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Transgene Rescue Identifies an Essential Function for *Drosophila* β Spectrin in the Nervous System and a Selective Requirement for Ankyrin-2–binding Activity

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The protein spectrin is ubiquitous in animal cells and is believed to play important roles in cell shape and membrane stability, cell polarity, and endomembrane traffic. Experiments here were undertaken to identify sites of essential β spectrin function in *Drosophila* and to determine whether spectrin and ankyrin function are strictly linked to one another. The Gal4-UAS system was used to drive tissue-specific overexpression of a β spectrin transgene or to knock down β spectrin expression with dsRNA. The results show that 1) overexpression of β spectrin in most of the cell types studied was lethal; 2) knockdown of β spectrin in most tissues had no detectable effect on growth or viability of the organism; and 3) nervous system-specific expression of a UAS-β spectrin transgene was sufficient to overcome the lethality of a loss-of-function β spectrin mutation. Thus β spectrin expression in other cells was not required for development of fertile adult males, although females lacking nonneuronal spectrin were sterile. Previous data indicated that binding of the DAnk1 isoform of ankyrin to spectrin was partially dispensable for viability. Domain swap experiments here uncovered a different requirement for neuronal DAnk2 binding to spectrin and establish that DAnk2-binding is critical for β spectrin function in vivo.

INTRODUCTION

The protein spectrin is part of a submembrane scaffold found at the plasma membrane of most animal cells. Spectrin is especially prominent in mammalian brain making up 2.4% of total protein (Davis and Bennett, 1983). It was first described in neurons as a high-molecular-weight doublet of axonally transported polypeptides (Levine and Willard, 1981). Before it was recognized as an isoform of spectrin it was named fodrin (from the Greek *fodros* for lining) to reflect its distribution in neurons and other cells where it appears to line the plasma membrane.

Three broad categories of function have emerged from genetic and biochemical analyses of spectrin. 1) Structure: Spectrin was originally identified as a structural determinant of human erythrocyte membrane shape and stability (Lux and Palek, 1995). Likewise, structural effects have been observed at the node of Ranvier in βIV spectrin mutant mice (Lacas-Gervais *et al.,* 2004) and also in *Caenorhabditis elegans* where a knockout of spectrin in the nervous system led to axon rupture in the course of normal body movements (Hammarlund *et al.,* 2007). 2) Polarity: In most cells spectrin is concentrated within specialized subdomains of the plasma membrane. Through the adapter ankyrin, the spectrin cytoskeleton is believed to capture and stabilize a host of interacting plasma membrane proteins at their sites of function in a variety of cell types (reviewed by Dubreuil, 2006). In one particularly striking example, silencing of ankyrin G expression in hippocampal neurons led to loss of Na_v channels, βIV spectrin, NF-186, and NrCam from the axon initial segment and ultimately to a loss of axonal identity (Hedstrom *et al.,* 2008). In addition to its structural effects, βIV spectrin knockouts also led to a failure of Na_v channel accumulation at the node of Ranvier (Komada and Soriano, 2002; Lacas-Gervais *et al.,* 2004). 3) Traffic: Spectrin and ankyrin mutations also appear to affect the trafficking of associated membrane proteins, resulting in a failure in their delivery to the plasma membrane. E-cadherin, the Na,K ATPase, and cyclic nucleotide receptors all accumulate at intracellular sites when their interaction with ankyrin is disrupted (Kizhatil *et al.,* 2007, 2009; Stabach *et al.,* 2008). The basis for this effect on traffic has not been established, although there is evidence for a link between the spectrin cytoskeleton and microtubule motors in the secretory pathway (Takeda *et al.,* 2000; Muresan *et al.,* 2001; Holleran *et al.,* 2001; Lorenzo *et al.,* 2010).

Genetic studies in *Drosophila* have also established important roles for αβ spectrin and ankyrin in development (Featherstone *et al.,* 2001; Pielage *et al.,* 2005; Garbe *et al.,* 2007; Hulsmeier *et al.,* 2007), and loss-of-function mutations are lethal (Lee *et al.,* 1993; Dubreuil *et al.,* 2000; Koch *et al.,* 2008; Pielage *et al.,* 2008). Much of the work has focused on the nervous system where phenotypes include effects on axon pathfinding, synaptic vesicle release, and synaptic stability. However, it has been difficult to ascertain the exact relationship between these *Drosophila* phenotypes and the three categories of function described in other systems. In the case of spectrin mutants, lethality has been attributed to an essential requirement for spectrin function outside the nervous system (Hulsmeier *et al.,* 2007). In contrast, the

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DAnk2 isoform of ankyrin appears to be essential within the nervous system (Koch *et al.*, 2008; Pielage *et al.*, 2008). These apparent differences in spectrin and ankyrin requirements raise the question of whether or not their functions are strictly coupled to one another in vivo.

Here we performed a series of experiments aimed at elucidating the site(s) of essential spectrin function in *Drosophila* and the functional interdependence of spectrin and ankyrin. We took advantage of the Gal4-UAS system to directly test the requirements for spectrin in neurons and other cells. A panel of Gal4 lines was used to drive expression of a UAS-dsRNA transgene that potently knocks down β spectrin expression in the nervous system (Pielage *et al.*, 2005) and, in parallel experiments, to drive overexpression of a UAS-β spectrin transgene in the same set of tissues. Ultimately, the Gal4-UAS system was used to demonstrate that neuronal expression of β spectrin was sufficient to rescue the lethality of a loss-of-function mutation. With that information it became relevant to ask if the essential function of spectrin in the nervous system was dependent on its interaction with DAnk2. A DAnk2-EGFP reporter transgene was produced to monitor the behavior of ankyrin in vivo and to ask how two specific modifications of the ankyrin-binding site of spectrin affected ankyrin behavior. The results revealed an unexpected difference in the way that the two isoforms of ankyrin associate with spectrin, and that the biological activity of spectrin strictly depends on its ability to bind to DAnk2.

