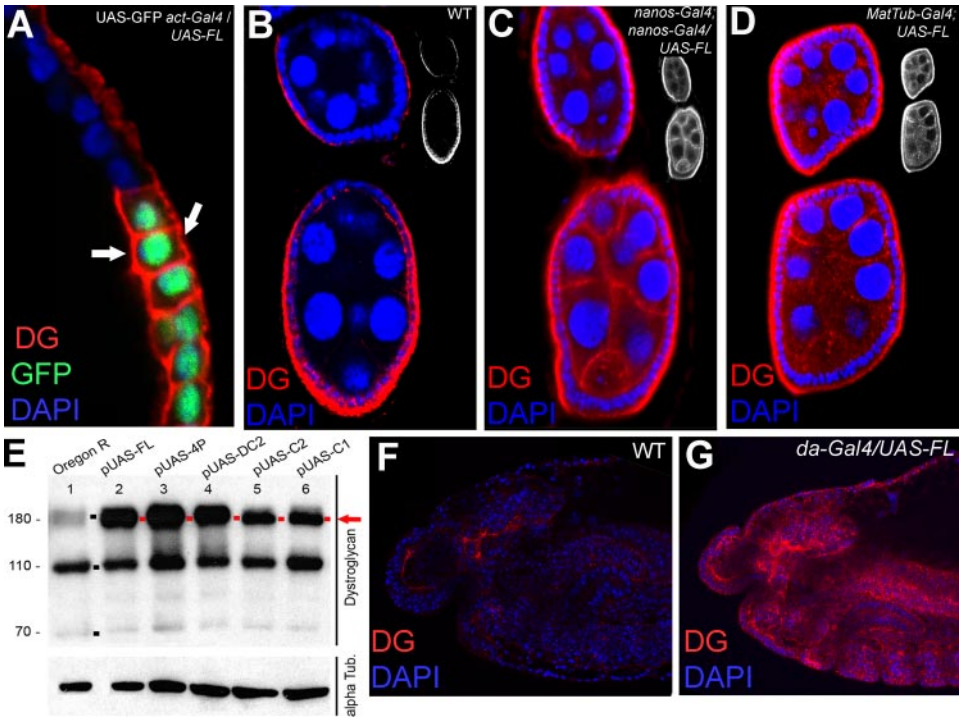


FIGURE 2. **Dg constructs used in this study and their expression in the follicle cells, germ line cells, and embryo.** Dg, *red*; GFP, *green*; DAPI, *blue*. *A,* Dg is localized to the basal membrane in wild type follicular epithelium, but when pUASp-Dg constructs are expressed in follicle cell clones, marked with GFP, Dg is found on both apical and basal membranes (*arrows, hsFlp; act < CD2 < Gal4/+; UAS-GFP/pUASp-FL*). *B,* wild type Dg expression in the germ line cells. *C, nanos-Gal4* driven pUASp-Dg expression in the germ line cells (*NGT40; P(w+: nanosGal4:VP-16)Ab-2/pUASp-FL*). *D, MatTub-Gal4*-driven pUASp-Dg expression in the germ line cells (*Mat-α4-Tub > Gal4-UP16; pUASp-FL*). *E,* Western blot of ovarian protein extracts probed with Dg antibody to detect overexpression in the germ line cells in different pUASp-Dg constructs driven by *Mat-α4-Tub > Gal4-UP16*. Three Dg bands of ~180, ~110, and ~70 kDa (marked by the *black dots*) are detected in wild type (OregonR, *lane 1*). When all Dg constructs are overexpressed, an ~180-kDa band is enriched (*lanes 2–6*, marked by *red dots, red arrow*). *F,* wild type Dg staining in the embryo at stage 13. *G, Daughterless-Gal4*-driven pUASp-Dg expression in the embryo at stage 13 (*Daughterless-Gal4/pUASp-FL*).

analyzed for each construct. The results represent mean values for experiments done with multiple independent insertion lines. All constructs expressed Dg at elevated levels compared with wild type (Fig. 2).

Dystroglycan Overproduction Disrupts Oocyte Polarity—Dg and Dys are required in the germ line for the early establishment of oocyte polarity (25, 26). To analyze how overproduction of Dg in the germ line affects oocyte polarity, we expressed Dg (FL) in the germ line cells using a germ line-specific driver (*MatTub-Gal4;* Fig. 2*D*, and supplemental Fig. S2*A*) and examined oocyte polarity using Orb as a marker. Orb marks the microtubular organizing center, which is localized at the anterior of the oocyte during stage 1, and then moves to the posterior by stage 3. Between stages 3 and 6, Orb is clearly localized to the posterior of the oocyte, making it an excellent marker to analyze the polarity of the oocyte (Fig. 3*A*–*A′* and supplemental Fig. S2, *A* and *B*). Absent or mislocalized Orb during these stages indicates a failure to establish early oocyte polarity. When FL is overproduced in the germ line Orb becomes mislocalized (Fig. 3, *B*–*B′*, *C,* and *G*); instead of being localized to the posterior, Orb surrounds the entire oocyte in a circle or accumulates in a clump at one side of the oocyte (48 ± 8%, n = 117). These early polarity defects resulted in abnormalities during the later stages of egg chamber development. In addition to defective appendages and necrosis, abnormal Orb staining was observed in later stage egg chambers. Instead of the smooth Orb staining throughout the oocyte, patchy and dotted staining was observed (80 ± 8%, n = 323; Fig. 3*H*). Therefore, dystroglycan, when expressed at elevated levels in the germ line cells, is sufficient to disrupt oocyte polarity in both early and late stages of oogenesis. Similarly, in vertebrates, overexpression of Dg has been shown to cause defects in neuromuscular junctions (37, 38).

The Region of the Dystroglycan C Terminus Containing WWbsII and SH3bsII Is Sufficient to Disrupt Oocyte Polarity—Because full-length Dg overproduction disturbs oocyte polarity, we analyzed which signaling molecule binding sites in Dg are required for this capacity, in the context of our assays. Each Dg construct (Fig. 1*B*) was expressed in the germ line cells using a *MatTub-Gal4* driver, and the percentage of stage 3–6 egg chambers with abnormal oocyte polarity was quantified (Fig. 3*G*).

Expression of C1 and C2 did not result in as high frequency of Orb mislocalization as the FL construct, suggesting that the C-terminal proline-rich region (absent in C1) is important for full Dg function and the WWbsI Dys-binding site alone is not sufficient to restore the activity of C1 to the wild type level (Figs. 1*B* and 3*G*). Therefore, other sites must act in conjunction with this WW domain binding site to regulate the Dg·Dys complex in the context of oocyte polarity.

