Functional dissection of phosphorylation of Dishevelled in *Drosophila*

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Abstract

Dishevelled/Dsh proteins (Dvl in mammals) are core components of both Wnt/Wg-signaling pathways: canonical β-catenin signaling and Frizzled (Fz)-planar cell polarity (PCP) signaling. Although Dsh is a key cytoplasmic component of both Wnt/Fz-pathways, regulation of its signaling specificity is not well understood. Dsh is phosphorylated, but the functional significance of its phosphorylation remains unclear. We have systematically investigated the phosphorylation of Dsh by combining mass-spectrometry analyses, biochemical studies, and *in vivo* genetic methods in *Drosophila*. Our approaches identified multiple phospho-residues of Dsh *in vivo*. Our data define three novel and unexpected conclusions: (1) Strikingly and in contrast to common assumptions, all conserved serines/threonines are non-essential for Dsh function in either pathway; (2) phosphorylation of conserved Tyrosine473 in the DEP domain is critical for PCP-signaling - Dsh^{Y473F} behaves like a PCP-specific allele; and (3) defects associated with the PCP specific *dsh^1* allele, Dsh^{K417M}, located within a putative Protein Kinase C consensus site, are likely due to a post-translational modification requirement of Lys417, rather than phosphorylation nearby. In summary, our combined data indicate that while many Ser/Thr and Tyr residues are indeed phosphorylated *in vivo*, strikingly most of these phosphorylation events are not critical for Dsh function with the exception of DshY473.

Keywords

Phospho-protein; Dishevelled; Wingless; Wnt-signaling; Signaling specificity; PCP

Introduction

Frizzled (Fz) family receptors act as transducers of the Wnt/Wg growth factor family, commonly signaling through the canonical Wnt (Wg)-Fz/β-catenin pathway (Logan and Nusse, 2004; Polakis, 2000). In addition, a distinct Wnt-Fz pathway regulates cellular polarity within the plane of an epithelium, referred to as non-canonical Wnt or Fz/planar cell

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polarity (PCP) signaling (Adler, 2002; Keller, 2002; Lawrence et al., 2007; Seifert and Mlodzik, 2007; Wang and Nathans, 2007; Wu and Mlodzik, 2009). Fz/PCP signaling diverges from the β-catenin pathway downstream of the cytoplasmic component Dishevelled (Dsh in *Drosophila*, Dvl 1–3 in mammals). Since both pathways require Fz and Dsh to transduce signaling information, a key question is how Fz and potential associated factors can differentially activate Dsh. In many tissues, both pathways act in the same cells and thus a tight regulation of Fz-Dsh signaling specificity is essential. In deregulated, mutant scenarios, the selection of the wrong Fz-Dsh pathway can lead to disease (i.e. polycystic kidney disease or cancer (Logan and Nusse, 2004; Polakis, 2000; Simons and Mlodzik, 2008)). Although a molecular framework of the two pathways has been established (Klein and Mlodzik, 2005; Logan and Nusse, 2004; Mlodzik, 2002; Strutt, 2003; Veeman et al., 2003), little is known about the molecular aspects regulating Fz-Dsh pathway selection and signaling specificity. Here we investigate this with focus on Dsh phosphorylation.

Drosophila is a well-suited model system to address Wnt-signaling specificity mechanisms. For example, in the fly eye, activation of the Fz/β-catenin pathway leads to the restriction of the eye field and later the removal of excess photoreceptor cells through apoptosis (Lin et al., 2004), whereas Fz/PCP signaling is required for correct induction of specific photoreceptor fates and their patterning (Mlodzik, 1999; Strutt and Strutt, 1999). Similarly, during wing development the two pathways have distinct non-overlapping functions: canonical Wg-signaling is required for correct wing blade/margin formation, while Fz/PCP signaling orients wing cells in the proximal-distal axis. Moreover, as Fz/PCP aspects are universal to metazoans (Adler, 2002; Keller, 2002; Lawrence et al., 2007; Seifert and Mlodzik, 2007; Wang and Nathans, 2007; Wu and Mlodzik, 2009), *Drosophila* is well suited to study Dsh function, because of lack of Dsh redundancy (a single *dsh* gene) and its function being testable in rescue assays of null mutants.