MATERIALS AND METHODS

Fly Stocks and Transgenes

The double-strand RNA (dsRNA) line carrying two autosomal β spectrin–specific inserts (UAS-β-$Spec^{dsRNA}$) was obtained from Dr. Graeme Davis (University of California, San Francisco, CA; Pielage *et al.*, 2005); the neuron-specific $elav^{c155}$-Gal4 driver, β-$Spec^{S012}$, and UAS-β-$Spec^{493}$ lines were obtained from Dr. Christian Klambt (Institute fur Neurobiologie, Muenster, Germany; Hulsmeier *et al.*, 2007); the DA-neuron driver *2-21*-Gal4 was from the Jan lab (University of California, San Francisco, CA; Grueber *et al.*, 2003); midgut *Mex*-Gal4 was from Dr. Graham Thomas (Penn. State University, University Park, PA; Philips and Thomas, 2006); prothoracic gland *phm*-Gal4 was from Dr. Pierre Leopold (IDBC Nice, France; Ono *et al.*, 2006). Cardia-specific *bab1*-Gal4 (BL6802), ubiquitous *tubulin*-Gal4 (BL5138), muscle *24B*-Gal4 (BL1767), wing-specific *MS1096*-Gal4 (BL8860), fat body *Cg*-Gal4 (BL7011), the heat-shock Gal4 line expressed in the salivary gland (BL1799), and *UAS-mCD8-GFP* reporter lines (BL5130 and BL 5136) were obtained from the Bloomington Stock Center (Bloomington, IL). β-$Spec^{em6}$, β-$Spec^{\alpha}$13, and UAS-DAnk1-EGFP were previously described (Dubreuil *et al.*, 2000; Das *et al.*, 2006).

We previously described the repetitive and nonrepetitive domains of α and β spectrin by their segment numbers (Das *et al.*, 2006). To avoid confusion with the growing mammalian spectrin literature, we adopt their convention of repeat numbering (where repeat 1 is the second structural domain in the linear sequence of β spectrin; Davis *et al.*, 2009; Ipsaro *et al.*, 2009; Ipsaro and Mondragon, 2010; Stabach *et al.*, 2009). We note however that by this convention the previously described β-$Spec^{\alpha}$13 transgene (Das *et al.*, 2006) actually contains the 12th repeat from α spectrin.

β-Spec$^{\alpha 8}$. The parent plasmid WUMB-β spectrin (Dubreuil *et al.*, 2000) was mutagenized to introduce a unique MluI restriction site at codon 1697 and a unique KpnI site at codon 1806, corresponding to the boundaries of repeat 14. The introduction of these sites created two single amino acid changes adjacent to repeat 14 (E1697A and E1807Y). Repeat 8 of α spectrin was amplified by PCR from *Drosophila* α spectrin cDNA N8 (Dubreuil *et al.*, 1989), with MluI and KpnI sites introduced via the primer sequences to allow in-phase replacement of β spectrin repeat 14.

UAS-α-Spec^{37} and UAS-β-Spec^{95}. The previously described myc-epitope–tagged coding sequences of α and β spectrin (Lee *et al.*, 1997; Dubreuil *et al.*, 2000, respectively) were recloned in the pUAST vector (Brand and Perrimon, 1993). The entire β spectrin insert was resequenced to rule out cloning errors as the basis for dominant negative phenotypes observed in vivo. Independent chromosomal insertions of the UAS-β Spectrin (UAS-β-$Spec^{62}$) and UAS-α Spectrin (UAS-α-$Spec^{27}$) transgenes were used in some experiments, as indicated.

UAS-Dank2S-EGFP. The coding sequence for the short form of *Drosophila* ankyrin 2 (DAnk2S) was produced using the BDGP cDNA RE03629 (Stapleton *et al.*, 2002), which is a member of the transcript class Ank2-RN in FlyBase (http://flybase.org) (Grumbling *et al.*, 2006). The coding sequence was modified by QuikChange mutagenesis (Stratagene, Santa Clara, CA) using the primers GACGGGCATTTTACCATTGGGAATTCTGAAAAAATGGTCAC (5′ EcoRI) and GCGCTTCTCCTCATTTGGGCCATGGACCAGGGCATTCATA (3′ NcoI). The NcoI site introduced at the stop codon allowed in-frame cloning of the DAnk2S coding sequence upstream of the enhanced green fluorescent protein (EGFP) coding sequence in pBluescript. Because of failure of the pBS construct to produce the expected product, we sequenced the insert and discovered a single-base deletion relative to the sequence reported in FlyBase at codon 552. After QuikChange mutagenesis to restore the missing base the construct yielded the desired IPTG-inducible fluorescent product. An EcoRI-NotI restriction fragment containing the complete coding sequence was subcloned in the pUAST vector to produce transgenic flies. A homozygous viable insertion on chromosome 3 was recombined with the heat-shock Gal4 chromosome to produce a line that constitutively expressed DAnk2S-EGFP in the salivary gland and midgut copper cells.

Microscopy

Whole flies were photographed with a Finepix F20 camera (Fujifilm, Valhalla, NY) on a dissecting microscope (MZF111; Leica Microsystems, Bannockburn, IL). Larval tissues were dissected and fixed as previously described (Dubreuil *et al.*, 2000) and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were captured using an FV500 confocal microscope (Olympus, Center Valley, PA) with a 40× Plan-Apo oil immersion objective and Fluoview 2.1 software. Brightness settings were adjusted for wild-type control specimens using the photomultiplier, and setting were kept constant for capturing data when samples were to be compared. Images were saved as "Experiments" in Fluoview and were converted to jpeg format by NIH ImageJ (http://rsb.info.nih.gov/ij/; Abramoff *et al.*, 2004). Montages were assembled using Photoshop 6.0 (Adobe Systems, San Jose, CA), and γ adjustments when required were performed for all panels simultaneously.

Antibodies

Rabbit anti-β spectrin serum (KCar; Dubreuil and Yu, 1994) was used for immunofluorescence and rabbit anti-β spectrin serum (337; Byers *et al.*, 1989) was used for Western blots. Myc-tag specific antibody 9E10 was from Sigma-Aldrich (St. Louis, MO) and mouse anti-GFP antibody was a gift from Dr. Nava Segev (University of Illinois, Chicago, IL). Protein kinase C antibody (sc-216) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immunofluorescent staining was carried out with Texas Red– (Zymed, South San Francisco, CA) or Cy3-labeled antibodies (Invitrogen, Carlsbad, CA) as indicated (Dubreuil *et al.*, 2000). Western blots were performed with alkaline phosphatase–coupled secondary antibodies (Zymed) and stained with bromochloroindolyl phosphate as previously described (Dubreuil and Yu, 1994). Filamentous actin was detected by incubation for 2 h at room temperature with rhodamine phalloidin (Invitrogen) diluted 1:1000 in Tris-buffered saline.

Rescue Cross

The neuronal driver *elav*-Gal4 was recombined onto the X chromosome of a female fly heterozygous for the spectrin loss-of-function mutation, β-$Spec^{em6}$. Heterozygous *elav*-Gal4-β-$Spec^{em6}$ female flies maintained over the *FM7* chromosomal balancer (which contains the wild-type β spectrin gene) were then outcrossed to male flies carrying the autosomal UAS-β spectrin wild-type transgene. Without rescue, the only surviving F_1 males are those that inherited the marked *FM7* balancer. The wild-type UAS-β spectrin transgenes rescued lethality at approximately the same frequency as a ubiquitin promoter-driven β spectrin transgene (Dubreuil *et al.*, 2000).

