

C1275 **Functional and Genetic Analysis  
of Spectraplakins in *Drosophila***

**Ines Hahn\*, Matthew Ronshaugen\*, Natalia Sánchez-Soriano<sup>†</sup>,  
Andreas Prokop\*,<sup>1</sup>**

\*Faculty of Life Sciences, Michael Smith Building, Manchester, United Kingdom

<sup>†</sup>Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Liverpool, United Kingdom

<sup>1</sup>Corresponding author: e-mail address: andreas.prokop@manchester.ac.uk

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**Abstract**

The cytoskeleton is a dynamic network of filamentous protein polymers required for virtually all cellular processes. It consists of three major classes, filamentous actin (F-actin), intermediate filaments, and microtubules, all displaying characteristic structural properties, functions, cellular distributions, and sets of interacting regulatory proteins. One unique class of proteins, the spectraplakins, bind, regulate, and integrate the functions of all three classes of cytoskeleton proteins. Spectraplakins are giant, evolutionary conserved multidomain proteins (spanning up to 9000 aa) that are true

members of the plakin, spectrin, and Gas2-like protein families. They have OMIM-listed disease links to epidermolysis bullosa and hereditary sensory and autonomic neuropathy. Their role in disease is likely underrepresented since studies in model animal systems have revealed critical roles in polarity, morphogenesis, differentiation and maintenance, migration, signaling, and intracellular trafficking in a variety of tissues. This enormous diversity of spectraplakin function is consistent with the numerous isoforms produced from single genomic loci that combine different sets of functional domains in distinct cellular contexts. To study the broad range of functions and complexity of these proteins, *Drosophila* is a powerful model. Thus, the fly spectraplakin, Short stop (Shot) acts as an actin–microtubule linker and plays important roles in many developmental processes, which provide experimentally amenable and relevant contexts in which to study spectraplakin functions. For these studies, a versatile range of relevant experimental resources that facilitate genetics and transgenic approaches, highly refined genomics tools, and an impressive set of spectraplakin-specific genetic and molecular tools are readily available. Here, we use the example of Shot to illustrate how the various tools and strategies available for *Drosophila* can be employed to decipher and dissect cellular roles and molecular mechanisms of spectraplakins.

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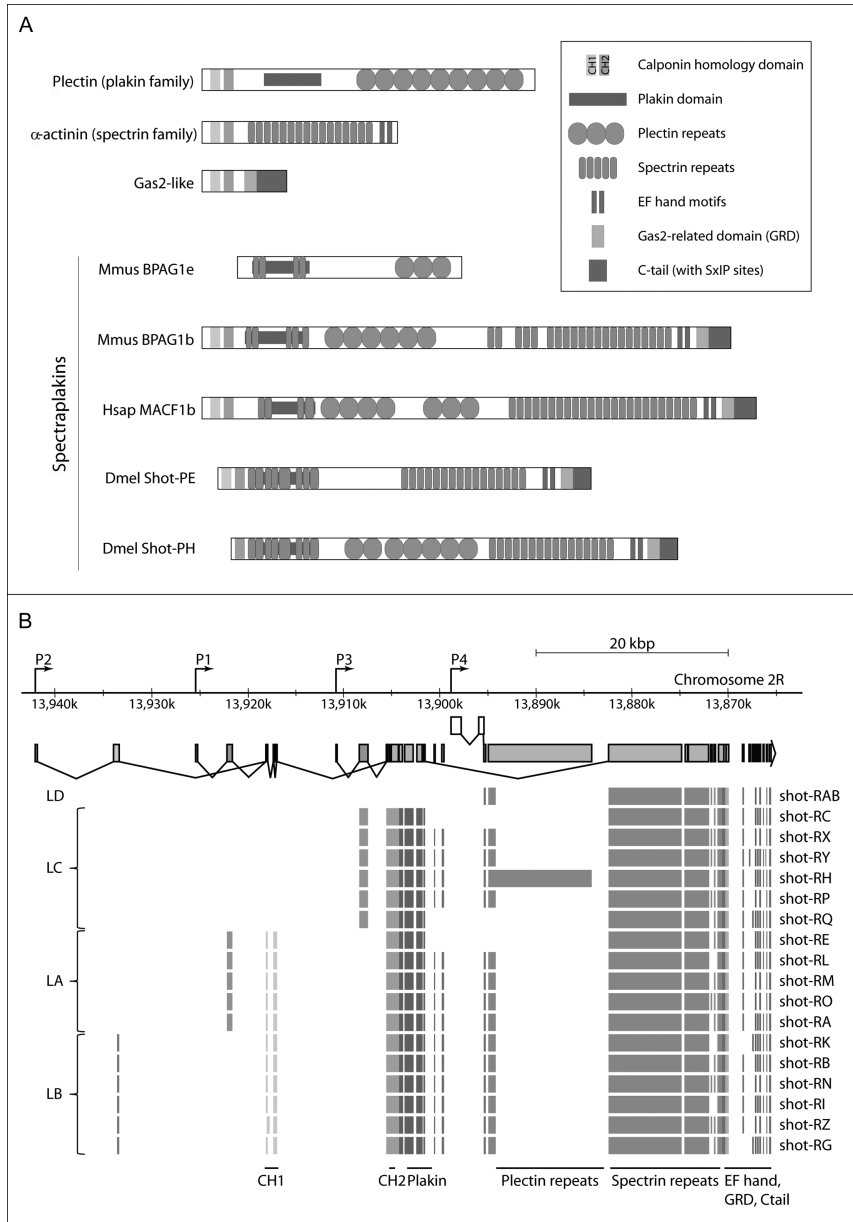
## 1. INTRODUCTION

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### 1.1 Structure, Roles, and Disease Links of Spectraplakins

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Spectraplakins are large proteins (up to 9000 aa, >400 nm) that are able to interact with all three classes of cytoskeletal filaments and link them either to each other or to other cellular structures, such as adhesion complexes. The best studied spectraplakins to date are Short stop (Shot) in *Drosophila*, Bullous Pemphigoid Antigen 1 (BPAG1/dystonin/BP320), and Microtubule–Actin Cross-linking Factor 1 (MACF1/Actin Cross-linking Family 7/ACF7) in mammals, as well as VAB-10 in *Caenorhabditis elegans*. As illustrated for Shot in Fig. 1A, the genomic loci encoding spectraplakins generate numerous isoforms with up to seven recognized functional domains that establish them as true members of three protein families, the plakins, spectrins, and Gas2-like proteins (Brown, 2008; Röper et al., 2002; Suozzi, Wu, & Fuchs, 2012): (1) The plakins (typical other members: plectin, desmoplakin, envoplakin, periplakin, and epiplakin) are cytoskeleton-associated scaffold proteins primarily at cell junctions crucial for maintaining tissues under mechanical stress (e.g., in the skin or heart; Sonnenberg & Liem, 2007). (2) The spectrins ( $\alpha$ -/ $\beta$ -spectrin,  $\alpha$ -actinin, dystrophin, and utrophin) are highly conserved actin-linked scaffolding proteins primarily at the cell cortex which form essential links between membrane and cytoplasmic proteins (Broderick & Winder,



**Figure 1** Family associations, homologues, functional domains, and isoforms of Shot. (A) Images show three typical representatives of the plakin, spectrin, and Gas2-like protein families, compared to specific isoforms of spectraplakins. Here shown are the shortest e- and longest b-isoform of mouse BPAG1 (Young, Pool, & Kothary, 2003), the longest b-isoform of human MACF1, and the long Shot-RE and Shot-RH isoforms (Continued)