Mutation of the conserved tyrosine of WWbsI to proline reduces binding affinity *in vitro* by an order of magnitude (from 7.6 to 172 μM in human and from 3.7 to 47 μM in *Drosophila*) (25). Expression of the 4P construct in *Drosophila* had the same ability to disrupt oocyte polarity as FL (46 ± 3%, n = 194; Fig. 3*G*), suggesting that either the reduced binding observed with the 4P construct is still enough to support functionality of the complex or that WWbsII is able to function in place of WWbsI. To probe this issue further, we expressed DC2, which only contains WWbsII and SH3bsII (Fig. 1*B*). Interestingly, DC2 was also able to disrupt oocyte polarity to the same extent as FL (53 ± 2%, n = 122; Fig. 3*G*), indicating that WWbsII indeed can function and that potential SH3 domain binding sites may play a role in the regulation of the Dg complex.

To test whether the putative SH3 domain binding sites are important for Dg function, we overexpressed the P → A construct, in which both SH3bsI and SH3bsII have been disrupted by proline to alanine substitutions (Fig. 1*B*). Importantly, this construct, in which only the two potential SH3 domain binding

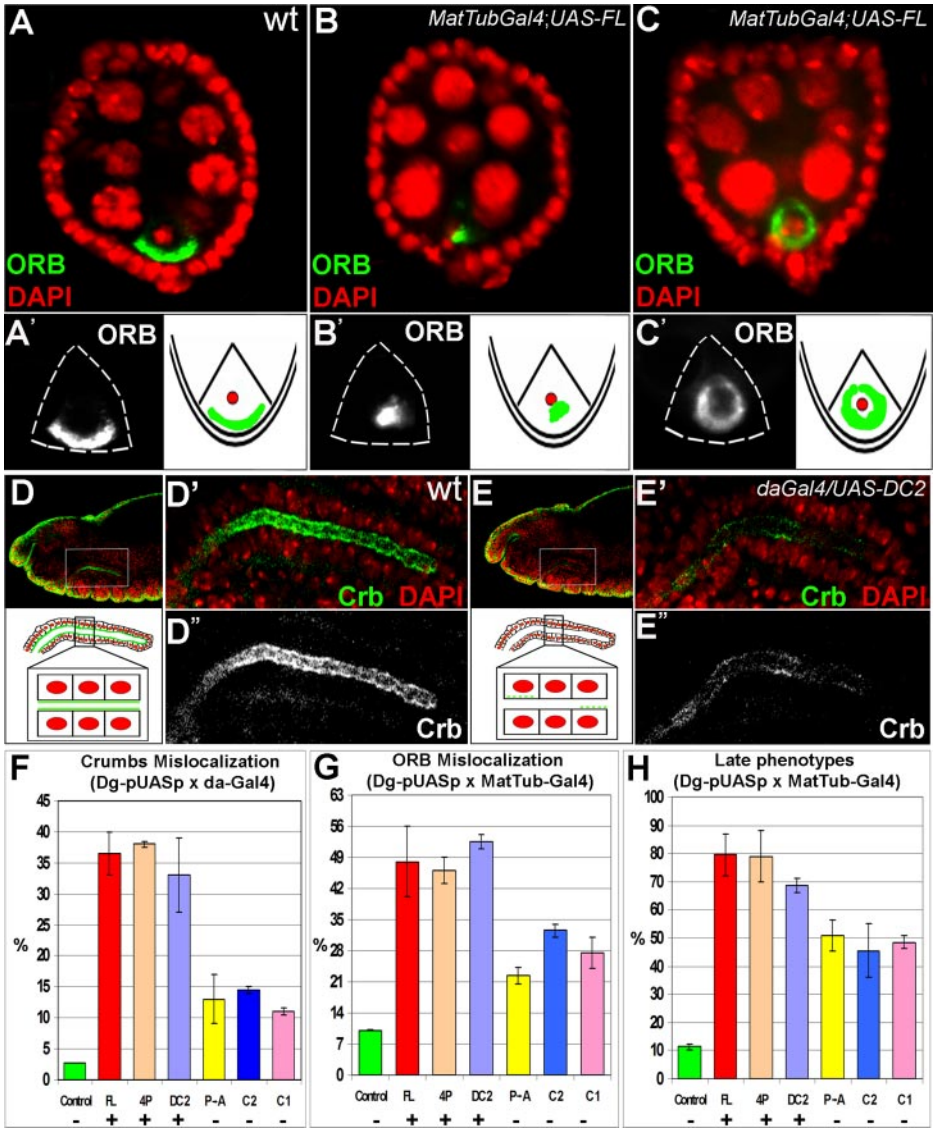


FIGURE 3. **Dg overproduction disrupts cellular polarity.** *A,* wild type (*wt*) stage 4 egg chamber shows Orb (*red*) correctly localized to the posterior of the oocyte. Orb localization is shown magnified in a single channel and schematically drawn below each photo (*A′–C′*). Orb, *green*; DAPI, *red*. *B* and *C,* when Dg is overproduced in the germ line cells, oocyte polarity is disrupted, resulting in mislocalized Orb (*Mat-α4-Tub > Gal4-UP16; pUASp-FL*). *D–D″,* a confocal image of the wild type salivary gland at stage 13 stained with Crb antibody that is localized to the apical side of the salivary gland epithelium. Crb, *green*; DAPI, *red*. *E–E″,* when Dg is overproduced in the embryo (*Daughterless-Gal4/pUASp-DC2*), Crb fails to localize properly to the apical side and has reduced levels in the salivary gland epithelium. Crb, *green*; DAPI, *red*. Schematic drawing of Crb localization in the salivary gland epithelium is shown below each photo (*D* and *E*). *F,* percentage of embryos with Crb mislocalization in salivary gland epithelium when Dg is overproduced using the *Daughterless-Gal4* driver (*Control,* 2.8, $n = 48$; *FL,* 37 ± 4, $n = 46$; *4P*, 38 ± 0.5, $n = 29$; *DC2*, 33 ± 6, $n = 15$; P → A, 13 ± 4, $n = 40$; *C2*,14.5 ± 0.5, $n = 27$; *C1*, 11 ± 0.5, $n = 28$). *G,* percentage of stage 3–6 egg chambers with mislocalized or absent Orb when pUASp-Dg is overproduced using the *MatTub-Gal4* driver (*Control*, 10 ± 0.1, $n = 125$; *FL*, 48 ± 8, $n = 117$; *4P*, 46 ± 3, $n = 194$; *DC2*, 53 ± 2, $n = 122$; P → A, 22 ± 2, $n = 140$; *C2*, 33 ± 2, $n = 135$; *C1*, 28 ± 4, $n = 118$). *H,* percentage of stage 6–10 egg chambers with late stage Orb defects when Dg is overproduced using the *MatTub-Gal4* driver (*Control,* 11 ± 1, $n = 74$; *FL*, 80 ± 8, $n = 323$; *4P*, 79 ± 9, $n = 492$; *DC2*, 69 ± 3, $n = 355$; P → A, 51 ± 6 $n = 330$; *C2*, 46 ± 10, $n = 264$; *C1*, 49 ± 3, $n = 359$). The *error bars* represent differences between different independent insertions lines.

sites had been mutated, has a reduced capacity to affect oocyte polarity, similar to the C1 construct, which lacks all the potential binding sites (Fig. 3*G*). This confirms that the putative SH3 domain binding sites are essential for the full function of the Dg protein in this assay.