RESULTS

Relative Effects of Spectrin Over- and Underexpression

The experiments reported here grew from the initial observation that overexpression of β spectrin was disruptive to wing development. When a UAS-β spectrin transgene (UAS-β-$Spec^{95}$) was overexpressed in the developing wing disk using *MS1096*-Gal4 there was a gross failure in wing formation (Figure 1A, bottom). In contrast to the effects of β spectrin overexpression, α spectrin overexpression with the same driver (Figure S1A) had no apparent effect on wing development (Figure 1A, top). Thus the disruptive effects were not a trivial consequence of overexpressing a large protein product.

Lethal effects were observed in several other tissues when UAS-β-$Spec^{95}$ was overexpressed in an otherwise wild-type background. UAS-β spectrin overexpression in the fat body, prothoracic gland, cardia, muscle, and the midgut was 100%

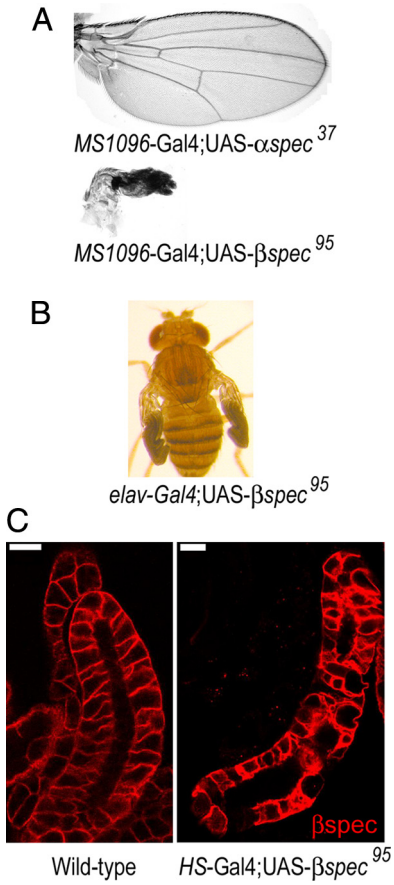


Figure 1. Overexpression of β spectrin in a wild-type background produced dominant negative effects in vivo. (A) Overexpression of UAS-α and UAS-β spectrin transgenes in the wing disk. Top, overexpression of the UAS-α-*Spec*^{37} transgene in the wing disk driven by *MS1096*-Gal4 did not affect wing development. Bottom, overexpression of the UAS-β-*Spec*^{95} transgene blocked normal wing development. (B) Overexpression of the UAS-β-*Spec*^{95} transgene driven by the neuronal *elav*-Gal4 caused defects in wing inflation/unfolding. (C) Overexpression of UAS-β-*Spec*^{95} in the salivary gland. Left, in control salivary glands, β spectrin was localized to the basolateral membranes of epithelial cells. β spectrin was detected with a rabbit anti-β spectrin antibody and a Cy3-labeled secondary antibody. Right, UAS-β-*Spec*^{95} was expressed in the salivary gland (sg) via basal activity of heat-shock Gal4, which resulted in perturbed epithelial cell polarity. β spectrin was aberrantly associated with the apical membrane domain, and some cells no longer showed cuboidal morphology. β-*Spec*^{95} was detected with mouse anti-myc antibody and Cy3-labeled secondary antibody. Scale bar, 10 μm.

larval lethal in each case (Table 1). Likewise, ubiquitous overexpression with the *tub*-Gal4 driver was lethal. Expression in the salivary gland did not affect viability, but did produce striking changes in cell shape and spectrin polarity (Figure 1C). β spectrin was strictly basolateral in wild-type cells (left panel). But with overexpression cells frequently lost their columnar morphology, and β spectrin became detectable on the apical as well as basolateral surfaces (right panel). Despite these effects on spectrin (and ankyrin) polarity, there were only modest effects of spectrin overexpression on other markers of epithelial polarity such as actin-rich microvilli and apical atypical protein kinase C (aPKC; Figure S2). Expression with the dendritic arborization (DA) neuron driver *2-21*-Gal4 or with *elav*-Gal4 produced little if any effect on viability. However, expression with these two drivers consistently interfered with wing inflation in the newly eclosed adult (Figure 1B). In contrast, overexpression with *pdf*-Gal4 did not produce a detectable effect on the circadian activity of the pdf neuron, on wing unfolding, or on viability (not shown).

In contrast to the dramatic effects observed with UAS-β-*Spec*^{95} overexpression, mutant phenotypes were rarely observed when β spectrin expression was knocked down with dsRNA (Table 1). As described previously (Pielage *et al.*, 2005), ubiquitous expression of two copies of the UAS-β spectrin dsRNA with *tub*-Gal4 produced a lethal phenotype, but *elav*-Gal4–driven expression of the dsRNA in neurons did not. Remarkably, none of the other Gal4 lines tested here produced a consistent lethal phenotype. In fact, the only conspicuous phenotype observed was in the fat body where there was a subtle change in the morphology of the plasma membrane, but without an effect on growth or viability of the organism (unpublished data). These results did not support the suggestion that β spectrin has a critical function outside of the nervous system (Hulsmeier *et al.*, 2007).

To rule out the possibility that the lack of a phenotype with dsRNA in nonneuronal tissues was simply due to poor dsRNA expression or efficiency, we performed immunofluorescent staining with an anti-β spectrin antibody (Figure 2). The efficacy of dsRNA knockdown of β spectrin in the nervous system was previously established (Pielage *et al.*, 2005). A comparable knockdown of β spectrin staining was observed in the salivary gland (Figure 2A) and in the fat body (Figure 2B). In both cases there was a dramatic tissue-specific loss of staining, whereas neighboring tissues not expressing dsRNA retained the normal level of staining with anti-β spectrin antibody. Thus, dsRNA expression in these tissues failed to produce a lethal phenotype despite a striking reduction in the abundance of β spectrin.