2005; Machnicka et al., 2014). (3) The Gas2-like proteins (Gas2, Gas2-like 1–3) act as linkers between MTs, end-binding (EB) proteins, and F-actin, important for cytoskeletal dynamics in cell division and development (Sharaby et al., 2014; Stroud et al., 2014; Wolter et al., 2012).

p0010 Notably, spectraplakins have maintained fundamental functions associated with all three protein families and, accordingly, the range of their functions is huge, and their loss often leads to early lethality. For example, the epidermal isoform of BPAG1/dystonin is an important constituent of hemidesmosomes in basal epithelial layers, particularly in the skin where autoimmune or hereditary functional aberration causes skin blistering of

**Figure 1—Cont'd** (compare B). The key (emboxed) shows the different functional domains: The N-terminus of Dystonin-a2 promotes localization to the nuclear envelope (Young & Kothary, 2007; Young et al., 2003). Two calponin homology domains (CH1 and CH2) form a classical actin-binding domain (ABD) essential for neuronal morphogenesis (Alves-Silva et al., 2012; Jefferson, Leung, & Liem, 2004; Kodama, Lechler, & Fuchs, 2004; Lee & Kolodziej, 2002b; Leung, Sun, Zheng, Knowles, & Liem, 1999). The plakin-homology domain (six to nine spectrin-like repeats and an SH3 domain embedded in the 5th repeat) links to transmembrane collagens and  $\beta 4$  integrin in BPAG1e (Aumailley, Has, Tunggal, & Bruckner-Tuderman, 2006; Koster, Geerts, Favre, Borradori, & Sonnenberg, 2003) and mediates compartmentalization of transmembrane proteins in fly neurons (Bottenberg et al., 2009). The spectrin/dystrophin repeat rod domain likely acts as spacer or in dimerization (Bottenberg et al., 2009; Leung et al., 1999; Machnicka et al., 2014; Röper, Gregory, & Brown, 2002). Plectin-repeat domains (PRD) typically bind intermediate filaments (Aumailley et al., 2006; Jefferson et al., 2004). The two helix-loop-helix EF-hand motifs bind calcium to regulate MT association (Kapur et al., 2012), the putative translational regulator Krasavietz/eIF5C (Kra) important for actin regulation during axonal pathfinding (Lee et al., 2007; Sánchez-Soriano et al., 2009), and intramolecularly to the CH domains to autoinhibit Shot functions (Applewhite, Grode, Duncan, & Rogers, 2013). The Gas2-related domain (GRD) binds MTs and protects them against destabilization. The flexible, positively charged Ctail region associates with MTs and contains one or two SxIP sites required for binding to end-binding proteins (EB1) which is essential for axon growth (Alves-Silva et al., 2012; Applewhite et al., 2010; Honnappa et al., 2009; Sun, Leung, & Liem, 2001). The C-terminus can be phosphorylated to regulate MT association (Wu et al., 2011) and is the region specifically deleted in human BPAG1/dystonin mutations linked to neurodegeneration (Edvardson et al., 2012). (B) According to FlyBase, the *shot* locus spans ~78 kb (genomic position 13,864,237–13,942,110) in cytogenetic map position 50C6–11 of chromosome 2R. The different isoforms Shot-R/P A–Z (R refers to transcript and P to protein isoforms) are generated from 4 transcriptional start sites (P1–4) giving rise to 4 N-terminal isoforms (termed Shot-LA-D as indicated on the left) and from the differential splicing of 39 different coding exons and 4 noncoding exons. *Modified from Röper and Brown (2003), Röper et al. (2002), and FlyBase (McQuilton, St Pierre, & Thurmond, 2012).*

the epidermolysis bullosa simplex type (Aumailley et al., 2006). Functional loss of the longer isoforms in humans as well as mice causes severe postnatal neurodegeneration classified as hereditary sensory and autonomic neuropathy type VI (HSAN6; Edvardson et al., 2012; #6627; Ferrier, Boyer, & Kothary, 2013; Young & Kothary, 2007). Furthermore, patient studies suggest potential roles in neural development (Giorda et al., 2004; Vincent et al., 2008), human melanoma (Shimbo et al., 2010), and the infection process of Herpes virus (Pasdeloup, McElwee, Beilstein, Labetoulle, & Rixon, 2013). Finally, studies of *dystonin* mutant mice reveal further defects in glial cells (Bernier, De Repentigny, Mathieu, David, & Kothary, 1998; Saulnier, De Repentigny, Yong, & Kothary, 2002) potentially linking to multiple sclerosis (Laffitte et al., 2005), and neuromuscular junction defects associated with intrinsic muscle weakness (Boyer, Bernstein, & Boudreau-Larivière, 2010; Dalpe et al., 1999; Poliakova et al., 2014).

p0015 Surprisingly, no human diseases or conditions have been associated with MACF1/ACF7, but mouse and zebrafish models suggest its involvement in a wide range of developmental processes, ranging from early embryogenesis (Chen et al., 2006; Gupta et al., 2010), cell migration of fibroblasts, or skin stem cells (Wu, Kodama, & Fuchs, 2008; Wu et al., 2011), as well as heart and brain development (Fassett et al., 2013; Goryunov, He, Lin, Leung, & Liem, 2010; Jorgensen et al., 2014; Sánchez-Soriano et al., 2009).

## s0015 **1.2 *Drosophila*: A Powerful Model for the Study of Spectraplakins Functions**

p0020 To assess the biological relevance of molecules and mechanisms, *in vivo* analyses are essential and, for this, genetic invertebrate model organisms such as *Drosophila* are particularly well suited.

p0025 First, *in vivo* work is relatively fast in flies, and it is not unusual that new experimental ideas yield experimental data within a few weeks. Notably, there are no legal requirements, meaning that experiments can be performed straight off the drawing board with little or no need for an extensive ethical approval process. Using simple classical genetic crosses allows the fast generation of animals combining multiple genetic aberrations or transgenic constructs which can then be applied for studies of spectraplakins functions in development and disease. The basic knowledge required for this work is readily accessible and has recently been put together in a comprehensive manual for newcomers to *Drosophila* research (Prokop, 2013b; Roote & Prokop, 2013). Also the generation of transgenic animals takes just 2 months from injection to first experimentation and can be outsourced at low cost.

p0030 Second, 100 years of intense research has led to excellent conceptual understanding of fundamental biology in *Drosophila* (Bate & Martínez-Arias, 1993; Bellen, Tong, & Tsuda, 2010; Demerec, 1950; Kohler, 1994). This provides a prolific context in which to perform *in vivo* experiments, further fueled by countless genetic tools, usually readily available (all listed in FlyBase and many obtainable from resource centers; Cherbas & Gong, 2014; Matthews, Kaufman, & Gelbart, 2005; McQuilton et al., 2012; St. Pierre, Ponting, Stefancsik, McQuilton, & FlyBase Consortium, 2014).

p0035 Finally, *Drosophila* is small enough that whole embryos, adult brains, or ovaries can be visualized under the microscope, even at high resolution and/or using live imaging techniques (Tomer, Khairy, & Keller, 2011). Embryos are small enough so that four specimens can be mounted in parallel in one block for electron microscopic analysis, while being large enough to be dissected via simple techniques to carry out detailed microscopy studies of specific tissues, physiological measurements, or the collection of samples that allow tissue-specific transcriptomic or proteomic approaches (Budnik, Gorczyca, & Prokop, 2006). Consequently, highly refined descriptions of virtually all tissues and organs of the fly are available (Hartenstein, 1993), providing a rich resource of *in vivo* readouts.

p0040 This article will explain resources and key strategies to the non-drosophilist, illustrating how flies can be used as an efficient and cost-effective model to address fundamental mechanisms of spectraplakins, generating knowledge that can then be used to instruct research in higher animals.