In Wnt-Fz/β-catenin signaling, the combined action of Fz, LRP5/6-Arrow (a Fz co-receptor), and Dsh is to antagonize the APC/GSK3/Axin complex that causes β-catenin (Arm in *Drosophila*) phosphorylation and degradation. Upon Wnt binding, the two transmembrane proteins associate, leading to a physical interaction of Dsh and Axin (via an interaction through their DIX domains) at the membrane receptor complex (Bilic et al., 2007; Zeng et al., 2008). This leads to the stabilization of β-catenin/Arm and allows its accumulation in the nucleus, where it interacts with TCF transcription factors on Wnt-signaling target sites (Logan and Nusse, 2004; Polakis, 2000). In contrast, during Fz/PCP signaling, Fz "activates" Dsh through an unknown mechanism in an LRP5/6-Arrow independent manner, with Dsh actng on a distinct set of effectors. The components of Wnt-Fz/β-catenin that act downstream of Dsh are not required in the Fz/PCP pathway, and vice versa (e.g. (Axelrod et al., 1998; Boutros et al., 1998)). The Fz-Dsh pair thus activates distinct pathways, a feature conserved between flies and vertebrates (Mlodzik, 2002; Sahai et al., 1998; Strutt, 2003; Veeman et al., 2003).

The regulation of signaling specificity remains largely obscure. Some insights have come from studies in *Drosophila*, with focus on the cytoplasmic (C-) tails of Fz and Fz2 identified as critical in regulating the specificity through their effects on subcellular localization (Boutros et al., 2000; Wu et al., 2004). Other domains of Fz and/or Fz2 (e.g. the Wnt interacting extracellular CRD) are also important in determining signaling outcome (Boutros et al., 2000; Rulifson et al., 2000; Strapps and Tomlinson, 2001; Wu and Mlodzik, 2008). All Dsh-family members (ranging from nematodes to humans) share three conserved domains: a DIX domain, a central PDZ domain and a C-terminal DEP domain, which have been implicated in protein-protein interactions, and thus Dsh likely serves as an adapter molecule (rev. in Boutros and Mlodzik, 1999; Wallingford and Habas, 2005). Dsh is recruited by Fz to the membrane (Boutros et al., 2000; Wu et al., 2004). Although a Fz-Dsh

interaction surface has been identified (Wong et al., 2003), additional mechanisms help to stabilize the membrane complex (Simons et al., 2009). In cell culture, when Dsh is not in a membrane-associated state, it tends to be in cytoplasmic aggregates with multimerization mediated by DIX domains (Schwarz-Romond et al., 2005). Regulation of Dsh activity and localization is therefore likely instrumental for signaling outcomes.

PCP specific Dsh recruitment appears more stable than its general basolateral membrane recruitment for β-catenin signaling (Wu et al., 2004), suggesting that Dsh receives different "local" input (for example through post-translational modification) for its membrane association (Metcalfe et al., 2010). Dsh/Dvl proteins also use different domains for downstream pathway functions: DIX and PDZ domains are essential for β-catenin signaling, while the PDZ and DEP-C terminal domains are critical for PCP signaling (rev. in Boutros and Mlodzik, 1999; Wallingford and Habas, 2005)(also Fig. 1A). Upon "activation", Dsh is subject to two major changes: (1) translocation from the cytosol to membrane and (2) hyper-phosphorylation, usually assessed by changes in gel mobility. Both aspects are often used as a measure of "Dsh activation", although what "activation" entails remains unclear. At least for phosphorylation, it is only a correlated phenomenon without a defined contribution to signaling function (Axelrod, 2001; Cong et al., 2004; Klein et al., 2006; Ossipova et al., 2005; Strutt et al., 2006). In *Drosophila*, both the *dsh[1]* and *fz-* mutants (with PCP specific defects) show markedly reduced Dsh phosphorylation as evident by band-shift (Axelrod, 2001). In vertebrate cell culture models, both canonical and non-canonical (PCP) Wnts, in particular Wnt3A and Wnt5A respectively, lead to induction of Dsh phosphorylation as detected with Dsh/Dvl band-shift assays. Multiple kinases have been suggested and shown to phosphorylate Dsh *in vitro*, with potential regulatory requirement(s) in overexpression experiments (Cong et al., 2004; Klein et al., 2006; Ossipova et al., 2005; Strutt et al., 2006).