We also examined the ability of the various Dg constructs to disrupt oocyte polarity at later stages and observed a similar trend; a higher frequency of phenotypes were observed for all constructs, but C1, C2, and P → A showed less of a capacity to disrupt Orb localization than FL, 4P, and DC2 (Fig. 3*H*). This indicates that presence of at least one pair of WW domain and putative SH3 domain binding sites can disrupt the oocyte polarity during early and late stages of oogenesis and is therefore important for Dg function in the context of this assay.

We also considered whether the amount of disrupted oocyte polarity was simply a result of the level of Dg protein overproduction, rather than the type of Dg construct. We compared the level of Dg production with the degree of Orb mislocalization and found no correlation. For example, different insertion lines with the DC2 construct exhibited a large range in the level of Dg overproduction when induced with the *MatTub-Gal4* driver (supplemental Fig. S1*A*); however, the range in the level of Orb mislocalization was very small (Fig. 3, *G* and *H*). This suggests that the amount of Dg overproduction in the oocyte beyond a 2-fold level is not responsible for the changes in oocyte polarity and, therefore, that the differences in disruption of oocyte polarity are the result of the presence of significant domain binding motifs.

The Region of the Dystroglycan C Terminus Containing WWbsII and SH3bsII Is Sufficient to Disrupt Salivary Gland Apical-Basal Polarity—Previous work has indicated that overproduction of Dg in the salivary gland is sufficient to disrupt epithelial cell apical-basal polarity (26). The salivary gland epithelium has a very clear cell polarity; in wild type embryos, the polarity marker Crumbs localizes to the apical side of the salivary gland (Fig. 3, *D–D″*). However, when Dg is overproduced in the embryo using the *Daughterless-Gal4* driver, Crumbs fails to localize normally in tissue (Fig. 3, *E–E″*, and *F*) (26).

To determine which potential Dg C-terminal signaling molecule binding sites are sufficient to disrupt salivary gland epithelium polarity, we expressed each Dg construct in the embryo using the *Daughterless-Gal4* driver and quantified the percent-

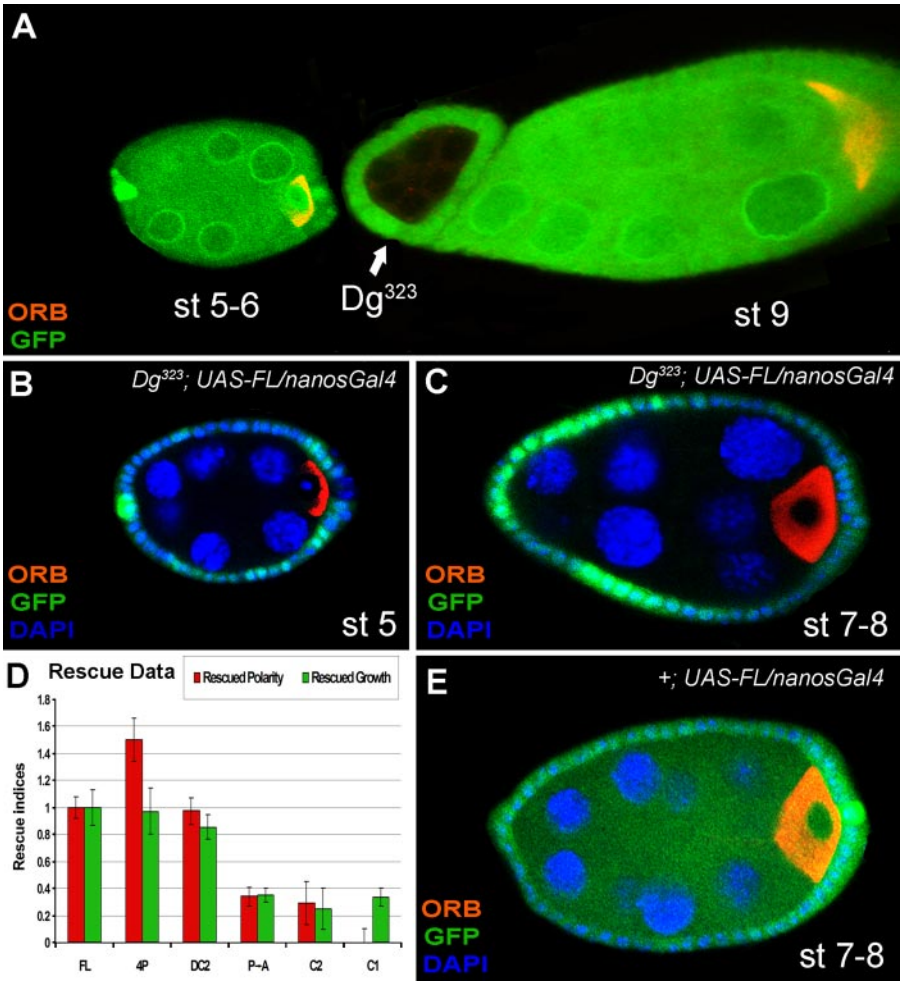


FIGURE 4. **Rescue of Dg loss-of-function germ line clones with expression of pUASp-Dg constructs.** Orb, *red*; GFP, *green*; DAPI, *blue*. *A,* Dg loss-of-function germ line clones (*black, white arrow; hsFLP; FRT42D Dg*^{323}) are arrested prior to stage 6 and have disrupted oocyte polarity (absent or mislocalized Orb). *B,* expression of pUASp-Dg with the *nanos-Gal4* driver in *Dg* clones rescues oocyte polarity in arrested clones stages 3–6 (as indicated by proper localization of Orb to the posterior of the oocyte (*hsFLP; FRT42D Dg*^{323}*; P(w+:nanosGal4:VP-16)Ab-2/pUASp-FL*)). *C,* expression of pUASp-Dg with the *nanos-Gal4* driver in Dg clones rescues oocyte growth to stage 7 or 8 (*hsFLP; FRT42D Dg*^{323}*; P(w+:nanosGal4:VP-16)Ab-2/pUASp-FL*). *D,* FL, 4P, and DC2 are able to rescue Dg loss-of-function phenotypes, whereas P → A, C2, and C1 do not (*red*, rescued polarity index; *green*, rescued growth index). The differences in rescue values between *FL, 4P, DC2, P→A, C2,* and *C1* are significant, and errors are based on independent experiments using independent insertion lines. *E,* although at earlier stages (4–6) the polarity marker Orb is normally localized at the posterior,and at later stages (7–8) Orb uniformly stains the oocyte cytoplasm (*hsFLP; FRT42D-UbiGFP; P(w+:nanosGal4:VP-16)Ab-2/pUASp-FL*).