## s0020 2. STRATEGIES TO MANIPULATE SHOT FUNCTION

### s0025 2.1 Available Gene Information and Classical Mutant Alleles for the *Drosophila shot* Gene

p0045 When searching for “shot” in FlyBase, a wealth of information is listed in ~17 categories in a logic, stringent, and well-linked-out manner (<http://flybase.org/reports/FBgn0013733.html>; McQuilton et al., 2012; St. Pierre et al., 2014). This information includes identifiers and location data (Prokop, 2013b), genomic maps, functional domains, gene ontology, expression and interaction data, orthologues, classical and transgenic alleles, external data, established fly models of human disease, available stocks and reagents, and a comprehensive list of over 200 publications in which *shot* is mentioned.

p0050 In the “Genomic Location/GBrowse” section of FlyBase, currently lists 22 different RNA/protein isoforms of *shot* (distinguished by an R/P suffix

code; Fig. 1B), and these data are in line with the ensembl.org data base. The isoforms are generated through the alternative splicing of 43 coding exons (Fig. 1B), as well as the presence of four alternative transcription start sites (Fig. 1B) which generate four different N-termini (often classified as Shot-LA, -LB, -LC, -LD, indicated on the left side in Fig. 1B; Bottenberg et al., 2009; Lee, Harris, Whittington, & Kolodziej, 2000; Lee & Kolodziej, 2002b). According to the information available, most isoforms share the plakin domain, the second CH domain, spectrin repeats, EF-hand domains, GRD, and Ctail, whereas the first CH domain (LA and LB isoforms) is present in only 60% of the isoforms, and a huge plectin repeat domain (~3000 aa encoded by one exon) is restricted to the *shot-RH*/Shot-PH isoform only (Fig. 1B).

p0055 The “Alleles and Phenotypes” section of FlyBase, currently lists 80 different classical mutant alleles for *shot*, many of which have been molecularly characterized. They are mostly *shot*-specific mutations but also include 6 deficiencies, 1 duplication, and 24 transgenic lines for the targeted expression of RNAi constructs, full-length or deletion constructs (Fig. 2), or carrying Shot isoforms genomically tagged with GFP (see end of Section 2.3). With one click on any of these entries, a wealth of allele- or transgene-specific information will be listed in a new window including publicly available fly stocks and literature where these alleles or transgenic lines were used. Stock availability can also be retrieved for all *shot*-related stocks at once in the “Stocks and Reagents” section of FlyBase, with currently 50 entries. Other stocks are often still kept by individual fly groups who used them in published work and tend to share them freely.

p0060 Of all mutant alleles described, the most frequently used for functional studies (Sections 3 and 4) are *shot*<sup>3</sup> and *shot*<sup>sf20</sup>. They are both considered null alleles and, accordingly, generate phenotypes comparable to smaller deficiencies uncovering the entire *shot* locus. Suitable deficiencies used in the literature are *Df(2R)MK1* [50B3–50B5; 50D4–50D7] (Sánchez-Soriano et al., 2009; Strumpf & Volk, 1998) or *Df(2R)BSC383* [50C6–50C6; 50D2–50D2] (Valakh, Walker, Skeath, & Diantonio, 2013), for which data in square brackets indicate the cytogenetic map position of their break points (i.e., the chromosomal area deleted). The genomic duplication *Dp(3R)6r35* has been used successfully to increase *shot* expression for immunohistochemical studies, thus complementing the analysis of loss-of-function (LOF) mutant alleles (Prokop, Uhler, Roote, & Bate, 1998). Another good example of how to make use of specific mutant alleles is the *shot*<sup>kakP2</sup> mutation. This allele affects the first two start sites of *shot* and generates only isoforms

UAS construct	Length [deleted region]	Isoform	Scheme	References
UAS-Shot-L(A)-GFP	5201 aa	shot-RE		1
UAS-Shot-L(A)-N'6myc	5201 aa	shot-RE		2
UAS-Shot-L(A)-N'6myc-C'GFP	5201 aa	shot-RE		2
UAS-Shot-L(C)-GFP	5160 aa	shot-RC		1
UAS-Shot-L(B)-GFP	5390 aa	shot-RB		1
UAS-Shot-L(A)-ΔPlakin-GFP	4364 aa [488–1325]	shot-RE		2
UAS-Shot-L(A)-Δrod1-GFP	1805 aa [1204–4600]	shot-RE		1
UAS-Shot-L(A)-Δrod2-GFP	4560 aa [1023–1664]	shot-RE		1
UAS-Shot-L(A)-Δrod3-GFP	4191aa [3589–4599]	shot-RE		1
UAS-Shot-L(A)-ΔEFhand-GFP	5063 aa [4711–4849]	shot-RE		1
UAS-Shot-L(A)-ΔGRD-GFP	5155 aa [4859–4905]	shot-RE		1
UAS-Shot-L(A)-ΔEB1-GFP	5165 aa [5154–5191]	shot-RE		2
UAS-Shot-L(C)-ΔGRD-GFP	4859–4905 aa of RE	shot-RC		2
UAS-Shot-RE-3MTLS*-GFP	5201 aa [4966–9, 5077-80, and 5141–4 replaced by AAAA]	shot-RE		4
UAS-Shot-RE-ΔCtail-GFP	4918 aa [4919–5201]	shot-RE		4
UAS-APT	1–1202 aa	shot-RE		5
UAS-PT	379–1202 aa	shot-RE		5
UAS-EF-GRD-Ctail-GFP	4780–5201 aa	shot-RE		5
UAS-GRD-GFP	4841–4923 aa	shot-RE		1
UAS-EB1-2HA	5140–5383 aa	shot-RE		2
UAS-Ctail	4918–5201 aa	shot-RE		4
UAS-Ctail-3MTLS*-GFP	4966–9, 5077–80, and 5141–4 replaced by AAAA	shot-RE		4

**Figure 2** Published transgenic fly lines carrying UAS-linked *shot* constructs. Published name of the construct (1st column), length in amino acids (2nd column), the respective mother construct (3rd column), and schematic representations of the constructs (4th column). The domain key is given in Fig. 1; asterisks indicate mutated sequences; blue (gray in the print version) circles, c-myc; green/red (light gray/dark gray in the print version) circles, GFP/RFP. References: (1) Lee and Kolodziej (2002a), (2) Bottenberg (2006), (3) Bottenberg et al. (2009), (4) Alves-Silva et al. (2012), (5) Subramanian et al. (2003).

LC and LD lacking the first CH domain (Fig. 1B); *shot*<sup>kakP2</sup> was therefore used for structure–function analyses, complementary to transgenic rescues strategies (see Section 3) with the Shot-LC construct similarly lacking the first CH domain (Bottenberg et al., 2009; Röper & Brown, 2003). Another important application of *shot* mutant alleles or transgenic tools is to capitalize on the ease of *Drosophila* mating schemes (Roote & Prokop, 2013) and combine them with mutant alleles of other genes. The key principle is to use partial LOF conditions for *shot* (e.g., a heterozygous condition for a null allele, such as *shot*<sup>f20/+</sup>) and for another gene, where neither of the two alone causes a phenotype. If these two conditions combined in the same animal generate a phenotype, this suggests they are likely to operate in the same



pathway or process. The nature of this interaction (e.g., sequential, parallel, in one complex) needs to be further investigated, for example, validated through biochemical studies. This strategy has worked well to illustrate functional and physical interactions of Shot with Eb1 and Krasavietz during cytoskeletal regulation required for axon growth (Alves-Silva et al., 2012; Lee et al., 2007; Sánchez-Soriano et al., 2009).