A comprehensive analysis of Dsh phosphorylation has been especially challenging since for example over 15% of Dsh residues are serines and threonines. Here we have explored mass-spectroscopic, biochemical, and genetic approaches to identify phosphorylated residues on Dsh and systematically define their functional significance in *in vivo* in rescue assays. Besides many Ser/Thr residues identified biochemically, our approach also identified tyrosine phosphorylation. Strikingly, in contrast to common assumptions and the fact that a phosorylation dependent band-shift of Dsh/Dvl-proteins is often used as pathway activation read-out (Axelrod, 2001; Cong et al., 2004; Klein et al., 2006; Ossipova et al., 2005; Strutt et al., 2006), all of the Ser/Thr residues tested (although conserved) are dispensable for Dsh function *in vivo*. We also show that the Dsh gel-shift can be uncoupled from Dsh activity. However, Tyr473 located within the DEP domain is essential for Dsh function for PCP signaling *in vivo*. Furthermore, dissection of the *dsh[1]* allele (Dsh[K417M]) suggests that K417 could undergo direct post-translational modification rather than affect a phosphorylation event in its flanking PKC site consensus. Our data indicate that while many Ser, Thr, and Tyr-residues are phosphorylated, most of these phosphorylation events are, unexpectedly, not critical for Dsh function.

Material and methods

DNA constructs, fly genetics, and Rescue Assay

dsh>DshGFP was kindly provided by Jeff Axelrod. Point mutagenesis of the Dsh ORF was performed by excising it with either KpnI (from 278nt of ORF to the end of polyA tail) or EcoRI (from about 135nt ahead of start codon to the end of polyA tail), subcloned into pBlueScript, and mutated by standard protocols. For in vivo purification, *dsh>Dsh-3XFlag* was made by replacing C-terminal GFP sequence from the original construct with 3XFlag sequence (Sigma). To analyze the potential requirements of S/T residues in the C-terminal part of Dsh, we have mutated them to Ala in three blocks (separated by introduced silent

restriction sites) indicated by purple, blue and red (Fig. 2). 2 cluster combinations, referred to as C-term#1-2, C-term#1-3 or C-term#2-3 in Table 1 were made by swapping combining individual clusters. The respective mutant clusters in the context of full-length Dsh were then tested in gel-shift assays after transfection into 293T cell with or without Fz. Finally, we also generated a Dsh that lacked all sequences downstream of the Blp1 site, referred to as ΔC-term (Table 1; note that the mutant constructs lack all but one Ser, while ΔC-term contains no Ser/Thr C-terminal to the DEP domain). A detailed cloning strategy is available upon request.

To estimate effects of mutants, *dsh>Dsh(mutant)GFP* strains were balanced these on second or third chromosomes and crossed to the *dsh^{v26}* null mutants. Note that the *dsh^{v26}* chromosome used was also marked with *forked (f)* to control for non-disjunction events. Rescue index was calculated as: rescue index (R.I.)=rescued/control. For each group of rescue assay, *dsh>Dsh^{wt}GFP* served as baseline control. R.I. of wild-type Dsh is in theory 1. Practical R.I. (wild-type) was used to calibrate R.I. of mutants.

y, w, f^{36a}, dsh^{V26}/FM6 was obtained from the Bloomington Stock center.