age of embryos with mislocalized Crumbs staining in the salivary gland (Fig. 3*F*). DC2 and 4P constructs were capable of disrupting salivary gland epithelium polarity as well as FL (Fig. 3, *E–E″* and *F*); however, C1, C2, and P → A constructs did not disrupt polarity to the same extent as FL (Fig. 3*F*). Nevertheless, in all the assays described, some phenotypes above the control level were observed even with the constructs that lack most of the C-terminal domain (*C1*; Fig. 3, *F–H*), indicating that the Dg extracellular domain alone might function in some capacity to regulate cellular polarity (similar to seen in other systems (30, 39).

The Region of the Dystroglycan C Terminus Containing WWbsII and SH3bsII Can Rescue Dg Loss-of-Function Defects in Oocyte—Because Dg is required for oocyte polarity and the overproduction of Dg is sufficient to alter polarity, we analyzed which part of the Dg C-terminal proline-rich region is required for this function. We tested the capacity of our constructs to rescue the polarity of *Dg* germ line mutants. To avoid the polarity defects caused by overexpression of Dg at high levels in the germ line (*MatTub-Gal4*, Fig. 3, *B, C, G,* and *H*), we chose a *nanos-Gal4* driver that induces only low levels of Dg expression at stages 2–6 in oogenesis (supplemental Fig. S2*B*),and causes lower percentage of polarity defects even in the presence of two copies of the *nanos-Gal4* driver (supplemental Fig. S1*B* and supplemental Fig. S2*C*). Dg constructs expressed by only one *nanos-Gal4* copy resulted in even lower levels of Orb mislocalization (data not shown). We analyzed the ability to rescue *Dg* loss-of-function oocyte polarity defects using Dg constructs (Fig. 1) driven by one copy of *nanos-Gal4*. Low levels of FL Dg can partially rescue the *Dg* mutant phenotype, whereas C1 and C2 constructs do not have the same rescue capacity (Fig. 4, *A–D,* and Table 1). As with the overexpression experiments, this indicates that the other C-terminal binding sites, in addition to WWbsI, play a role in the establishment of oocyte polarity. Interestingly, the construct with defective SH3 domain binding sites (P → A) was also unable to rescue polarity to the FL levels (Fig. 4*D,* Table 1) further supporting the idea that SH3 domain binding sites in Dg play an important role in the oocyte polarity. DC2 and 4P, however, rescued oocyte polarity at the same level as the FL (Fig. 4*D* and Table 1), indicating that a single WW domain binding site and a single putative SH3 domain binding site on Dg C terminus are sufficient to partially rescue the establishment of oocyte polarity prior to stage 6, more specifically the anterior to posterior translocation of the microtubular organizing center during stages 1–3.

The Region of the Dystroglycan C Terminus Containing WWbsII and SH3bsII Can Rescue Egg Chamber Growth—In addition to oocyte polarity, Dg is required for egg chamber growth and development; most Dg mutant clones arrest by stage 6 (Fig. 4*A*) (26). To test which proline-rich sites in the C terminus of Dg, if any, are required to rescue egg chamber growth, we expressed the different Dg constructs (Fig. 1*B*) in *Dg* mutant clones using the *nanos-Gal4* driver expressing only one copy of *nanos-Gal4*, as was done for the polarity assay, and we tested how far these egg chambers developed.

TABLE 1
Dg constructs containing putative SH3- and WW-binding sites can rescue Dg loss-of-function phenotypes

Genotype	Egg chambers with growth rescue[a]	Egg chambers with normal Orb localization
Oregon	100%	90 ± 0.1%, $n = 125$
Dg^{323}[b]	12 ± 2%, $n = 54$[c,d]	8 ± 5%, $n = 58$
Dg^{323}; nanosGal4/pUASp-FL	47 ± 7%, $n = 40$	28 ± 2%, $n = 116$
Dg^{323}; nanosGal4/pUASp-4P	46 ± 8%, $n = 38$	38 ± 4.6%, $n = 68$
Dg^{323}; nanosGal4/pUASp-DC2	42 ± 3%, $n = 48$	28 ± 3%, $n = 68$
Dg^{323}; nanosGal4/pUASp-P → A	25 ± 3%, $n = 49$	15 ± 2%, $n = 49$
Dg^{323}; nanosGal4/pUASp-C2	21 ± 7%, $n = 46$	14 ± 5%, $n = 43$
Dg^{323}; nanosGal4/pUASp-C1	24 ± 3%, $n = 11$	8 ± 3%, $n = 40$
Dg^{323}; nanosGal4/pUASp-AATA	NA[e]	41 ± 1%, $n = 35$
Dg^{323}; nanosGal4/pUASp-ALLP	NA	16 ± 5%, $n = 45$

[a] Growth rescue data represent the percentage of mutant clones stage 6 and above from all clones that have reached at least stage 4.
[b] Dg^{323} indicates *hsFLP; FRT42D* Dg^{323}.
[c] ± indicates the average deviation calculated from analysis of independent insertion lines.
[d] *n* indicates the number of counted egg chambers with the correct genotype.
[e] NA indicates not analyzed.

To determine the degree to which full-length Dg could rescue egg chamber growth using this assay, we first expressed the FL construct in Dg clones and observed that FL was partially able to rescue growth (Fig. 4, *C* and *D*; Table 1). These "rescued" clones were classified as stage 7–8 based on the size of their egg chambers. However, in many cases, the oocyte was smaller than in wild type stage 7–8 oocytes.