## s0030 **2.2 Genomic Engineering Technologies to Manipulate the shot Locus**

p0065 In the past, mutant alleles in *Drosophila* had to be generated through fairly random procedures using X-ray, chemical or transposon mutagenesis, all well suited for forward genetics approaches (Prokop, 2013b). Now, also reverse genetics has become highly efficient through the advent of genomic engineering strategies.

p0070 *TALEN* (transcription activator-like effector nuclease)-induced strategies have been successfully applied in *Drosophila* in a couple of cases to modify genes endogenously (Kondo et al., 2014; Liu et al., 2012). This strategy is based on tailor-made site-specific DNA endonucleases which introduce double-strand breaks at specific loci causing errors or deletions leading to LOF alleles, or the brakes are used to introduce any DNA of choice via gap repair.

p0075 Before *TALEN* could be fully developed in flies, *CRISPR* (clustered regularly interspaced palindromic repeats) strategies were introduced which have clearly taken over as the key method for future genomic engineering approaches. Similar to *TALEN*, *CRISPR* allows the targeted deletion and/or replacement of genomic sequences but through a different mechanism. In *CRISPR*, the *Streptococcus*-derived RNA-guided DNA endonuclease Cas9 (CRISPR-associated protein 9) induces DNA double-strand breaks at sites for which complementary RNA sequences of 20 nucleotide length are provided in the form of small guide RNA (sgRNA) constructs. If two Cas9-dependent double-strand breaks are induced, this can be used to generate targeted deletions (Bassett, Tibbit, Ponting, & Liu, 2013; Gratz et al., 2013). If a construct for homology-directed repair (containing homology regions of around 1 kb) is provided in parallel, the cutout genomic section can be replaced by any sequence of choice. This strategy provides a powerful means to insert designed mutations, protein tags, or attP sites (see next paragraph) into the gene locus of choice (Bassett & Liu, 2014a, 2014b; Gratz et al., 2014). Through *CRISPR* technology, gene loci can be rapidly modified within ~2 months (Gokcezade, Sienski, & Duchek,

2014). The simplest method is to use standard injection procedures for early embryos (Prokop & Technau, 1993) to bring *cas9* plasmid DNA, the sgRNA(s) and, if homology-directed repair is intended, also the repair sequence into germline cells. If suitable markers were inserted during the process, successful targeting events can be screened ~10 days after injection when the individual flies eclose. Further improvements of CRISPR technology in flies include transgenic *nanos-Cas9* fly lines (stably expressing Cas9 in the germ line, so that Cas9 RNA or plasmid no longer needs to be coinjected; Ren et al., 2013), optimized sgRNA expression vectors (Port, Chen, Lee, & Bullock, 2014), and the development of online resources to facilitate construct design and prediction of potential off-target sites (Bassett et al., 2013; Gratz et al., 2013; Hsu et al., 2013; Ren et al., 2013; Yu et al., 2013). To keep up to date with CRISPR technology in *Drosophila*, a number of dedicated Web pages are available (<http://www.flycrispr.molbio.wisc.edu>, <http://crispr.genome-engineering.org/>, <http://www.crisprflydesign.org>).

p0080 *φC31 recombinase*-mediated strategies make use of targeted recombination involving two specific target sequences, attP and attB. When attB-bearing vectors are injected into *φC31*-expressing fly strains carrying attP sites at a chosen genomic location, >90% of integration events occur site-directed at the attP sites (Bischof, Maeda, Hediger, Karch, & Basler, 2007). This strategy can be used to insert transgenes always in the same genomic position and avoid uncontrollable position effects, so that all transgenes will show comparable expression properties as a constant parameter during their functional comparison. Currently, ca. 130 different fly lines with defined attP landing sites are readily available from stock centers.

p0085 *Recombinase-mediated cassette exchange (RMCE)* provides a powerful strategy to replace chosen endogenous gene sequences in a targeted approach (Huang, Zhou, Dong, Watson, & Hong, 2009; Nagarkar-Jaiswal et al., 2015). In RMCE, first two attP sites flanking a gene or part of it are introduced, for example, using CRISPR/Cas9 technology (see previous paragraph). This flanked segment can then be substituted through *φC31*-mediated cassette exchange, providing vectors carrying attB-flanked sequences of mutated, tagged, or otherwise nonhomologous DNA, and in this way whole series of endogenous gene variants can be produced (Gratz et al., 2014). Clearly, the advent of CRISPR/*φC31* in combination with RMCE has generated a step change in the precision of genetic manipulation and analysis possible in the fly.

### s0035 **2.3 Transgenic Ways of Depleting *shot***

- p0090 A number of targeted approaches to deplete intrinsic gene products in a time-, tissue-, or even cell-specific manner can be used in *Drosophila*, thus providing a powerful complementary means to classical genetic methods. All these methods are based on well-established systems of targeted gene expression in the fly, in particular the GAL4/UAS system (Section 2.4.1).
- p0095 Gene silencing by double-stranded RNA (RNAi, RNA interference) allows specific knockdown of any transcripts or their individual isoforms (Dykxhoorn, Novina, & Sharp, 2003). RNAi-mediated gene silencing is very effective in flies and has been used in a huge number of systematic genetic screens identifying regulators of various processes (Ejsmont & Hassan, 2014). In *Drosophila*, there are stable transgenic lines for the targeted knockdown of virtually every gene, most of which are readily available from public stock collections in Bloomington (USA; <http://flystocks.bio.indiana.edu>), Vienna (Austria; <http://stockcenter.vdrc.at/control/main>), and Mishima (Japan; <http://www.shigen.nig.ac.jp/fly/nigfly>). RNAi technology in flies has undergone gradual developments and improvements (Dietzl et al., 2007; Schmid, Schindelholtz, & Zinn, 2002), and the newest versions of The Transgenic RNAi project (TRiP) combine a couple of important features: (1) All constructs are inserted into a defined attP landing site ensuring even expression levels across the collection (Section 2.2). (2) Insulator sequences were added to make expression levels independent of influences from the surrounding genome. (3) Highly efficient small shRNAs (22 bp length) are employed to deliver siRNAs which use the endogenous microRNA pathway (Ni et al., 2008, 2009, 2011). In particular, the newly introduced use of small shRNAs, as compared to long double-stranded RNA, makes it now possible to generate rescue constructs with codon modifications within the targeted sequence that are immune to the knockdown.
- p0100 There are various sources of *shot* RNAi lines: Subramanian et al. successfully used the *UAS-dskakRNA* line which reproduced *shot* mutant phenotypes in tendon cells and the nervous system (Alves-Silva et al., 2008, 2012; Subramanian et al., 2003). A second-generation *shot* TRiP line (*GL01286*; Bloomington stock #41858) was shown to reduce *shot* transcript levels in the nervous system when expressed via *elav-GAL4* (Valakh et al., 2013). A first-generation TRiP line (*JF02971*; Bloomington stock #28336) exists, but seems not to have been used yet in published work. The efficiency of RNAi knockdown can be further enhanced by coexpression of the enzyme Dicer (a rate-limiting component of the RNAi machinery; Dietzl et al., 2007) or

by rearing flies at higher temperatures, usually 29 °C, thus capitalizing on the temperature sensitivity of the UAS/GAL4 system (Section 2.4.1; Ni et al., 2009).