Nervous System Requirement for β Spectrin Function

The failure to phenocopy lethality by dsRNA knockdown of β spectrin in nonneuronal cells together with the recent demonstration that neuronal ankyrin was required for viability in *Drosophila* (Koch *et al.*, 2008; Pielage *et al.*, 2008) led us to revisit the question of whether or not the essential function of β spectrin resides in the nervous system. In previous mutant rescue experiments we scored the ability of a ubiquitously expressed autosomal β spectrin transgene to rescue F_1 males carrying the lethal β spectrin allele on X (Das *et al.*, 2006). The same single-generation rescue strategy was used here to rescue males carrying the lethal β spectrin allele by expressing UAS-β-*Spec*^{95} with the neuronal driver *elav*-Gal4. The *elav*-Gal4 insertion is also X-linked; therefore, we produced a recombinant chromosome carrying both the lethal β-*Spec*^{em6} mutation and *elav*-Gal4 (Figure 3A). Heterozygous females carrying the recombinant mutant chromosome over a wild-type balancer chromosome (*FM7*) were crossed to homozygous UAS-β-*Spec*^{95} males. If neuronal expression of UAS-β-*Spec*^{95} was not sufficient to rescue lethality, then all of the surviving F_1 male progeny should have carried the balancer chromosome with its wild-type copy of the β spectrin gene. However, nervous system expression of UAS-β-*Spec*^{95} was sufficient to allow a high rate of survival of F_1 males carrying the β-*Spec*^{em6}-*elav*-Gal4 chromosome (Figure 3, B and C). In initial experiments where flies were reared at 25°C the rescued males appeared somewhat unhealthy (crumpled wings, rough eyes, small size; Figure 3B) and were short-lived. Nevertheless, these males were fertile and thus represent a significant rescue of the embryonic lethality normally associated with the β spectrin loss-of-function mutation. The nervous system–specific expression of *elav* in the

Table 1. Relative effects of β spectrin overexpression and RNA interference knockdown

No.	Gal4 driver	*UAS-β-Spec*^{95}	*UAS-β-Spec dsRNA*
1	Ubiquitous (*tub*-Gal4)	Larval lethal	Larval lethal
2	Neurons (*elav*-Gal4)	Wing unfolding defect/partial lethality	No visible effect
3	DA neurons (*2-21*-Gal4)	Wing unfolding defect/partial lethality[a]	No visible effect
4	Pdf neurons (*pdf*-Gal4)	No effect	No visible effect
5	Prothoracic gland (*Phm*-Gal4)	Larval lethal[b]	Partial lethal[c]
6	Fat body (*Cg*-Gal4)	Larval lethal	Altered lipid droplets—not lethal
7	Cardia (*bab1*-Gal4)	Larval lethal	No visible effect
8	Muscle (*24B*-Gal4)	Larval lethal (29C)	No visible effect
9	Larval midgut (*Mex*-Gal4)	Larval lethal	No visible effect
10	Wing disc (*MS1096*-Gal4)	Failed wing development	No visible effect
11	Salivary gland (heat-shock Gal4)	Cell shape and polarity defects	No visible effect
12	Proneural (*Rotund*-Gal4)	Wing and bristle abnormalities	No visible effect

[a] These phenotypes were observed with an independent transgene stock *UAS-β-Spec*^{62} *in which β spectrin is expressed at a higher level. No defects were observed with 2-21* Gal4 and *UAS-β-Spec*^{95}.

[b] Lower-level expression in the prothoracic gland using the driver *P0206*-Gal4 led to the development of unusually large flies, presumably by delaying gland function rather than destroying activity.

[c] Lethality observed here was highly variable from experiment to experiment, but was noteworthy as the only example of lethality observed with tissue-specific β spectrin dsRNA expression.

embryo has been rigorously characterized (Berger *et al.*, 2007). Control experiments using the reporter UAS-mCD8-GFP verified that expression of *elav*-Gal4 was limited to the nervous system during larval development, except for promiscuous expression in the salivary gland (Figure S3).

One possible explanation for the apparent poor health of the mutants rescued by expression of β spectrin in the nervous system was that *β-Spec*^{95} expression was too limited; however, unexpectedly, we found that the opposite was true. The appearance of the rescued mutants improved when the level of β spectrin transgene expression was reduced. First, we repeated the rescue experiments using an independently produced UAS-β spectrin transgene (UAS-*β-Spec*^{493}; Hulsmeier *et al.*, 2007) that failed to produce the dominant negative phenotypes observed with UAS-*β-Spec*^{95}, because it is expressed at a lower level (data not shown). When rescue experiments were carried out with *elav*-Gal4–driven UAS-*β-Spec*^{493} expression the rescue flies appeared as healthy as controls, having properly unfolded wings and a full-size thorax and abdomen (Figure 3B). Thus the defects observed in initial experiments were actually due to β spectrin overexpression, rather than insufficient expression. On the basis of these observations, we reexamined the rescue activity of UAS-*β-Spec*^{95} expression by carrying out the rescue cross at reduced temperature (22°C). The level of transgene expression was reduced (Figure S1B), and we found that once again the appearance of the rescued flies was normal (Figure 3B). The flies were fertile, and it has been possible to propagate them as a stable line for many generations. Nevertheless, although the rescue flies appeared healthier when transgene expression level was lowered, their lifespan was still reduced to about the same extent as with UAS-*β-Spec*^{95} rescue at 25°C (Figure 4). This effect was not a trivial consequence of β spectrin overexpression, because reduction of lifespan was not observed when either UAS transgene was expressed in a wild-type background (not shown).

β Spectrin Function Relies on Its Ability to Bind to Neuronal DAnk2

Previous studies established that the general ankyrin isoform in *Drosophila* (DAnk1) binds to the 15th repeat unit of β spectrin and that ankyrin assembles downstream of β spectrin (Das *et al.*, 2006). A mutation affecting the ankyrin-