Similar to what has been seen with the overexpression and polarity loss-of-function experiments, C1, C2, and P → A constructs were not able to rescue the growth to the FL levels (Fig. 4, *A–D*; Table 1). This indicates that the other C-terminal binding sites, in addition to WwbsI, play a role in the egg chamber growth and that putative SH3 domain binding sites are essential for full Dg function in the context of this assay. Nevertheless, some rescue above the control level was observed even with the construct that lacks most of the C-terminal domain (*C1*, Fig. 4*D*; Table 1), indicating that, as discussed earlier, the Dg extracellular domain alone might function in some capacity to regulate egg chamber growth. This is similar to what is seen with skeletal myotubes (39). DC2 and 4P, however, rescued egg chamber growth at the same level as the FL (Fig. 4*D*; Table 1), indicating that a single WW domain binding site in addition to a single putative SH3 domain binding site are sufficient for the function of Dg proline-rich C terminus for egg chamber growth.

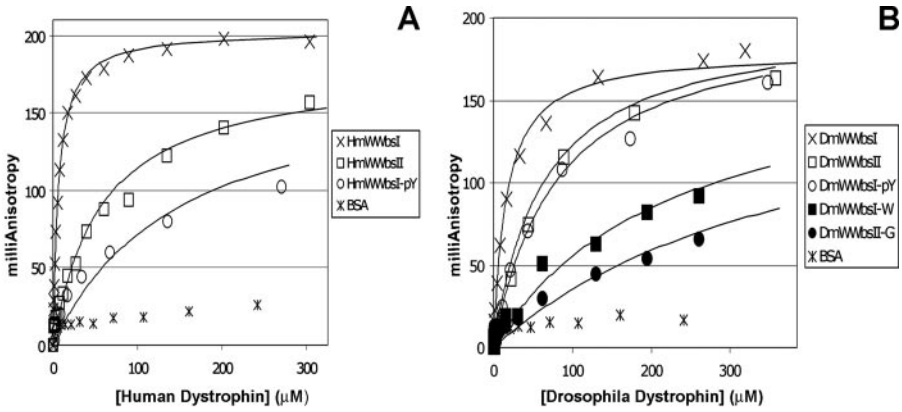
Again, we considered whether the ability to rescue growth and polarity was simply a result of the level of Dg protein overproduction, rather than the type of Dg construct. We compared the level of Dg production with the degree of polarity and growth rescue and found no correlation (supplemental Fig. S1, *C–D*). For example, FL expression varied between 2 and 4 times greater than wild type. However, the level of polarity or growth rescue did not correlate with the level of protein. This suggests that the amount of Dg overproduction in the oocyte is not responsible for differences in the degree of rescue and, therefore, that the differences in ability to rescue polarity and growth are a result of the presence of significant binding motifs.

Drosophila and Human Dystrophin Bind to Dystroglycan WWbsI and WWbsII in Vitro—Both overproduction and rescue experiments indicate that DC2 is able to affect cellular polarity to a similar extent as FL. DC2 includes one putative SH3 domain binding motif (SH3bsII) and one WW domain binding motif (WWbsII). Previous work in our laboratory has established the effectiveness of using a fluorescence polarization assay to measure binding of dystrophin (WW + EF regions) to the first WW domain binding motif (WWbsI) of dystroglycan (25). To assess the ability of dystrophin to bind this second WW domain binding motif (WWbsII), a second *Drosophila* dystroglycan peptide (DmWWbsII) that includes both the SH3bsII and WWbsII domain binding motifs present in DC2 was used. *Drosophila* dystrophin binds to DmWWbsII with a K_d of 46 ± 18 μM (Fig. 5, *A–C*). The affinity of this interaction is lower than that of *Drosophila* dystrophin and WWbsI (16 ± 4 μM), but it is still well within the range of reported dissociation constants for class I WW domains (49). In contrast, mutations predicted to abolish the WW (but not the SH3) binding domain resulted in much lower affinities (DmWWbsI-W, 178 μM and DmWWbsII-G,147 μM; Fig. 5, *B* and *C*). These values are comparable with the K_d value observed with a negative control for the assay (K_d for an unrelated peptide, p53 is 248 μM; Fig. 5*C*).

The second WW domain binding motif is conserved in human Dg, and the unexpected result above prompted us to investigate the same interaction between human dystrophin and human dystroglycan. Again, a second human dystroglycan peptide (HsWWbsII) was assayed for binding to human dystrophin. Human dystrophin binds to HsWWbsII with a K_d of 81 ± 11 μM, demonstrating that this interaction first seen with *Drosophila* peptides can also be seen with the corresponding human peptides (Fig. 5, *A–C*).

To further examine the specificity of the interaction between dystrophin and the second WW domain of dystroglycan, we tested the ability of human dystrophin to bind *Drosophila* dystroglycan peptide and vice versa. Human dystrophin does not bind to DmWWbsII (K_d 282 ± 18 μM); however, *Drosophila* dystrophin does bind to HsWWbsII (59 ± 10 μM; Fig. 5, *A–C*).

The observed importance of the SH3 domain binding sites in Dg (Fig. 1*B*, Fig. 3, *F–H*, and Fig. 4*D*) brings up the possibility that the SH3 domain of a tyrosine kinase could dock on that site, phosphorylate the tyrosine in WW-binding sites, and thereby affect dystrophin WW domain binding to this site. To test on what level tyrosine phosphorylation affects the WW domain binding in this assay, we tested dystrophin binding to Dg peptides that are tyrosine-phosphorylated (DmWWbsI-pY, DmWWbsII-pY, and HmWWbsI-pY). In both *Drosophila* and humans, tyrosine phosphorylation dramatically reduced WW domain binding (86 μM



C **Dissociation Constants (K_d, μM) for Dystrophin-Dystroglycan Interactions in human and *Drosophila***

Dystroglycan WWbs	Sequence	Dystrophin WW + EF Hand	
		Human	Drosophila
HmWWbsI	KNMTPYRSPPPYVSP	7.6 ± 1.6 (×)	3.7 ± 0.3
HmWWbsII	PLILQEEKAPLPPPEYSN	81 ± 11 (□)	59 ± 10
HmWWbsI-pY	KNMTPYRSPPP**pY**VSP	100 (○)	ND
DmWWbsI	GKSPATPYRKPPPYVSP	24 ± 8	16 ± 4 (×)
DmWWbsII	PVILKDEKPPLLPPSYNT	282 ± 18	46 ± 18 (□)
DmWWbsI-pY	GKSPATPYRKPPP**pY**VSP	ND	86 ± 10 (○)
DmWWbsII-pY	PVILKDEKPPLLPPS**pY**NT	ND	112 ± 5
DmWWbsI-W	GKSPATPYRK**W**PPYVSP	ND	178 ± 81 (■)
DmWWbsII-G	PVILKDEKPPLLPPS**G**NT	ND	147 ± 34 (●)
DmSH3bsII-A	PVILKDEKP**A**LLPPSYNT	ND	54 ± 20
p53	SQETFSDLWLLPEN		248