p0105 In recent years, additional strategies emerged to deplete gene function also at the protein level which, unlike RNAi, can overcome the problem of slow protein turnover rates. These strategies make use of camelid single-chain variable fragments (ScFv or nanobodies) which can be cloned and expressed in cell lines or used to generate stable transgenic fly lines. Nanobodies generated against a protein of interest retain their immune specificity in *Drosophila* cells and can functionally deplete the (cytoplasmic) proteins they were raised against (Layalle et al., 2011). The degrade Green Fluorescent Protein (deGradFP) system takes this strategy a step further by using an anti-GFP nanobody fused to a proteasome targeting signal. This fusion protein, when expressed in cells, rapidly depletes cytoplasmic pools of GFP- as well as YFP-tagged proteins (Caussinus, Kanca, & Affolter, 2012). Furthermore, deGradFP is temperature sensitive and, by simply shifting flies from 18 to 28 °C, protein pools can be depleted, and then restored again by reverting to 18 °C (Nagarkar-Jaiswal et al., 2015). This system can easily be combined with a huge variety of readily available protein trap lines where endogenous gene loci are genomically tagged with GFP or YFP. Such lines were systematically raised by the Flytrap, the MiMIC (Minos-Mediated Integration Cassette) and the Cambridge Protein Trap Insertion project (Kelso et al., 2004; Lowe et al., 2014; Nagarkar-Jaiswal et al., 2015; Venken et al., 2011). For Shot, a number of GFP trap lines exist [*shot*<sup>CPTI001962</sup>, *shot*<sup>CPTI-003653</sup> (Lowe et al., 2014), *Mi{MIC}shot*<sup>MI01617</sup>, *Mi{MIC}shot*<sup>MI03583</sup> (Venken et al., 2011)] which are all readily available for deGradFP knockdown experiments but also for live imaging studies (Section 3.2).

## s0040 2.4 Transgenic *shot* Constructs

### s0045 2.4.1 Various *Drosophila* Expression Systems Enable Spatiotemporal Expression of Transgenes

p0110 The GAL4/UAS system is considered the “Swiss Army knife” of *Drosophila* geneticists. It is a highly versatile means for the targeted spatiotemporal expression of genes or genetically encoded tools (Brand & Perrimon, 1993; del Valle Rodriguez, Didiano, & Desplan, 2012; Duffy, 2002). There is hardly a *Drosophila* publication where this system has not been applied and, in the case of *shot*, it has been used widely to drive deletion constructs (Section 2.4.2), to label specific cells or perform live imaging in *shot* mutant

backgrounds (Sections 3 and 4), to knock down *shot* (Section 2.3), or to perform MARCM analysis (Section 3.1). GAL4/UAS is a binary system with two main components: The yeast transcription factor GAL4 (expressed in a tissue and/or time-specific pattern) activates transgenes under the control of a UAS (upstream activating sequence) promoter, thus enabling spatiotemporal specific transgene expression. Thousands of different *GAL4* fly lines have been raised, so that virtually any tissue and developmental stage can be targeted. To refine this approach, a number of tricks can be used to achieve even higher spatial or temporal resolution (del Valle Rodriguez et al., 2012; Pfeiffer et al., 2010): (1) The temperature-sensitive GAL4 repressor *Gal80<sup>ts</sup>* can be coexpressed, so that GAL4-induced expression is active only during periods when the experimental animals are shifted to 30 °C (McGuire, Mao, & Davis, 2004). (2) The GeneSwitch system uses tissue-specific GAL4 lines which are inactive under normal conditions but can be activated by supplementing the food with the steroid hormone mifepristone (Nicholson et al., 2008). (3) In the SplitGAL4 system, GAL4 is split into two halves which are expressed by two independent enhancers, so that only the very refined set of cells which has coincident expression of both halves shows GAL4 activity. A huge number of such lines are available from the Fly Light Split-GAL4 Driver Collection (<http://splitgal4.janelia.org/cgi-bin/splitgal4.cgi>; Aso et al., 2014; del Valle Rodriguez et al., 2012).

p0115 Finally, other binary expression systems exist, so that gene expression via the GAL4/UAS system can be combined with the expression of different genes or tools under a completely independent control (del Valle Rodriguez et al., 2012). First, in the *colE1*-derived LexA/LexAop system, the transcriptional activator LexA binds and activates the LexA operator (LexAop; Szuts & Bienz, 2000), and respective transgenic fly lines are readily available at the Bloomington stock center ([http://flystocks.bio.indiana.edu/Browse/lexA/lexA\\_main.htm](http://flystocks.bio.indiana.edu/Browse/lexA/lexA_main.htm)). Second, in the *Neurospora crassa*-derived QF/QUAS system, QF is the activator and can be repressed when coexpressing its antagonist QS (Riabina et al., 2015), and respective fly lines are also available at the Bloomington stock center (<http://flystocks.bio.indiana.edu/Browse/Qsystem/Qintro.htm>). Since QS can be inhibited by quinic acid supplemented to the fly food, it can be used as a conditional system just like *Gal80<sup>ts</sup>* or GeneSwitch (Potter, Tasic, Russler, Liang, & Luo, 2010).

#### s0050 **2.4.2 Generation of *shot* Transgenes for Structure–Function Analysis**

p0120 A huge variety of *shot* constructs and transgenes have been generated (Fig. 2) and used in a variety of contexts to carry out structure–function analyses and

unravel the mechanistic basis of the various Shot functions (see Sections 3 and 4). For most of them, the Shot-PE isoform (Lee et al., 2000; Fig. 1B) has been used as the template. Shot-PE has become the gold standard for domain deletion studies because it is able to fully or partially rescue all *shot* mutant phenotypes tested so far (Section 3). Apart from Shot-PE, the Shot-PH isoform might need future consideration because it contains an additional ~3000 aa plectin repeat domain encoded by one exon (Fig. 1B). Shot-PH is strongly expressed in the nervous system (Section 2.1), expected to play roles in epithelial maintenance (Roper & Brown, 2003), and it provides a potential path to understand the still unknown roles played by plectin/plakin-repeat-containing isoforms of the mammalian Shot homologue BPAG1/dystonin (Fig. 1A). One feasible path to generate *shot-RH* constructs is the use of BAC clones containing the entire genomic *shot* region (*P[acman] BAC CH321-44 M03*; listed at [pacmanfly.org](http://pacmanfly.org) and distributed via [bacpac.chori.org](http://bacpac.chori.org)) which are designed for the use of recombineering technology.

p0125 Through the advent of BAC recombineering technology, in combination with site-directed attP/attB mutagenesis, the handling of the enormously large *shot* coding region (~16,000 bp for *shot-RE*) has become an efficient procedure when generating *shot* constructs or transgenic animals. For work with *shot* constructs, we normally use the *P[acman]* vector derivative *M-6-attB-UAS-1-3-4* which displays a number of helpful features (Alves-Silva et al., 2012; Venken, He, Hoskins, & Bellen, 2006): (1) It is generated on a BAC backbone suitable for the cloning and transgenesis of ≥100 kb DNA fragments. (2) The MCS-2 multiple cloning site contains rare restriction sites (we use *AscI* as 5' and *PacI* as 3' restriction sites) allowing digestion/ligation transfers of full-length *shot-RE* in one step. (3) Cloning of two homology arms via the MCS allows versatile construct mutagenesis or tagging through recombineering. (4) A 5xUAS fragment drives GAL4-induced expression in transgenic animals (Section 2.4.1). (5) Via an attB site, transgenic animals can be generated through  $\phi$ C31-mediated position-specific integration at defined genomic attP sites (Section 2.2).