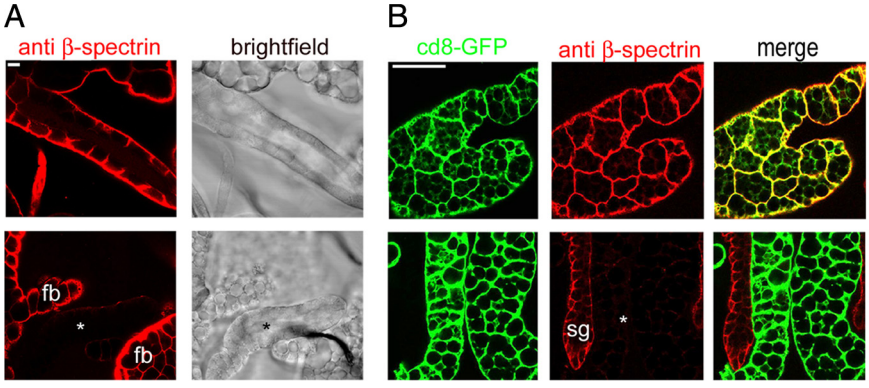
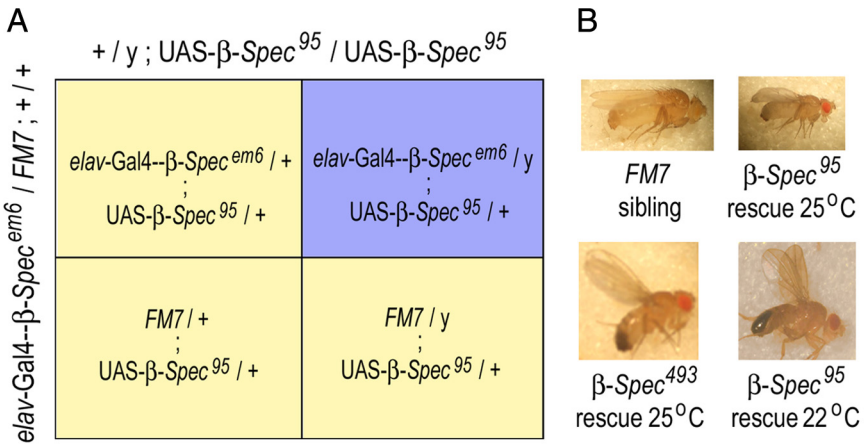


Figure 2. β spectrin knockdown with dsRNA. β spectrin was detected with a rabbit anti-β spectrin antibody and a Cy3-labeled secondary antibody. (A) Expression of UAS-β spectrin dsRNA in the salivary gland (using heat-shock Gal4 as in Figure 1) reduced β spectrin abundance. Top, β spectrin staining of wild-type salivary gland and fat body cells. Bottom, β spectrin dsRNA expression in the salivary gland reduced spectrin staining to background levels (*). Fat body cells (fb) not expressing the dsRNA construct remained strongly stained for β spectrin. Scale bar, 10 μm. (B) Expression of UAS-β spectrin dsRNA in the fat body with *Cg*-Gal4. Top, wild-type fat body cells, demonstrating that β spectrin (red) colocalized with the plasma membrane marker UAS-mCD8-GFP (green). Bottom, β spectrin immunofluorescence in the fat body was reduced to background levels by *Cg* -Gal4–driven expression of UAS-β spectrin dsRNA in the fat body (*). A salivary gland (sg) beside the fat body still expressed β spectrin at wild-type levels. Scale bar, 50 μm.



C

Transgene tested	Total females	*FM7* siblings	Rescue males	Total
UAS-β-*Spec*^{95}	189	32	26	247 (5 independent crosses)
UAS-β-*Spec*^{493}	130	82	46	258 (3 independent crosses)
UAS-α-*Spec*^{27}	49	21	0	70 (1 cross)

Figure 3. Neuronal expression of UAS-β-*Spec*^{95} was sufficient to rescue the lethality of β spectrin mutants. (A) Rescue cross. The neuronal driver *elav*-Gal4 was recombined onto the X chromosome of a female fly heterozygous for the spectrin loss-of-function mutation β-*Spec*^{em6}. Heterozygous *elav*-Gal4-β-*Spec*^{em6}/FM7; +/+ female flies were then crossed to male flies carrying the myc-tagged wild-type transgene (+/Y; UAS-β-*Spec*^{95}/UAS-β-*Spec*^{95}). Successful rescue was scored by the presence of a non-*FM7* F_1 male class (which lacks endogenous β spectrin). (B) Adult rescue flies. Flies rescued with the UAS-β-*Spec*^{95} transgene at 25°C were generally smaller than wild-type siblings (FM7/Y) and had wing unfolding defects similar to wild-type flies that overexpressed UAS-β-*Spec*^{95} (Table 1). In contrast, flies rescued with UAS-β-*Spec*^{493} at any temperature or with UAS-β-*Spec*^{95} at 22°C did not exhibit wing unfolding or body size defects. (C) Quantitative analysis of rescue. Neuronal expression of either the UAS-β-*Spec*^{95} or the UAS-β-*Spec*^{493} transgenes (both at 25°C) efficiently rescued the lethal β-*Spec*^{em6} mutation at about the same rate as a ubiquitously expressed β spectrin transgene described previously (Dubreuil *et al.*, 2000). Expression of UAS-α-*Spec*^{27} (negative control) did not produce the rescue class of male progeny.

binding activity of β spectrin repeat 15 did not affect the association of spectrin with the plasma membrane, but it shifted the distribution of DAnk1-EGFP from the plasma membrane to the cytoplasm. Here we tested the contribution of repeat 14 to ankyrin-binding activity by producing a similarly modified β spectrin transgene (Figure 5A). The coding sequence for β spectrin repeat 14 was replaced with divergent repeat 8 from α spectrin. The recombinant product was expressed from the ubiquitin promoter and examined for expression, targeting, and function in mutant rescue experiments. The myc epitope–tagged transgene product (β-Spec$^{\alpha 8}$) was readily detected in Western blots of total adult fly proteins (Figure 5B, lane 1) and was similar in abundance to the previously described β-Spec$^{\alpha 13}$ product (lane 2). Immunofluorescent staining of the transgene product in the larval salivary gland, in a mutant lacking endogenous β spectrin, revealed a bright pattern of staining of lateral contacts between cells (Figure 5C). The pattern was indistinguishable from that observed with other functional β spectrin transgene products. Thus the replacement of repeat 14 did not affect the stability or targeting of the protein in polarized epithelial cells. Nevertheless, the β-Spec$^{\alpha 8}$ transgene product altogether lacked biological activity in β-*Spec*^{em6} rescue experiments (data not shown). Thus, unlike β-*Spec*$^{\alpha 13}$, which frequently survives to adulthood, the repeat replacement in β-*Spec*$^{\alpha 8}$ was incompatible with spectrin function in vivo.

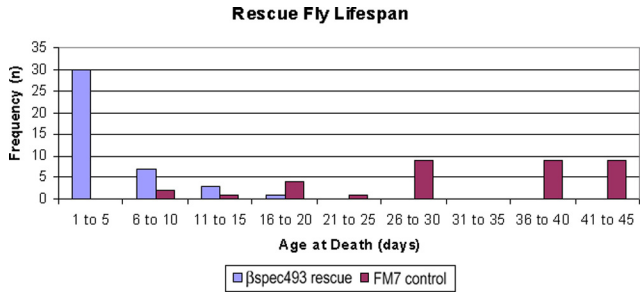


Figure 4. Lifespan of UAS-β-*Spec*^{493}-rescued β-*Spec*^{em6} mutants. Age at death was plotted for β-*Spec*^{em6} mutants rescued by the β-*Spec*^{493} transgene (blue) compared with *FM7* controls (red) all reared at 25°C. Similar results were obtained with the β-*Spec*^{95} transgene (not shown). Adult progeny from a representative rescue cross were scored daily to determine posteclosion age at time of death. Neuron-specific expression of β-*Spec*^{493} was sufficient to allow development of β spectrin mutants to adulthood, but the rescued flies were unusually short-lived.