ND - not determined

FIGURE 5. Human and *Drosophila* dystrophin binds both dystroglycan WW-binding sites *in vitro* (*A and B*). Fluorescence polarization analysis of human dystrophin binding to 200 nM HmWWbsI (×), HmWWbsII (□), and HmWWbsI-pY (○) reveals binding to all peptides but shows significantly higher affinity for HmWWbsI and HmWWbsII compared with HmWWbsI-pY. *B, Drosophila* dystrophin binds to both the DmWWbsI (×) and DmWWbsII (□) but shows a significant loss of binding when these sites are mutated in DmWWbsI-W (■) and DmWWbsII-G (●) peptides. Tyrosine phosphorylation of WWbsI (DmWWbsI-pY) (○) results in reduced binding compared with nonphosphorylated DmWWbsI (×). Bovine serum albumin (*BSA*; *) binding by HmWWbsI (*A*) or DmWWbsI (*B*) serves as a nonspecific protein binding control. *C, table* that represents the dissociation constants (K_d, μM) for dystrophin-dystroglycan interactions in human and *Drosophila*.

compared to 16 μM, 112 μM to 46 μM and 100 μM to 7.6 μM, respectively; Fig. 5, *A–C*).

A Putative SH3 Domain Binding Motif Is Critical for Dg Function in Oocyte Polarity—Both overexpression and loss-of-function experiments revealed that SH3 domain binding sites in the Dg C terminus are essential for its function in cellular polarity. To further dissect which of the two SH3 domain binding sites is critical, we disrupted each site independently by proline to alanine substitutions and tested the capacity of the mutant proteins to affect oocyte polarity in both loss-of-function and overexpression analyses. Two proline to alanine substitutions in the SH3bsI caused no reduction in the activity of the wild type protein in overexpression and loss-of-function experiments (Fig. 6, *B–D*, *AATA*; Table 1). In sharp contrast, a single proline to alanine substitution in SH3bsII is functionally equivalent to deletion of the whole proline-rich region (Fig. 6, *B–D*, *ALLP*; Table 1). Thus, SH3bsII but not SH3bsI appears to be required for Dg function in oocyte polarity. Furthermore, the mutation apparently interferes specifically with SH3 domain binding and not WW domain binding because dystrophin WW domain still binds to the Dg peptide with this mutation *in vitro* (DmSH3bsII-A, K_d = 54 μM; Fig. 5*C*).

DISCUSSION

Unexpectedly, we find that the WW domain binding site at the very C terminus of dystroglycan, which has been previously implicated in dystrophin binding, is not essential for the function of the Dg·Dys complex in cellular polarity in *Drosophila*. Instead, an internal region of the dystroglycan C terminus containing a second WW domain binding site and a putative SH3 domain binding site appears to be sufficient for function in this context. We also find that mutating a single proline to alanine within this conserved, putative SH3 domain binding site dramatically reduces the functionality of this protein when compared with full-length Dg in these assays (Fig. 6, *B–D*, *ALLP*; Table 1). Finally, we show that dystrophin binds both the C-terminal and the internal WW domain binding site *in vitro* and that these interactions are conserved between humans and flies (Fig. 6*A*). Taken together, these results suggest that the internal WW domain binding site can mediate interactions with dystrophin, and presently unidentified SH3 domain containing protein(s) may functionally interact with a conserved region of the dystroglycan C terminus.

Previous studies (16–18) have indicated that dystrophin primarily binds to the first but not the second PP*X*Y motif of dystroglycan. In contrast, here we show that dystrophin can indeed bind this second WW domain binding motif (WWbsII), and we suggest that from the C-terminal proline-rich region this site in combination with an SH3 domain binding site is sufficient for Dg C-terminal function in the establishment of cellular polarity in *Drosophila*. *In vitro*, dystrophin binds WWbsII with lower affinity than WWbsI (46 μM compared with 16 μM). It appears that WWbsII functions just as well as WWbsI in our *in vivo* assays, because the DC2 phenotype is similar to the FL phenotype in both overproduction and loss-of-function experiments.

The *in vitro* interaction between dystrophin and the Dg WWbsII is conserved in humans. This is interesting in light of the fact that mutations in Dg are not observed clinically in patients with MD; instead, mutations in Dys, Dg-modifying enzymes, or extracellular matrix proteins result in MD (1). Because Dg knockouts die during embryonic development in mice and as an oocyte in *Drosophila,* it was assumed that the