p0130 The pool of *shot* constructs (Fig. 2) can be employed for time- and tissue-specific overexpression experiments using the GAL4/UAS system (Section 2.4.1). Such experiments can be performed as gain-of-function analyses where constructs are overexpressed to assess their localization or potential dominant phenotypes (e.g., Alves-Silva et al., 2012; Bottenberg et al., 2009). Alternatively, in LOF analyses, expression of deletion constructs can be used to assess potential failure to rescue mutant phenotypes, thus determining the requirements of the various functional

domains (Lee & Kolodziej, 2002b; Sánchez-Soriano et al., 2009). The very flexible use of these constructs for the study of *shot* mutations will keep them a powerful tool for the future, even in times of CRISPR technology (Section 2.2).

s0055

p0135

### 3. FUNCTIONAL ANALYSES OF *SHOT* IN VIVO

One important strength of the fly model is the ease with which mechanisms and roles of spectraplakins can be analyzed in biological contexts *in vivo*, revealing relevant mechanistic concepts that can then be used for studies in higher organisms (Prokop, Beaven, Qu, & Sánchez-Soriano, 2013). To illustrate these points, we will focus here on a number of *in vivo* readouts used for functional studies of *shot*.

s0060

#### 3.1 Analysis of *shot* Function in the Nervous System

p0140

The first *shot* mutant phenotype reported consisted of reduced sensory axon growth in the embryo (hence the name *short stop*; Kolodziej, Jan, & Jan, 1995) which was later extended to motor axons, dendrites, and synaptic terminals (Gao, Brenman, Jan, & Jan, 1999; Lee et al., 2000; Löhr, Godenschwege, Buchner, & Prokop, 2002; Prokop, Uhler, et al., 1998). A key prerequisite for detecting and working with these phenotypes is the existence of enormously detailed descriptions of all embryonic motor and sensory neurons including their axonal and dendritic morphology (Landgraf, Bossing, Technau, & Bate, 1997; Merritt & Whittington, 1995; Rickert, Kunz, Harris, Whittington, & Technau, 2011). For functional analyses, they can either be stained with antibodies [all neurons (anti-HRP), (subsets of) sensory (22C10, Mab49C4) or motor axons (anti-FasII, anti-FasIII)] or using GAL4/UAS-based (Chiang et al., 2011) as well as manual single-cell labeling strategies (Hoang & Chiba, 2001; Landgraf et al., 1997). Simple strategies based on anti-HRP-labeled histological landmarks were developed to measure the extent of axon/nerve growth toward their respective target areas, thus generating quantitative data for mutant or experimental analyses (Bottenberg et al., 2009). Studies of motornerves in parallel to work with primary neurons (Section 4.2) have been used for very detailed structure–function analyses, making neurons the cellular context in which Shot’s evolutionary conserved role as an actin–microtubule linker is currently best understood (Alves-Silva et al., 2012; Bottenberg et al., 2009; Lee & Kolodziej, 2002b; Sánchez-Soriano et al., 2009). The understanding of this role is

further enhanced through additional work unraveling the functional interfaces of Shot with other actin- and MT-regulating proteins (Prokop et al., 2013).

p0145 Importantly, analysis of *shot* LOF can be extended into larval or adult stages, although this condition is embryonic lethal. For this, the expression of RNAi constructs can be targeted exclusively to specific neurons to knock down *shot* in these cells (Valakh et al., 2013), and this knockdown can be spatially and temporally refined as explained earlier (Section 2.4.1). Complementary to this, *shot* LOF mutant alleles can be used to the same end via the MARCM technique (mosaic analysis with a repressible cell marker). MARCM uses genetically induced Flippase/FRT-mediated homologous recombination between chromosome arms to generate clones of homozygous mutant neurons marked by a reporter gene, whereas the rest of the brain remains heterozygous mutant and is not marked (Wu & Luo, 2006). In this way, only the mutant neurons are labeled and their morphology can be analyzed. Notably, the *shot*<sup>3</sup> mutant allele was the first ever mutant allele used for MARCM analyses, which have revealed Shot requirements during axon growth and neuronal polarity at pupal stages (Lee & Luo, 1999; Reuter, 2003: #1933).

### s0065 3.2 Functional Studies of *shot* in Tendon Cells

p0150 Shot has also been extensively analyzed in specialized epidermal cells, called tendon cells, the nature of which is briefly outlined here. *Drosophila* has an exoskeleton in the form of an extracellular matrix (ECM) called cuticle which is secreted from the apical surface of the epidermis (Broadie, Baumgartner, & Prokop, 2011), whereas muscle tips anchor to the opposite basal surface of the epidermis via integrin- and ECM-dependent junctions (Prokop, Martín-Bermudo, Bate, & Brown, 1998). To withstand the enormous muscular pulling forces, the epidermis forms specialized tendon cells at these anchor points which contain dense arrays of actin and MT filaments linking the basally attached muscles to the apical cuticle on the other side (Alves-Silva et al., 2008). This organization is functionally analogous to basal keratinocytes where keratin filament networks link basal integrin-dependent hemidesmosomes to desmosomes on lateral and apical surfaces of the same cells (Aumailley et al., 2006). In both cell types, Shot and BPAG1/dystonin are required for linking the basal, integrin-dependent junctional complexes to the cytoskeleton, and their functional loss causes intracellular rupture at these junctions, leading to epidermolysis bullosa



simplex in the case of BPAG1 (Aumailley et al., 2006; Prokop, Uhler, et al., 1998). Notably, epidermal isoforms of BPAG1/dystonin are short containing Plectin repeats (Fig. 1), whereas *Drosophila* Shot isoforms in tendon cells are long and bind MTs.

p0155 Roles of Shot in tendon cells become apparent not before the last third of embryogenesis (Campos-Ortega & Hartenstein, 1997). At this stage, functional studies are facilitated by the easy visualization and highly characteristic segmental pattern of tendon cells in the epidermis. Visualization is further facilitated by markers that can be used to specifically label tendon cells. These markers include endogenous proteins, such as integrins and their associated adhesion complex components; ECM proteins such as Tigrin and Thrombospondin; the transcription factors Stripe, F-actin which is highly enriched in tendon cells, or the  $\beta$ 1-tubulin isoform which is exclusive to the nervous system and tendon cells (Alves-Silva et al., 2008; Buttgerit, Leiss, Michiels, & Renkawitz-Pohl, 1991; Fogerty et al., 1994; Subramanian, Wayburn, Bunch, & Volk, 2007; Volohonsky, Edenfeld, Klambt, & Volk, 2007). For the detection of these proteins, antibodies or genomically GFP-tagged gene versions (Section 2.3) can be used, and also phalloidin works extremely well (Alves-Silva et al., 2008). In addition, *stripe-GAL4* can be used to drive any gene constructs of choice specifically in tendon cells, for example, to label them or to perform structure–function analyses with *shot* deletion constructs in *shot* mutant embryos (Alves-Silva et al., 2008; Bottenberg et al., 2009). Specimens can be either analyzed live, cut open, and dissected flat or injected with a fixation/labeling solution containing paraformaldehyde, phalloidin, and Triton-X (Alves-Silva et al., 2008; Budnik et al., 2006; Prokop & Technau, 1993). In flat dissected animals, collagenase or trypsin treatments can be used to dissolve ECM and detach muscles from tendon cells, thus providing an unhindered view at tendon cells in the absence of major mechanical forces (Alves-Silva et al., 2008). EM analyses at embryonic or larval stages are best performed in the sagittal plane close to the ventral midline which gives the best perspective of tendon cell architecture (Alves-Silva et al., 2008; Budnik et al., 2006; Prokop, Martín-Bermudo, et al., 1998).