Peptides and antibodies

The phospho-peptide sequences used as antigens were:

pS36: CSAQV(pT)LRDFK

pY280: RGGDGGI(pY)VGSI

pS451: CRREARRIV(pS)AMLR

pY473: GGEQC(pY)YVVNE

Peptides were synthesized by ProSci and Tufts University Mass-Spectrometry Core Facility, and antisera were generated by ProSci (California). Anti-Fmi antiserum was from Developmental Studies Hybridoma Bank

Collection of fly samples and protein purification

There is no documented setup to collect *Drosophila* larvae and pupae at large scale. We modified a regular trash-can to a "larval farm". 15–20 bottles of fly cultures (larval stages) were transferred to the "farm". Small amount of water was added to make it moist and a thin layer of yeast pellets was spread on top, the surface was increased with plastic foil/film to allow larvae to crawl up and covered with a glass panel and wet towel. The setup was kept at room temperature and inspected every 12 hours to adjust for moisture. 48 hours after set-up, plastic films were taken out to collect larvae/pupae (about a 1:1 ration of pupae and larvae were the preferred stage). A larval "farm" produced about 20g sample each time.

To detect phosphorylation of Dsh protein *in vivo*, Dsh protein needed to be purified from >500 larvae/pupae. The sample was homogenized in PBS with 1% SDS, 1% Triton X-100, 1mM DTT, 1:100 dilution of protease inhibitor cocktail, phosphatase inhibitor cocktail I and II (from Sigma). Lysate was spun at 4°C 14,000g for 10min, supernatant filtered with Whatman paper, followed by (NH4)2SO4 precipitation (Dsh-3Xflag was enriched in 5–10% saturated fraction). 50–100ng of pure Dsh-3XFlag was obtained from 50g larva/pupae.

Transfected Dsh-3Xflag in HEK293T cells was immunoprecipitated with M2-agarose gel (Sigma). Cell lysis buffer (same for immunoprecipitation) contained 50mM Tris-HCl(pH7.4), 140mM NaCl, 1% Triton X-100, 1:100 dilution of protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktails I and II (both Sigma). Protein was eluted with 0.1M glycine (pH2.8) and precipitated with methanol/chloroform.

Mass spectrometry

Mass spectrometry was performed at the Tufts University Protein Core Facility. Excised bands were subjected to in-gel reduction, alkylation, and enzymatic digestion (Roche Applied Science, Indianapolis) in a HEPA-filtered hood to reduce keratin background. LC/MS/MS analysis was performed on the in-gel digest extracts using Agilent (Santa Clara, CA) 1100 binary pump directly coupled to a mass spectrometer. 2–8ul of sample was injected on column using a LC Packings (Sunnyvale, CA) FAMOS autosampler. Nanopore electrospray columns were constructed from 360mm o.d., 75 mm i.d. fused silica capillary with the column tip tapered to a 15 mm opening (New Objective, Woburn, MA). The columns were packed with 200 Å 5 um C18 beads (Michrom BioResources. Auburn, CA.), a reverse-phase packing material, to a length of 10 cm. The flow through the column was split precolumn to achieve a flow rate of 300 nL/min. The mobile phase used for gradient elution consisted of (A) 0.3% acetic acid 99.7 % water and (B) 0.3% acetic acid 99.7 % Acetonitrile. Tandem mass spectra (LC/MS/MS) were acquired on a Thermo LTQ ion trap mass spectrometer (Thermo Corp., San Jose, CA). Needle voltage was set to 3 kV, isolation width was 3 Da, relative collision energy was 30%, and dynamic exclusion was used to excluded recurring ions. Ion signals above a predetermined threshold automatically triggered the instrument to switch from MS to MS/MS mode for generating fragmentation spectra.

Histology and confocal microscopy

Wings of adult flies were collected and soaked in PBS with 0.1% Triton X-100 for 4 hours. The wings were carefully mounted in 80% glycerol PBS solution. Eye sections were performed by standard protocols (Gaengel and Mlodzik, 2008).

Pupae were dissected at 30APF, and fixed with 4% formaldehyde for 1 hour. Dissected wing disc samples were treated with 5% FBS for 30min followed by antibody staining (1:10 anti-Fmi; 1:500 anti-GFP) overnight at 4°C. Following several washes, pupal wings were mounted in Vectashield (Vector Labs), and imaged with a Zeiss LSM5 confocal microscope. Single optical sections were analyzed with the Zeiss Image Browser and Adobe Photoshop software.