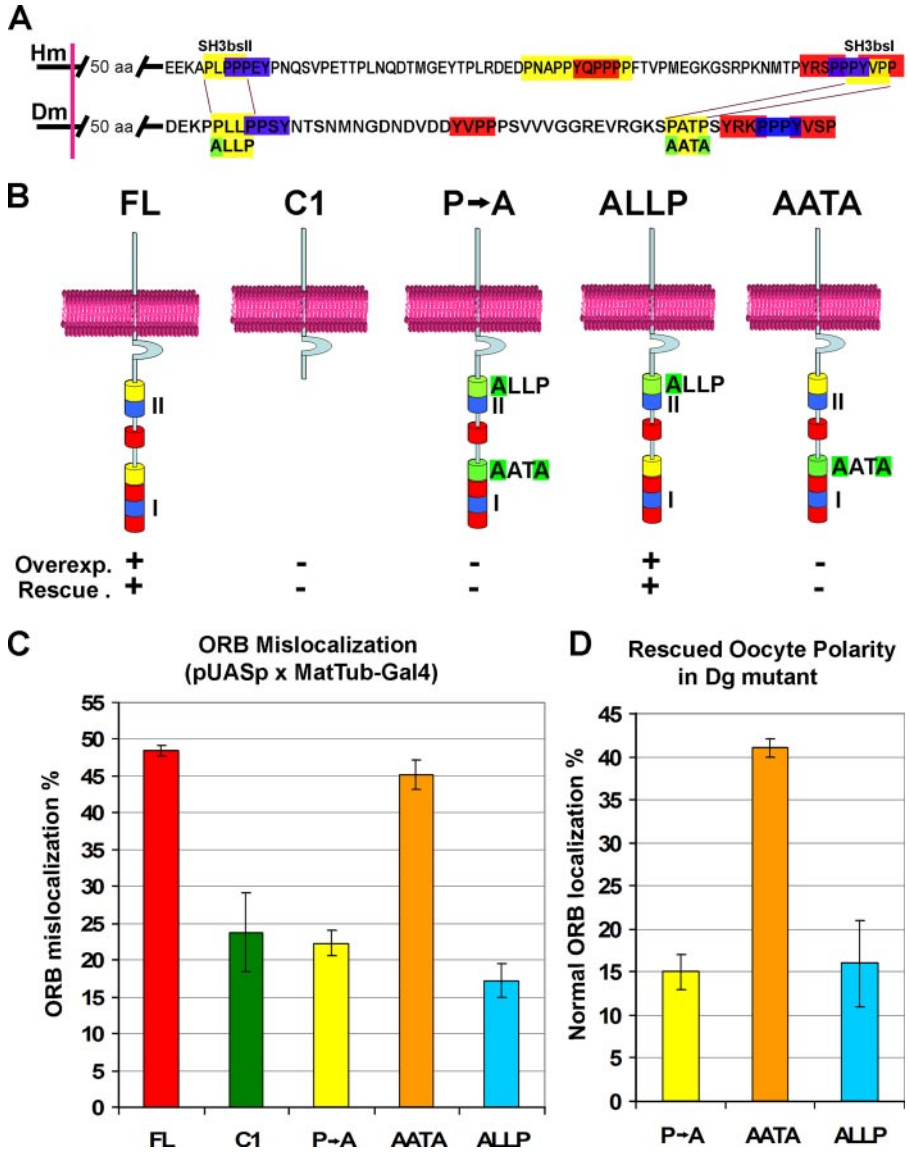


FIGURE 6. **SH3bsII but not SH3bsI is required for Dg function.** Two proline to alanine substitutions in the SH3bsI (*AATA*; *A* and *B*) cause no reduction in the activity of the wild type protein in overexpression (*C*, *Mat-α4-Tub > Gal4-UP16; pUASp-Dg*) or loss-of-function rescue (*D*, *hsFLP; FRT42D Dg*^{323}; *P(w+:nanosGal4:VP-16)Ab-2/p-pDg*) assays. In sharp contrast, a single proline to alanine substitution in SH3bsII (*ALLP*, *A* and *B*) completely eliminates the activity and is functionally equivalent to deletion of the whole proline-rich region (*C* and *D*). SH3bsII is conserved between human (*Hm*) and *Drosophila* (*Dm*) proline-rich C terminus of Dg (SH3bsII, *A*). SH3bs, *yellow*; SH3bs, *red*; WWbs, *blue*.

lack of MD patients with any mutations in Dg could be explained by its lethality. However, the results presented suggest a potentially new explanation; perhaps WWbsI and WWbsII are redundant. Perhaps humans with a mutation in WWbsI exist, but they do not show any MD phenotypes because WWbsII can substitute in place of the mutant WW domain binding site.

As discussed previously, *in vitro* work suggests that when the tyrosine of the PPPY motif (WWbsI) is phosphorylated, the binding between Dg and Dys is abolished (19), signifying that the Dg·Dys complex may be regulated in a tyrosine phosphorylation-dependent manner. In this study, we show using a quantitative assay that tyrosine phosphorylation of either of the two WW domain binding sites, PPPY motif or PPSY motif, does reduce the binding affinity (Fig. 5, *A*–*C*), suggestive of fine regulation. In addition, our *in vivo* work indicates that a putative SH3 domain binding site in Dg is required for proper function of the protein. These data suggest a more specific mechanism of regulation. One possibility is that an SH3 domain containing tyrosine kinase may dock to the SH3 domain binding site in Dg. This may result in a kinase activation and phosphorylation of the WW domain binding site in Dg thereby reducing dystrophin binding to this site.

The evidence thus far regarding the regulation of the Dg-Dys interaction depicts a model that strikingly resembles what we know about integrin-talin interactions (40–42). Integrins are heterodimeric, transmembrane proteins that like dystroglycan link the extracellular matrix to the intracellular cytoskeleton. The NP*X*Y motif on the integrin β subunit interacts with talin, an actin-binding protein, via the F3 subdomain within the FERM domain of talin, a PTB-like domain (43). Talin plays a role analogous to dystrophin by binding the NP*X*Y motif on integrin β cytoplasmic tails and linking integrins to the actin cytoskeleton. Binding of Talin to the NP*X*Y motif is required for energy-dependent activation of integrins (44). In addition to performing analogous structural roles, a similar regulatory mechanism may exist. It is known that integrin-talin interaction is mediated in a phosphorylation-dependent manner. When the tyrosine of the NP*X*Y motif is phosphorylated, binding of the integrin to talin is abolished (43, 45–47). Focal adhesion kinase and integrin-linked kinase bind to integrins *in vitro* and may regulate integrin-talin interaction, although this remains to be demonstrated *in vivo*. Furthermore, several other proteins, including platelet myosin, SHC, and Grb2, have been shown to bind integrins in their phosphorylated state *in vitro* (48). This study provides evidence that a similar mechanism may act to regulate Dg-Dys interaction. We have shown that a P*X*XP motif, which may be an SH3 domain binding site, is important for Dg function, opening up the possibility that an SH3 domain containing kinase may bind to Dg and phosphorylate the tyrosine on the WW domain binding site. Other signaling molecules may then interact with Dg in a phosphorylation-dependent manner.

We have identified two putative signaling molecule binding sites, the second WW domain binding site and a putative SH3

domain binding site, that are important for the regulation of the Dg complex. The key question now is to identify the signaling molecules that bind to these sites. Previous work has revealed SH3 domain-mediated interaction between Dg and Grb2 (21). However, we have not been able to observe direct binding between Dg and Drk, a *Drosophila* homologue of Grb2 (K_d = 480 μM), suggesting a different candidate for the SH3 domain interaction in *Drosophila*. Identification of the critical molecule that will associate with the putative SH3 domain binding site in Dg will further our understanding of the role Dg plays in signaling and may provide new insights into the pathogenesis of muscular dystrophy.

Acknowledgments—We thank Dr. Volodya Shcherbatyy for injections to produce the transgenic lines; Mona Radheshwar for help with protein quantification; David Chang for assistance with protein purification and fluorescence polarization analysis; and Mario Pantoja for critical reading of the manuscript.