p0160 Studies in tendon cells so far revealed that Shot is required for the integrity of MT arrays but not actin arrays (Alves-Silva et al., 2008). This function is absolutely dependent on the MT-binding C-terminal GRD and Ctail, partly the spectrin-repeat rod, but not at all the ABD or plakin-like domain (Fig. 1A; Alves-Silva et al., 2012; Bottenberg et al., 2009). Unlike BPAG1 at hemidesmosomes which binds keratin through its C-terminus and  $\beta$ 4

integrin and collagen XVII through its plakin-like domain (Aumailley et al., 2006), a role of Shot as a physical linker in tendon cells remains debatable. Its primary roles may lie in the development and maintenance of these arrays, consistent also with findings that Shot promotes tubulin expression in tendon cells downstream of neuregulin signaling (Strumpf & Volk, 1998).

p0165 A number of other *in vivo* contexts have so far been less well studied but provide powerful readouts for studies of Shot. These include development of the foregut (where Shot is involved in Notch signaling; Fuss, Josten, Feix, & Hoch, 2004), the tubular transpiration system (called tracheae; where Shot is involved in cellular fusion; Lee & Kolodziej, 2002a), salivary glands (where Shot anchors minus ends of MTs; Booth, Blanchard, Adams, & Röper, 2014), epithelial junctions (Röper & Brown, 2003), the compound eye (where Shot is involved in junctional morphogenesis; Mui, Lubczyk, & Nam, 2011), and oocytes (where Shot is required for MT linkage to an internal membrane structure, the fusome, and subsequent oocyte specification; Röper & Brown, 2004), as well as the structural maintenance of muscle nuclei (where Shot, EB1 and nesprin protect nuclei from intrinsic or extrinsic forces; Wang, Reuveny, & Volk, 2015).

s0070

## 4. FUNCTIONAL ANALYSES OF SHOT IN CELL CULTURE

p0170

Complementary to *in vivo* analyses of Shot, cell culture studies offer efficient strategies to refine and speed up the investigation or provide alternative experimental means. Such studies can make use of ~200 existing *Drosophila* cell lines readily available from a dedicated stock center (<https://dgrc.cgb.indiana.edu/cells/Catalog>; Cherbas & Gong, 2014), primary cell cultures (cells directly harvested from the organism) in particular neurons and hemocytes (immune cells of the body fluid; Prokop, Küppers-Munther, & Sánchez-Soriano, 2012; Sampson & Williams, 2012), and also heterologous studies in mammalian cell lines are a feasible and often helpful strategy (Alves-Silva et al., 2012).

s0075

### 4.1 Studying Shot in S2 Cells

p0175

Studies in S2 cells have a long history in *Drosophila* with major contributions to genetic mechanisms of cell division (Moutinho-Pereira, Matos, & Maiato, 2010), and detailed protocols are readily available ([www.flyrnai.org/DRSC-PRC.html](http://www.flyrnai.org/DRSC-PRC.html); Ceriani, 2007; Rogers & Rogers, 2008). S2 cells are extremely easy to keep in Schneider's *Drosophila* Medium (Life Technologies), with passages being required every 3–4 days. With an average

diameter of 10  $\mu\text{m}$ , S2 cells are far smaller than fibroblasts but they present many helpful cytoskeletal readouts that can be used for studies of Shot: they contain acentrosomal microtubule arrays (Nye, Buster, & Rogers, 2014) and have actin-rich lamellipodia in the periphery but no stress fibers (although these can be artificially induced when manipulating formins; Lammel et al., 2014). Transfection of S2 cells is easy (Armknecht et al., 2005). Usually genes to be expressed are cloned into the *pAc5.1* vector (Life Technologies) containing the constitutively active actin promoter. Alternatively, any constructs readily available on *pUAST*-plasmids (the most commonly used in the *Drosophila* field) can be cotransfected with a *pMT-GAL4* plasmid (which induces GAL4 expression upon 100 mM  $\text{CuSO}_4$  supplementation to the medium) or *actin-GAL4* (driving constitutively). Over recent years, cell lines with defined genomic attP landing sites for  $\phi\text{C31}$ -mediated construct integration have been generated, ideal to establish stable S2 cell lines (Groth, Fish, Nusse, & Calos, 2004), and CRISPR technology is now being used to manipulate S2 cells through genomic engineering (Bassett, Tibbit, Ponting, & Liu, 2014). However, S2 cells cannot be combined with classical genetics approaches, and knockdown strategies have to be used instead. S2 cells were originally generated from primary cultures of late-stage (20–24 h old) embryos and display a number of properties typical of hemocytes (Ribeiro, D'Ambrosio, & Vale, 2014). However, they do not adhere to ECM and are not very motile in the culture dish, so that the biological meaning of changes in cytoskeletal organization and dynamics is often not clear. Nevertheless, S2 cells are a powerful tool to unravel new molecular mechanisms which can then be studied in other contexts. In this way, S2 cells have provided us with first descriptions of mechanisms underlying the recruitment of Shot to MT plus ends (Applewhite et al., 2010; Slep et al., 2005) and a novel intramolecular autoinhibition mechanism (Applewhite et al., 2013).

## s0080 **4.2 Using Primary Cultures Combines Detailed Readouts with Powerful Genetics**

p0180 Primary cell cultures have to be generated for each experiment anew, but, in contrast to cell lines, they offer full access to versatile *Drosophila* genetics and can be directly compared to *in vivo* contexts using animals with the identical genetic constellation. For example, hemocytes are a potential alternative to S2 cells. Their harvest from embryos or larvae is effortless (Sampson & Williams, 2012), and their study in culture can be complemented by work *in vivo*, including live imaging of cytoskeletal dynamics in developmental or

wound healing contexts (Beaven et al., 2015; Evans, Zanet, Wood, & Stramer, 2010; Stramer et al., 2010). Surprisingly, Shot has not yet been investigated in these cells.

p0185 Instead, extensive studies of Shot have been performed in primary neurons. As explained and discussed in detail elsewhere (Prokop et al., 2012; Sánchez-Soriano et al., 2010), these neuron cultures can be generated from animals at all stages of development and a variety of different culture media can be used. Work on Shot has mostly been performed in primary neurons cultured in Schneider's medium and derived from mid-stage embryos (when most neurons are born and are about to grow axons; Campos-Ortega & Hartenstein, 1997). Neurons cultured in this way reproduce an impressive range of neuronal *in vivo-like* properties, including characteristic cytoskeletal organizations and dynamics or physiologically active presynaptic structures, both closely resembling descriptions from vertebrate neurons (Küppers, Sánchez-Soriano, Letzkus, Technau, & Prokop, 2003; Küppers-Munther et al., 2004; Sánchez-Soriano et al., 2010). Since the same combinatorial genetic strategies to combine mutations and/or transgenic elements can be applied in primary neurons just like *in vivo* (Beaven et al., 2015; Prokop et al., 2013), cultures offer important additional experimental opportunities: First, some of the readouts available in primary neurons are far more detailed than can be achieved *in vivo*, increasing the resolution of mechanistic studies. Second, genetic manipulations especially of the cytoskeleton often cause *in vivo* phenotypes so severe that they cannot be interpreted, whereas neurons cultures from such embryos still allow sensible experimentation. The only prerequisite is that embryos carrying these constellations develop neuronal precursor cells, so that primary neurons can be harvested from them and grown in culture. In addition to using transgenic manipulations, cells can be transfected, as has been demonstrated for RNAi constructs (Bai, Sepp, & Perrimon, 2009) as well as for larger constructs (our unpublished results).