Results

Identification of Dsh phosphorylation sites by mass-spectrometry

Dsh phosphorylation is detected in many systems, ranging from *in vivo* assays to cell transfection experiments and is readily observed on gels by an associated change in mobility (e.g Axelrod, 2001; Cong et al., 2004; Klein et al., 2006). Although some phosphorylated residues have been identified *in vitro* (Cong et al., 2004; Klein et al., 2006; Ossipova et al., 2005; Strutt et al., 2006; also Fig. 1A), Dsh phosphorylation has not been addressed comprehensively. Moreover, although the phosphorylation-associated slower migration (band-shift) of Dsh/Dvl proteins is often used as read-out for pathway activation, its functional significance has never been demonstrated.

To systematically address Dsh phosphoryation, we first decided to identify phospho-residues through mass-spectroscopic analyses of purified Dsh from different cell culture conditions. HEK293T cells appeared well suited as they could be transfected efficiently (in contrast to *Drosophila* S2). After transfection into HEK293T cells alone or with Fz or dFz2, tagged Dsh was purified by immunoprecipitation (Fig. 1B; see Methods). The slower migrating hyper-phosphorylated bands were cut out and eluted (see Methods). The phospho-Dsh proteins from the three states tested: Dsh alone (representing a "naïve" Dsh), Dsh plus Fz (representing a Fz/PCP associated state), and Dsh plus Fz2 (likely representing non PCP membrane association) were analyzed by a tandem mass-spectrometry/HPLC approach of

tryptic fragments. This identified multiple phosphorylation events on Ser/Thr as well as Tyrosine residues (Fig. 1C), suggesting wide-spread phosphorylation of Dsh.

The phospho-residues thus identified were distributed throughout the protein, including the conserved DIX, PDZ, and DEP domains. In addition, the less conserved C-terminal Dsh region was found to harbor many phosphorylated residues. There was a noticeable and reproducible difference among the samples from the three distinct Dsh transfections/ "states", suggesting that several phosphorylation events were due to distinct "signaling"-response(s). Despite the potential overlap between Fz and dFz2 in canonical signaling *in vivo* (canonical Wg-signaling is controlled redundantly by both Fz and dFz2; Bhanot et al., 1999; Bhat, 1998; Chen and Struhl, 1999; Mueller et al., 1999), dFz2 appears to be the dedicated receptor for canonical signaling (Boutros et al., 2000). Accordingly, distinct phosphorylation patterns induced by the two Fz-receptors suggested distinct function(s) in this cell culture system, further confirmed by the fact that the mechanism of membrane recruitment of Dsh mediated by Fz and Fz2 is distinct in cell culture (Simons et al., 2009; T.J. Klein and MM unpublished).

Among three independent rounds of mass-spectrometry analysis, consistent data was obtained with respect to the phosphorylated residues throughout Dsh (Fig. 1C). Nevertheless, due to the pattern of tryptic fragments, the S/T-rich area between the basic-region and PDZ domain (known to be phosphorylated *in vitro*) (Cong et al., 2004; Klein et al., 2006; Ossipova et al., 2005; Strutt et al., 2006) and part of the DEP domain could not be covered well by this approach. However, as it already has been documented that the S/T-rich region just upstream of the PDZ domain does not require major phosphorylation for function *in vivo* (Strutt et al., 2006; also Fig. 1A), we did not analyze this region *in vivo* in further detail. Moreover, our biochemical data demonstrate that Ala-substitutions within this region (Dsh-ST124 and Dsh-ST45 in Fig 1A and 2A; Penton et al., 2002 for original description) did not affect Dsh phosphorylation as detected by Fz or dFz2 induced band-shifts (Fig. 2A).

Although the mass-spectrometry approach provided a useful framework for subsequent *in vivo* studies (see below), it was clear that it could not substitute for equivalent physiological analyses. We therefore complemented and confirmed the mass-spectrometry data by functional studies and specific antibodies raised against individual phosphorylated residues (see below).

Residue specific phosphorylation of Dsh *in vivo*

The phospho-residue mapping via mass-spectrometry was performed on cell culture samples for technical reasons, making it possible that some candidate phosphorylation sites were due to overexpression.