REFERENCES

1. Davies, K. E., and Nowak, K. J. (2006) *Nat. Rev. Mol. Cell Biol.* **7,** 762–773
2. Barresi, R., and Campbell, K. P. (2006) *J. Cell Sci.* **119,** 199–207
3. Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G., and Campbell, K. P. (1990) *Nature* **345,** 315–319
4. Lapidos, K. A., Kakkar, R., and McNally, E. M. (2004) *Circ. Res.* **94,** 1023–1031
5. Langenbach, K. J., and Rando, T. A. (2002) *Muscle Nerve* **26,** 644–653
6. Cohn, R. D., Henry, M. D., Michele, D. E., Barresi, R., Saito, F., Moore, S. A., Flanagan, J. D., Skwarchuk, M. W., Robbins, M. E., Mendell, J. R., Williamson, R. A., and Campbell, K. P. (2002) *Cell* **110,** 639–648
7. Moore, S. A., Saito, F., Chen, J., Michele, D. E., Henry, M. D., Messing, A., Cohn, R. D., Ross-Barta, S. E., Westra, S., Williamson, R. A., Hoshi, T., and Campbell, K. P. (2002) *Nature* **418,** 422–425
8. Cohn, R. D. (2005) *Neuromuscul. Disord.* **15,** 207–217
9. Martin-Rendon, E., and Blake, D. J. (2003) *Trends Pharmacol. Sci.* **24,** 178–183
10. Martin, P. T. (2003) *J. Neurocytol.* **32,** 915–929
11. Martin, P. T., and Freeze, H. H. (2003) *Glycobiology* **13,** R67–R75
12. Muntoni, F., Brockington, M., Torelli, S., and Brown, S. C. (2004) *Curr. Opin. Neurol.* **17,** 205–209
13. Sgambato, A., and Brancaccio, A. (2005) *J. Cell. Physiol.* **205,** 163–169
14. Henry, M. D., Cohen, M. B., and Campbell, K. P. (2001) *Hum. Pathol.* **32,** 791–795
15. Muschler, J., Levy, D., Boudreau, R., Henry, M., Campbell, K., and Bissell, M. J. (2002) *Cancer Res.* **62,** 7102–7109
16. Jung, D., Yang, B., Meyer, J., Chamberlain, J. S., and Campbell, K. P. (1995) *J. Biol. Chem.* **270,** 27305–27310
17. Rentschler, S., Linn, H., Deininger, K., Bedford, M. T., Espanel, X., and Sudol, M. (1999) *Biol. Chem.* **380,** 431–442
18. Huang, X., Poy, F., Zhang, R., Joachimiak, A., Sudol, M., and Eck, M. J. (2000) *Nat. Struct. Biol.* **7,** 634–638
19. Sotgia, F., Lee, H., Bedford, M. T., Petrucci, T., Sudol, M., and Lisanti, M. P. (2001) *Biochemistry* **40,** 14585–14592
20. Ilsley, J. L., Sudol, M., and Winder, S. J. (2002) *Cell. Signal.* **14,** 183–189
21. Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G., and Campbell, K. P. (1995) *J. Biol. Chem.* **270,** 11711–11714
22. Spence, H. J., Dhillon, A. S., James, M., and Winder, S. J. (2004) *EMBO Rep.* **5,** 484–489
23. Zhou, Y. W., Thomason, D. B., Gullberg, D., and Jarrett, H. W. (2006) *Biochemistry* **45,** 2042–2052
24. Kay, B. K., Williamson, M. P., and Sudol, M. (2000) *FASEB J.* **14,** 231–241
25. Shcherbata, H. R., Yatsenko, A. S., Patterson, L., Sood, V. D., Nudel, U., Yaffe, D., Baker, D., and Hannele Ruohola-Baker, D. (2007) *EMBO J.* **26,** 481–493
26. Deng, W. M., Schneider, M., Frock, R., Castillejo-Lopez, C., Gaman, E. A., Baumgartner, S., and Ruohola-Baker, H. (2003) *Development* (*Camb.*) **130,** 173–184
27. Schneider, M., Khalil, A. A., Poulton, J., Castillejo-Lopez, C., Egger-Adam, D., Wodarz, A., Deng, W. M., and Baumgartner, S. (2006) *Development* (*Camb.*) **133,** 3805–3815
28. Poulton, J. S., and Deng, W. M. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 12775–12780
29. van der Plas, M. C., Pilgram, G. S., Plomp, J. J., de Jong, A., Fradkin, L. G., and Noordermeer, J. N. (2006) *J. Neurosci.* **26,** 333–344
30. Weir, M. L., Oppizzi, M. L., Henry, M. D., Onishi, A., Campbell, K. P., Bissell, M. J., and Muschler, J. L. (2006) *J. Cell Sci.* **119,** 4047–4058
31. Tracey, W. D., Jr., Ning, X., Klingler, M., Kramer, S. G., and Gergen, J. P. (2000) *Genetics* **154,** 273–284
32. Van Doren, M., Williamson, A. L., and Lehmann, R. (1998) *Curr. Biol.* **8,** 243–246
33. Hacker, U., and Perrimon, N. (1998) *Genes Dev.* **12,** 274–284
34. Wodarz, A., Hinz, U., Engelbert, M., and Knust, E. (1995) *Cell* **82,** 67–76
35. Pignoni, F., and Zipursky, S. L. (1997) *Development* (*Camb.*) **124,** 271–278
36. Rorth, P. (1998) *Mech. Dev.* **78,** 113–118
37. Kahl, J., and Campanelli, J. T. (2003) *J. Neurosci.* **23,** 392–402
38. Heathcote, R. D., Ekman, J. M., Campbell, K. P., and Godfrey, E. W. (2000) *Dev. Biol.* **227,** 595–605
39. Tremblay, M. R., and Carbonetto, S. (2006) *J. Biol. Chem.* **281,** 13365–13373
40. Brown, N. H., Gregory, S. L., Rickoll, W. L., Fessler, L. I., Prout, M., White, R. A., and Fristrom, J. W. (2002) *Dev. Cell* **3,** 569–579
41. Carman, C. V., and Springer, T. A. (2003) *Curr. Opin. Cell Biol.* **15,** 547–556
42. Kinashi, T. (2005) *Nat. Rev. Immunol.* **5,** 546–559
43. Campbell, I. D., and Ginsberg, M. H. (2004) *Trends Biochem. Sci.* **29,** 429–435
44. Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H., and Calderwood, D. A. (2003) *Science* **302,** 103–106
45. Tapley, P., Horwitz, A., Buck, C., Duggan, K., and Rohrschneider, L. (1989) *Oncogene* **4,** 325–333
46. Ling, K., Doughman, R. L., Iyer, V. V., Firestone, A. J., Bairstow, S. F., Mosher, D. F., Schaller, M. D., and Anderson, R. A. (2003) *J. Cell Biol.* **163,** 1339–1349
47. Calderwood, D. A. (2004) *Biochem. Soc. Trans.* **32,** 434–437
48. Liu, S., Calderwood, D. A., and Ginsberg, M. H. (2000) *J. Cell Sci.* **113,** 3563–3571
49. Kato, Y., Ito, M., Kawai, K., Nagata, K., and Tanokura, M. (2002) *J. Biol. Chem.* **277,** 10173–10177