p0190 To generate embryonic primary neuron cultures (Prokop et al., 2012), whole stage 11 embryos are selected for the right genotypes using standard fluorescent dissecting microscopes in combination with green balancer chromosomes (Casso, Ramirez-Weber, & Kornberg, 2000; Halfon et al., 2002). Embryos are cleaned and cells are mechanically extracted using a pistil, followed by chemical dispersion for 4 min, washes in fresh medium (involving centrifugation), their final dilution (ca. 4 embryos per 15 µl), and subsequent transfer of 30 µl drops into special culture chambers made from a slide with a drilled hole of 15 mm diameter glued onto another intact

slide (both made from lead-free glass). Chambers are sealed with a cover slip and a bit of Vaseline and turned upside-down after 1.5 h (hanging drop culture) to avoid debris around the growing neurons. Mechanisms of axon growth are best analyzed in 6 to 8 h cultures, and synapses can be analyzed after ~15 h. Furthermore, neurons can be kept for up to a month in order to assess neurodegenerative phenotypes. Analyses of these neurons can be performed with live imaging using suitable fluorescently tagged constructs (Alves-Silva et al., 2012), or cells are fixed and stained using standard immunocytochemistry.

p0195 As summarized elsewhere (Prokop et al., 2013), work on *shot* performed in this system so far included detailed localization studies, LOF analyses, and structure–function approaches with *shot* deletion constructs. Furthermore, genetic interaction studies with other factors (e.g., *eb1* or *kra*; Section 2.1) were used complementary to biochemical binding studies to unravel the functional networks in which Shot operates (Alves-Silva et al., 2012; Sánchez-Soriano et al., 2009). These studies yielded understanding of novel spectraplakins roles in actin regulation, and molecular and cellular mechanisms underpinning roles of Shot in MT stabilization and guidance, which also provide simple conceptual explanations for neurodegenerative phenotypes observed in dystonin mutant mice and patients (Edvardson et al., 2012; Prokop, 2013a).

### s0085 **4.3 Analysing Shot Domain Requirements in NIH3T3 Fibroblasts**

p0200 Finally, for the analysis of Shot constructs, NIH3T3 mouse fibroblasts provide a well-established and convenient system, offering highly refined cell biological descriptions while being easy to maintain and transfect (Blair, 2001; Jainchill, Aaronson, & Todaro, 1969; Todaro & Green, 1963). For example, GFP-tagged Shot constructs of ~16.5 kb length transfect with a ~3% success rate (Alves-Silva et al., 2012). For this, fibroblasts are cultured in DMEM (Sigma-Aldrich; supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% FCS in a humidified incubator at 5% CO<sub>2</sub>) and passaged in a 1:10 dilution every 3 days. For transient DNA transfections, Lipofectamine and Plus reagent (Invitrogen) can be used according to the manufacturer's instructions, and cells are replated 5 h after transfection at 40% confluence in glass-bottom dishes (MatTek Corporation) coated with 10 g/ml bovine plasma fibronectin (Sigma-Aldrich). Immunohistochemistry follows standard protocols (Alves-Silva et al., 2012). For live imaging, cells are maintained in Ham's F-10 medium (Sigma-Aldrich)

supplemented with 4% FCS. As explained in Section 2.3, we have most of our *shot* constructs flanked by *PacI* and *Ascl* restriction sites. Using a modified version of the *pcDNA3.1* vector (Invitrogen) containing these restrictions sites, constructs can therefore be cloned over in a one-step process, making fibroblast studies an easily accessible part of the analysis process. Importantly, *shot* constructs in fibroblasts revealed localization and functional features reminiscent of comparable constructs in neurons and of ACF7/MACF1 or BPAG1/dystonin constructs in mammalian cells (Alves-Silva et al., 2012), but distinct from observations in S2 cells (Applewhite et al., 2010). They are therefore an efficient and valuable addition for work on Shot.

s0090



## 5. INFORMATICS APPROACHES TO SHOT PROTEIN FUNCTION

p0205

Whole-genome and large-scale “omics” approaches have generated a wealth of data that are often used to address specific biological questions. These extensive datasets constitute a resource that can be mined through the use of informatics tools to gain insight into regulatory processes. Much of this information has been aggregated and integrated into Web-based resources such as FlyBase that facilitate its use (Mohr, Hu, Kim, Housden, & Perrimon, 2014). One of the greatest explosions in big data has come from the development of next-generation RNA-sequencing approaches and the subsequent generation of transcriptomic profiles and profiles for DNA-binding and chromatin-interacting proteins.

p0210

As explained in greater detail elsewhere (McQuilton et al., 2012), FlyBase aggregates many available genome wide datasets (>60 transcriptomic datasets in the current release FB2015\_02). These enable the examination and dissection of overall *shot* expression and isoform-specific expression levels throughout development, in specific tissues, and in a diverse array of *Drosophila*-derived cell lines. Much of these data are derived from the modENCODE consortia and is graphically accessible through the implementation of a generic genome browser (GBrowse), allowing diverse datasets to be examined simultaneously (Boley, Wan, Bickel, & Celniker, 2014). This transcriptomic data as well as a vast array of additional genomic annotations (more than 800 individual ChIP-chip and ChIP-seq experiments) can be displayed and customized through the selection of specific data under the GBrowse “Select Tracks” tab on FlyBase (datasets, descriptions and usage are reviewed elsewhere; Boley et al., 2014; Ejsmont & Hassan, 2014). For example, the inspection of *shot* transcription in whole

embryos during development reveals that *shot* isoforms containing the large plectin repeat-encoding exon (Fig. 1B) are virtually absent in the early embryo prior to the initiation of zygotic transcription, but become predominant within the pool of zygotic transcript isoforms. Furthermore, tissue-specific transcriptomic datasets find the inclusion of this exon to be low in neuronal stem cells (neuroblasts) during larval stages, but greatly enriched in differentiated neurons at the same stage. The “Expression data/External data” section of FlyBase, also aggregates tissue-specific expression data revealed through *in situ* analysis of embryos and microarray-based profiles.

p0215 Beyond these diverse whole-genome ChIP-Seq and RNA-seq resources, FlyBase integrates links to additional external gene and protein interaction databases that aggregate physical and genetic interactions to help identify the network of components involved in a biological process. These resources are linked out in the “Interactions and Pathways/External data” section of FlyBase. For example, the BioGRID database, which collects physical and genetic interaction datasets for a number of diverse organisms (<http://thebiogrid.org>), suggests a number of physical contacts for *shot* that have not been explored experimentally yet. They include p115, an armadillo domain-containing protein that plays a role in vesicle transport and exocytosis (Kondylis & Rabouille, 2003), CG7379 a relatively uncharacterized zinc finger protein, and Kat60 an AAA-type ATPase component of the microtubule severing Katanin complex (Mao et al., 2014).

s0095

## 6. CONCLUSION

p0220

Given the enormous complexity of spectraplakins functions, work on the *Drosophila* spectraplakins Shot offers powerful opportunities to decipher their roles in cellular and tissue contexts. Such studies can capitalize on efficient combinatorial genetics available in the fly, an excellent infrastructure providing easy access to information and a rich pool of molecular tools, as well as the ease of *in vivo* work complementary to culture studies. Notably, understanding gained from studies on Shot can instruct work in vertebrates or mammals, thus accelerating research and often revealing a high degree of mechanistic conservation (Bellen et al., 2010).

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## Non-Print Items

**Keywords:** *Drosophila*, Cytoskeleton, Microtubules, Actin, Genetics, Spectraplakins, Neurons, Cell culture

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