We thus next employed *in vivo* assays to further dissect the role of potential phosphorylation sites in Dsh. First, we wished to confirm that the sites in question were indeed phosphorylated *in vivo* at the relevant stages of development during PCP signaling. To address this, phospho-residue specific antibodies to Thr36, Tyr280, Ser451 and Tyr473 were generated (Fig. 1C). Peptides containing the phospho-residues were used to immunize rabbits (see Methods). The respective anti-sera were first characterized with dot-blots by comparing sensitivity between phospho-peptides and their non-phosphorylated counterparts (see Suppl. Fig. S1 for example). Batches of responsive sera were affinity purified using phospho-peptide conjugated columns. Purified antibodies were further tested on cell culture samples: most of the phospho-peptide antibodies detected specific bands on Dsh co-transfected with Fz (Suppl. Fig. S2 shows an example of anti-pS451). *In vitro* kinase assays were used as positive controls for Tyrosine specific antibodies (the Src kinase could phosphorylate the PDZ domain *in vitro*; Supp Fig. S3A). The sole (and phosphorylated)

Tyrosine residue within the Dsh PDZ domain were Tyr280 and anti-pTyr280 (anti-pY280) antibodies detected specific bands on Src-treated Dsh protein (Suppl. Fig. S3), indicating its specificity.

Next, endogenous Dsh protein samples were analyzed for phosphorylation of these residues using *dsh-Dsh3XFlag, dsh^{V26}* rescued animals (Table 1 and below). As expected from dot blot assays (Suppl. Fig S1), a direct Western blot approach was not sensitive enough, and thus Dsh protein was enriched from late larval/pupal stages by immunoprecipitation (the 3XFlag tag allows efficient affinity purification; see Supp. Data/Methods for details). Distinct Dsh protein banding patterns were reproducibly detected with phospho-specific antisera on Western blots (probing immunoprecipitated Dsh from *dsh-Dsh3XFlag, dsh^{V26}* animals; Fig. 3), which ran as several major bands in a Fz-dependent manner (Fig. 3, lane 1), consistent with previous work (e.g. Cong et al., 2004; Klein et al., 2006). Strikingly, anti-pY280, anti-pS451, and anti-pY473 recognized the top band, while anti-pS36 stained all three bands (Fig. 3, lanes 2, 3, 4 and 5; as anti-pY473 and anti-pS451 stained specifically only one of the Dsh bands, the other Dsh bands served as controls demonstrating the specificity of the antibodies). These data confirm that several residues, S36, Y280, T451, and Y473, were phosphorylated *in vivo*. Although the antisera work on Western-blots (Fig. 3) a large amount of Dsh protein needs to be enriched for these studies, and thus the sensitivity of these sera is not high enough to detect protein in situ.

The detection of phosphorylation states of individual residues *in vivo* is physiologically relevant, and comparing it with our mass-spectrometry data, these data confirm many consistent phosphorylation events between the two assays. This approach also helped to confirm phosphorylated residues that were detected by mass-spectrometry only in some experiments, such as Y473 (Fig. 1C). Furthermore, these data also confirmed our analysis of Abl-mediated DshY473 phosphorylation and function (Singh et al., 2010, see also below).

A quantitative Dsh rescue assay

To address the functional relevance of phosphorylation of individual residues or phosphorylated clusters, we established an *in vivo* rescue assay of a *dsh* null allele. Although cell culture based reporter assays (such as Top-Flash activation) or *in vivo* over-expression assays are fast and gather initial insights into functional questions, altered expression levels of Dsh can cause increased and/or aberrant signaling. Thus, to compare Dsh-activity levels accurately, a functional rescue assay that faithfully represented the endogenous physiological situation was necessary. A similar transgenic approach using endogenous *dsh* control sequences (*dsh-DshGFP)* has been used to illustrate asymmetric membrane localization of Dsh (Axelrod, 2001).