The β-Spec$^{\alpha 8}$ mutant was also tested for its effects on the behavior of the previously described DAnk1-EGFP reporter and a new DAnk2-EGFP reporter. The recently described *Drosophila DAnk2* gene shares a number of features with *DAnk1* and their vertebrate counterparts (Koch *et al.*, 2008; Pielage *et al.*, 2008). Both proteins have an N-terminal membrane-binding domain comprised of 24 ankyrin repeats (Figure 5A). DAnk1 includes a central spectrin-binding domain followed by a small C-terminal domain of undefined function. In contrast, the short isoform (DAnk2S) studied here has its C-terminus within a truncated spectrin-binding domain. The UAS-*DAnk2-EGFP* transgene was produced by converting the stop codon of *DAnk2S* into an NcoI restriction site and then splicing it in-frame to the coding sequence of EGFP. A single-base deletion found in the initial construct, relative to the reported DAnk2 sequence in FlyBase, was repaired by QuikChange mutagenesis. The insertion was verified by DNA sequencing and inducible expression of the DAnk2S-EGFP fusion in *Escherichia coli* (Figure 5B, lane 4) was also verified before production of the *Drosophila* transformation vector.

The effects of β spectrin mutations on the interaction between ankyrin and spectrin can be assessed in vivo by simultaneously expressing the mutant β spectrin transgene

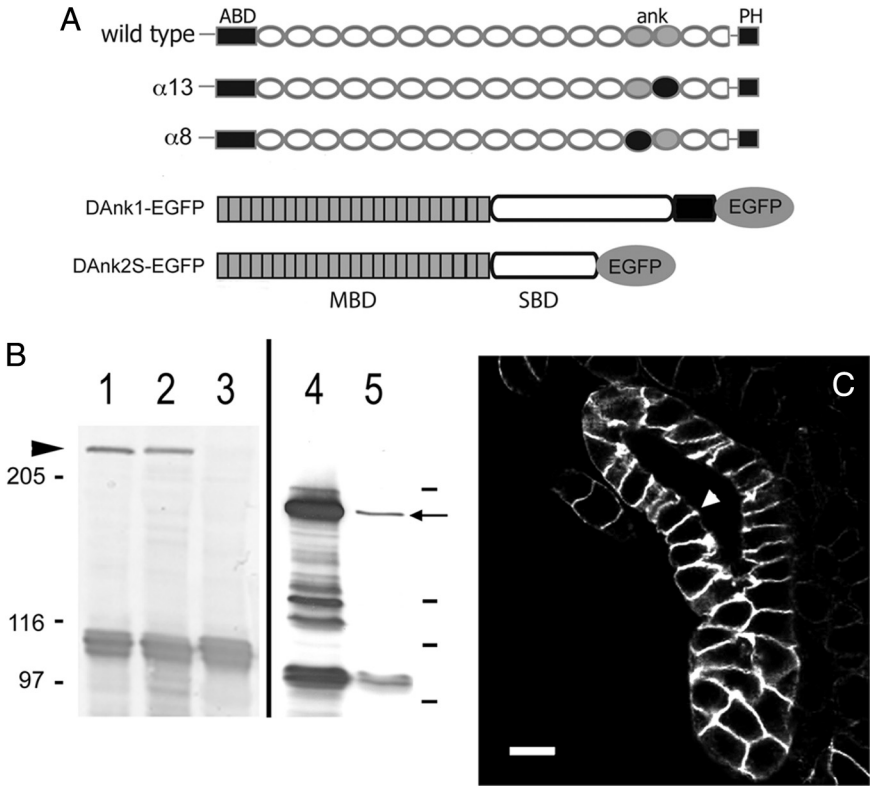


Figure 5. Production of modified β *spectrin* and *DAnk2* transgenes. (A) Top, β spectrin is divided into discrete structural domains, including an N-terminal actin-binding domain (ABD), a C-terminal pleckstrin homology (PH) domain, 16 degenerate ~106-amino acid–long spectrin repeats (ellipses), and one partial repeat near the C-terminus (Bennett and Baines, 2001). Two of the degenerate repeats (14 and 15) have been implicated in ankyrin (ank)-binding activity. Modified transgenes include α13 (ankyrin-binding repeat 15 of β spectrin replaced with repeat 12 of α spectrin; Das *et al.*, 2006) and α8 (ankyrin-binding repeat 14 of β spectrin replaced with repeat 8 of α spectrin). Bottom, conventional ankyrins are divided into three major structural domains: an N-terminal membrane binding domain (MBD) with 24 copies of a 33-amino acid ankyrin repeat, a central spectrin-binding domain (SBD), and a C-terminal domain that diverges widely among ankyrin isoforms (Bennett and Baines, 2001). UAS-DAnk transgenes were engineered with a C-terminal EGFP tag. The DAnk2S isoform produced here contains the N-terminal ankyrin repeat domain and a truncated spectrin-binding domain. The C-terminal domain found in other Dank2 isoforms (and DAnk1) is absent in Dank2S. (B) Western blots demonstrated that the transgenes are stably expressed and are the expected size. Total protein from flies constitutively expressing β-*Spec*$^{\alpha 8}$ (lane 1, arrowhead), β-*Spec*$^{\alpha 13}$ (lane 2), or no transgene (lane 3) was probed with mouse anti-myc antibody and rat anti-α actinin as a loading control. Total protein from induced (lane 4) or uninduced (lane 5) bacteria carrying a DAnk2S-EGFP insert was probed with anti-EGFP antibody. IPTG induction strongly elevated expression of the expected DAnk2S-EGFP product (arrow). Molecular-weight standards are indicated to the left in kDa (additional 66-kDa standard indicated for different percentage gel on right). (C) Mouse anti-myc antibody staining of the β-*Spec*$^{\alpha 8}$ transgene product in the salivary gland of a mutant larva lacking endogenous β spectrin. A goat anti-mouse Texas Red–conjugated secondary antibody was used. The β-*Spec*$^{\alpha 8}$ exhibited the same polarized basolateral distribution as wild-type β spectrin (arrowhead). Scale bar, 10 μm.

together with GFP-tagged ankyrin in a genetic background lacking endogenous wild-type β spectrin. Using this approach, we observed that DAnk1-EGFP targeting to the plasma membrane (Figure 6A) was reduced to near background levels in the absence of β spectrin (Figure 6B) or when repeat 15 was replaced (Figure 6C). The same loss of DAnk1-EGFP targeting was observed when repeat 14 was replaced (Figure 6D). The variable expression of the Gal4 driver used in these experiments (Das *et al.*, 2006) helps to establish that membrane targeting was lost at all expression levels.