First confirmed that *dsh-DshGFP* was indeed expressed at endogenous levels (Suppl. Fig. S4) and that the *dsh-DshGFP* transgene was capable to fully rescue *dsh-* null animals. *dsh^{V26}* (a.k.a. *dsh^3*) animals were fully rescued by all *dsh-DshGFP* transgenic lines (Table 1) and indistinguishable from wild type demonstrating that canonical Wg/Wnt signaling required for patterning is fully functional. They displayed a wild-type PCP pattern in all tissues analyzed (not shown and below). Similarly, the equivalent transgene with a different tag, *dsh-Dsh3XFlag* (which provides an advantage for immunoprecipitation and biochemical studies, see above) also fully rescued the *dsh^{V26}* null (Table 1 and not shown).

Analysis of Serine/Threonine phosphorylation mutants of Dsh *in vivo*

THis *in vivo dsh⁻* rescue assay was then used to stringently test whether the respective phospho-residues play a physiological role in Dsh signaling. We focused on phospho-residues that were either highly conserved or reproducibly detected in the mass-spectrometry

analyses or both (Fig. 1C; due to the large number of candidate S/T residues, 110 in total in Dsh, the mass-spectrometry pre-selection was essential). We first targeted 14 conserved S/T residues in the DIX, PDZ, DEP and proline rich domains as individual mutations and the conserved tyrosine residues located in the PDZ (Y280) and DEP domains (Y473, Y474). Strikingly, in the dsh^{V26}, dsh-$DshGFP*$ rescue assay (*indicating the individual mutations), none of the S/T residues, when mutated to Ala (A), had a detectable effect on either viability and patterning (canonical Wg-signaling) or PCP (Table 1; all mutants have been analyzed in multiple independent lines and expression levels were confirmed with the GFP-tag; see below and not shown). The extent of rescue by the individual transgenes/mutants was quantified (Table 1 and not shown). It is worth noting that, even though the PCP specific dsh^1 allele is thought not to affect canonical Wnt-signaling (and does not in cell culture studies; see below), the dsh-Dsh^1 transgene rescued dsh^{V26} null background to viability with only a reduced recovery of adult flies (Table 1 and not shown; the rescue level denoted "+" in Table 1 allowed keeping of a homozygous stock). This observation is consistent with the behavior of the dsh^1 allele that also displays reduced viability (Boutros et al., 1998).

As none of the individual S/T mutations tested affected phenotypic aspects of Dsh function, we mutated groups of related S/T-residues. We focused on the DEP domain and the C-terminal region of Dsh, since the DEP domain/C-terminal regions have been linked to PCP signaling and are required for Fz-dependent Dsh membrane recruitment (e.g. Boutros et al., 1998; Klein et al., 2006; Rothbächer et al., 1998). Within the DEP domain, several PKC phosphorylation consensus sites are present and a PKC member has been implicated in Dsh membrane association in *Xenopus* (Kinoshita et al., 2003). We thus mutated several S/T-residues within potential PKC substrate sites (Table 1; also Fig. 1C), including a mutant with all S/T sites in the DEP domain mutated (Dsh^{DEP6A}; Table 1). Strikingly, the Dsh^{DEP6A} mutant fully rescued all aspects of dsh function to wild type (Table 1 and not shown).

Besides the three conserved domains, Dsh/Dvl proteins also have a C-terminal region (C-terminal to DEP domain) with many conserved S/T residues (Fig. 1C and 2C). This region has been implicated in Dsh function in PCP signaling (Park et al., 2008). Moreover, we have detected several of these S/T-residues to be indeed phosphorylated in the mass-spectrometry studies (Fig. 1C). We thus targeted S/T-clusters in the C-terminal region by first establishing three mutant clusters individually and then combining them in different combinations (Fig. 2B, C). Strikingly, mutating these clusters to Ala affected the band-shift of Dsh as induced by Fz co-transfections (Fig. 2B, C). When all three of these clusters were mutated the Dsh shift was almost completely lost (Fig. 2C, lanes labeled "Mut"). Similarly, single mutant clusters also affected the level of the band shift (Fig. 2C, a notable exception being the most proximal cluster labeled in pink in Fig. 2B; see Fig. 2C legend for details). Despite these strong effects in biochemical assays, none of the three clusters affected Dsh function in either viability or PCP signaling, when tested in pairs in the dsh-$DshGFP*$ rescue assays (Table 1 and not shown). Strikingly, even when this entire region was deleted (dsh-$Dsh\Delta C$; deletion from residue 505 onward, generating a truncated protein that lacks all of the 29 C-terminal S/T-residues) the mutant isoform fully rescued both canonical Wg-signaling requirements and all PCP aspects (Table 1; Figure 4E, J; and not shown). These data demonstrated that phosphorylation of the C-tail or the C-tail of Dsh *per se* is not essential for its function *in vivo* (see also Discussion).