We went on to analyze the behavior of DAnk2-EGFP in the same four backgrounds. Although *DAnk2* expression has not been detected outside the nervous system by in situ hybridization analysis of embryos (Bouley *et al.*, 2000; Koch *et al.*, 2008), we nevertheless observed robust DAnk2-EGFP accumulation at lateral contacts in the salivary gland of wild-type larvae (Figure 6E, cell marked by arrowhead). The targeting of DAnk2-EGFP was dependent on β spectrin because the plasma membrane signal was greatly diminished in salivary glands from β-*Spec*^{em6} mutant embryos (Figure 6F). There was a low-level nuclear signal in the DAnk2S-EGFP–expressing tissues that was often more conspicuous in the β spectrin mutants (e.g., Figure 6F, arrowhead). Unlike DAnk1-EGFP, much of the DAnk2-EGFP signal remained at the lateral plasma membrane in salivary glands from β-*Spec*$^{\alpha 13}$ mutants, where β spectrin repeat 15 is missing (Figure 6G). In contrast, the lateral signal was nearly eliminated in the β-*Spec*$^{\alpha 8}$ mutants, which lack β spectrin repeat 14 (Figure 6H). Thus it appears that β spectrin repeat 14 affects the binding of both ankyrin isoforms to β spectrin, but repeat 15 primarily affects the interaction with DAnk1. However, recruitment of both ankyrin isoforms to the plasma membrane was strictly dependent on the presence of functional β spectrin.

DISCUSSION

The results of β spectrin over- and underexpression in *Drosophila* provide some surprising new insights into spectrin biology in this model organism. First, expression of a UAS-β spectrin transgene in the nervous system was sufficient to overcome the lethality of a loss-of-function mutation in the endogenous β spectrin gene. Thus spectrin does not appear to be absolutely required for viability in most of the tissues where it is normally expressed. Second, spectrin activity was dependent on its ability to interact with DAnk2, indicating that spectrin and ankyrin function are intimately linked to one another. Previous studies had suggested that spectrin and DAnk1 function were not strictly linked to one another (Das *et al.*, 2006).

Spectrin Function in the Nervous System

The finding that spectrin expression in the nervous system is sufficient to rescue the lethality of loss-of-function mutations fits well with the recent demonstration that neuronal ankyrin is also an essential gene in *Drosophila* (Koch *et al.*, 2008; Pielage *et al.*, 2008). However, the present results differ

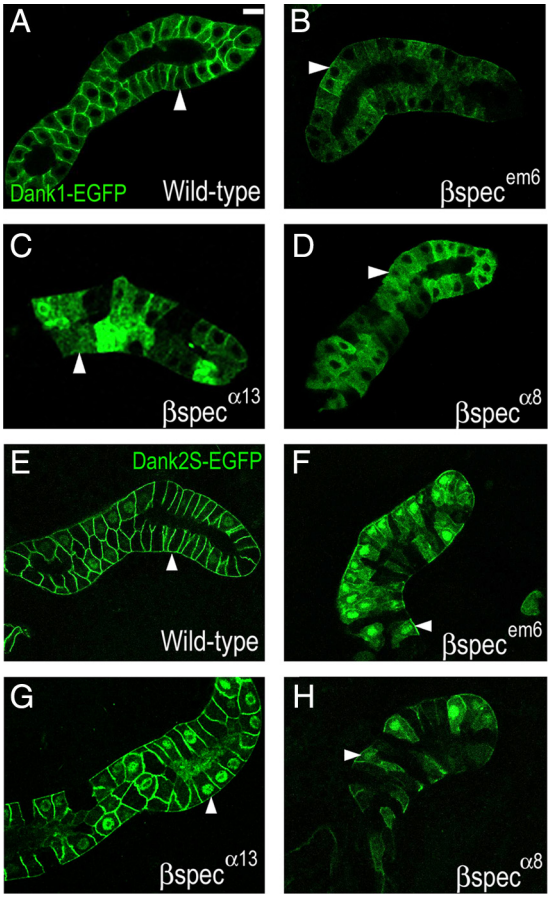


Figure 6. Targeting of DAnk1-EGFP and Dank2S-EGFP to the plasma membrane in β spectrin mutants. Transgene expression was driven in the salivary gland by heat-shock Gal4 and was detected by EGFP fluorescence and confocal microscopy. (A–D) DAnk1-EGFP. (E–H) DAnk2-EGFP. In wild-type controls (A and E) both DAnk-EGFP isoforms were prominently enriched at the basolateral surface of the salivary gland epithelial cells. In the absence of endogenous β spectrin (β-*Spec*^{em6} mutant) the membrane-labeling pattern was reduced to near background level (B and F). Note the variable level of DAnk-EGFP expression from cell to cell, resulting in strong cytoplasmic signal in some cells. Both spectrin repeat modifications (β-*Spec*$^{\alpha}$13, C; β-*Spec*$^{\alpha}$8, D) had a similar effect on the behavior of DAnk1-EGFP, reducing the membrane-specific signal to near background levels. The β-*Spec*$^{\alpha}$8 repeat modification had a comparable effect on the behavior of DAnk2S-EGFP (H). In contrast, DAnk2S-EGFP recruitment to the plasma membrane was left largely intact in the β-*Spec*$^{\alpha}$13 mutant (G). A nuclear signal (presumably an artifact of the DAnk2S-EGFP fusion) that was barely detectable in wild-type (E) became more conspicuous in mutant cells with high-level expression (F–H, arrowheads) but was never observed with DAnk1-EGFP (A–D).

from a previous study in which neuronal expression of a UAS-β spectrin transgene rescued an axonal patterning phenotype, but not the lethality of a loss-of-function mutant (Hulsmeier *et al.*, 2007). It is possible that rescue activity may have been missed in those studies because of the short lifespan of the rescued adults (~4 d). However, we were also unable to rescue the lethality of a representative mutant allele from the Hulsmeier study with either of the UAS-β spectrin transgenes (data not shown). It is formally possible that there is an allelic difference between β-*Spec*^{em6} and β-*Spec*^{S012} that allows the former but not the latter to be rescued. β-*Spec*^{em6} encodes a truncated protein that lacks major functional sites including the ankyrin-binding repeats and the tetramer formation site (Dubreuil *et al.*, 2000), and it has a greatly reduced abundance compared with wild type (Hulsmeier *et al.*, 2007). In contrast the β-*Spec*^{S012} allele is a protein null, and it is formally possible that β-*Spec*^{em6} retains some partial function that makes it compatible with rescue, even though it has the same embryonic lethal phenotype as β-*Spec*^{S012}. However, the small quantity of β-*Spec*^{em6} protein and its inability to form a spectrin network argue against this possibility. An alternative explanation is that there is an unidentified second site mutation or other background genetic effect that prevents transgene rescue of β-*Spec*^{S012} for reasons that are unrelated to spectrin.

It is intriguing that the rescued adults in our experiments are so short-lived. One strong possibility is that the effect on lifespan is linked to the loss of spectrin function outside the nervous system. For example, loss of spectrin may compromise immune system function, making the fly more susceptible to infection. Or, given the link between the spectrin cytoskeleton and membrane transport in mammals it is possible that loss of spectrin affects the behavior of an important transporter that normally prevents the accumulation of toxic compounds in wild-type flies.

Interdependence of Spectrin and Ankyrin

Another important issue that was resolved by the experiments here is the relationship between spectrin and ankyrin. We previously concluded that some functions of spectrin are independent of ankyrin, based on the observation that a mutation of repeat 15 of β spectrin blocked its ability to bind to DAnk1, but spectrin retained significant function in mutant rescue experiments (Das *et al.*, 2006). The finding here that 1) the previously described β-*Spec*$^{\alpha 13}$ mutant (repeat 15) retained significant DAnk2 binding activity, 2) that the β-*Spec*$^{\alpha 8}$ mutation in repeat 14 disrupted DAnk2 binding activity, and 3) that the loss of DAnk2 binding activity was incompatible with biological activity establishes that essential function of spectrin is critically dependent upon ankyrin-binding activity in neurons. These observations are generally consistent with recent structural studies showing that spectrin repeats 14 and 15 both contribute to the interaction between spectrin and ankyrin in mammals (Stabach *et al.*, 2009; Ipsaro *et al.*, 2009; Ipsaro and Mondragon, 2010; Davis *et al.*, 2009). With the current results showing that spectrin is not absolutely required outside the nervous system, it is now easy to see why binding to the nonneuronal DAnk1 appeared to be dispensable for spectrin function in previous studies (Das *et al.*, 2006): spectrin itself is not required for survival to adulthood in most *Drosophila* cells.

What is the ankyrin-dependent role of spectrin in neurons? Results from mammalian systems have focused attention on the role of ankyrin as an adapter that links the spectrin scaffold to interacting integral membrane proteins such as ion pumps and channels (reviewed by Dubreuil, 2006). Recent evidence suggests that this function may have been exploited to a greater extent in vertebrate evolution, because many of these ankyrin-membrane interactions do not appear to be conserved in the fly (Pan *et al.*, 2006; Dubreuil and Grushko, 1999; our unpublished observations). Phenotype analysis of DAnk2 mutants uncovered a role for ankyrin in the attachment of microtubules to the presynaptic membrane of neuromuscular junctions in *Drosophila* larvae (Koch *et al.*, 2008; Pielage *et al.*, 2008). Thus spectrin and ankyrin may exert important "inward" effects on cytoplasmic organization in addition to their "outward" effects on plasma membrane composition. It is intriguing that the *Drosophila* DAnk2 mutant phenotype includes

effects on axonal transport (Koch *et al.*, 2008), pointing to the possibility that the spectrin–ankyrin complex plays an additional role in intracellular traffic.

Is Spectrin Function Redundant?

We and others have envisioned *Drosophila* spectrin as forming a cross-linked network beneath the plasma membrane (e.g., Deng *et al.*, 1995; Pielage *et al.*, 2006), comparable to the hexagonal network of spectrin and actin found in human erythrocytes (Byers and Branton, 1985). This view is supported by the estimated quantity of spectrin per cell in the *Drosophila* embryo ($\sim 3 \times 10^5$ dimers), which is comparable to what is found in the human erythrocyte (Pesacreta *et al.*, 1989). At this density the spectrin network is believed to provide mechanical support to the plasma membrane (structure) and to provide a large number of potential docking sites to stabilize populations of interacting membrane proteins (polarity). Now it appears that this network can be greatly reduced or eliminated in most *Drosophila* cells without affecting survival to adulthood.

Yet, spectrin does not appear to be without function in nonneuronal tissues. Loss-of-function α and β spectrin mutations produce subtle defects in cell shape and organization in a number of different *Drosophila* tissues (Lee *et al.*, 1993, 1997; Dubreuil *et al.*, 2000; Pielage *et al.*, 2006), and in this study we observed a conspicuous change in the morphology of the plasma membrane with β spectrin knockdown in the fat body. The strong dominant negative effect of overexpression of β spectrin further suggests that spectrin is not without function. The lack of a lethal phenotype when spectrin is eliminated or greatly reduced may be due to the presence of a compensating activity with overlapping function. If so, this redundant activity is not likely to be another spectrin, because the only other spectrin in *Drosophila* ($\alpha\beta_H$) has markedly different properties and does not codistribute with conventional $\alpha\beta$ spectrin in most cells (Dubreuil and Grushko, 1998; Thomas, 2001).

Implications for Other Systems

Here we used survival to adulthood as a readout for spectrin function, leading to the conclusion that the embryonic lethality of *Drosophila* spectrin mutants is due solely to the loss of spectrin function in the nervous system. However, it is noteworthy that the animals that were scored as "viable" are not the same as wild type, and a human with the same genotype would probably be considered quite ill. These observations distinguish two distinct tiers of spectrin function in *Drosophila*. There is an essential function that resides in the nervous system, accounting for invariable lethality. In addition there are nonessential functions outside the nervous system (and perhaps within the nervous system too) that more subtly affect the quality and length of life. These nonessential functions (which are nevertheless important) may be most relevant to understanding the disease processes caused by spectrin and ankyrin defects in humans (reviewed by Bennett and Healy, 2008). We speculate that the nonneuronal phenotypes in *Drosophila* and disease phenotypes in humans are both influenced by the presence of redundant factors with at least partially overlapping function to circumvent lethality of spectrin mutations early in development. The genetic tools available in *Drosophila* make it an ideally suited model system with which to identify the genes responsible for redundant function.

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