In summary, all S/T mutants tested, including the S/T cluster mutants in the DEP domain and C-terminal region, were fully functional (Table 1). Detailed analyses of such animals for potential visible defects, PCP or other, also revealed no aberrant phenotypes. We thus conclude that all S/T-residues tested were non-essential for either canonical Wg or PCP signaling. Previous work analyzing the S/T clusters between the basic region and the PDZ

domain also revealed that these sites, although phosphorylated, were not essential for Wg-signaling (Penton et al., 2002; Strutt et al., 2006) and only a minor PCP requirement was seen when at least 8 of these were mutated (Strutt et al., 2006). Thus to date, including our data here for the C-tail, no S/T phosphorylation on Dsh has been shown to be essential for canonical Wnt/Wg-signaling.

Dsh Tyrosine473 is required for PCP signaling

Although previous work has suggested that Dsh is mainly phosphorylated on S/T residues and not tyrosines (Willert et al., 1997a), we have detected Tyr(Y)-phosphorylation in our mass-spectrometry studies and, importantly, confirmed Y280 and Y473 to be phosphorylated *in vivo* (Figs. 1 and 3). We thus tested for a potential physiological role of the highly conserved Y280 (PDZ domain) and Y473, Y474 residues (DEP domain). DshY280 to phenylalanine (Dsh[Y280F]) substitution had no effects on either viability or PCP (Table 1). In contrast, Dsh[Y473F] displayed the strongest effect in the viability rescue in the same range as the Dsh[1] mutant (Table 1), although a rescued (homozygous) stock was viable, consistent with our previous data in the Abl context (Singh et al., 2010). We did not, however, detect defects in Wg-signaling target gene expression or signaling strength during development, and also a quantitative cell culture assay did not reveal a significant difference of DshY473F as compared to Dsh[WT] (Fig. 4). We thus conclude that the Y473 is not required for canonical signaling and the reduced viability might have to do with other Dsh functions not yet described in *Drosophila*.

Strikingly, the *dsh[Y473F]* flies displayed strong PCP defects in all tissues analyzed (Table 1 and Fig. 5C, H). The neighboring Y474 had no effect and the double mutant (Dsh[Y473, 474F]) behaved like Dsh[Y473F] alone (Table 1 and not shown). As these PCP defects were strikingly similar to the *dsh[1]* allele, we compared their phenotypic PCP defects in more detail. In both cases, *dsh[1]* and *dsh[Y473F]*, the rescued individuals displayed classical PCP defects in the thorax and wings (Figure 5A–C′ and not shown), and had rough eyes, with randomized chirality, symmetrical clusters, and misrotations again resembling *dsh[1]* and classical PCP mutants in general (Figure 5F–H). Moreover, *dsh-Dsh[Y473F]* did not rescue the PCP defects of the *dsh[1]* allele (not shown). Taken together, these data indicate that Dsh[Y473F] behaves like a strong PCP specific *dsh* allele and thus Y473 appears to be required for PCP regulation.

These data are consistent with our observation that Y473 can be phosphorylated by the Abl kinase, which is also required for PCP signaling but not canonical Wnt-signaling (Singh et al., 2010). The neighboring and downstream Tyr residues can also be phosphorylated by Abl *in vitro* (Singh et al., 2010), and thus we asked whether any of these could be functionally important in the absence Y473 in a redundant manner. We thus tested for potential phenotypic consequences of *dsh-*, *dsh-DshY473F* flies in an *abl−/+* background. Neither viability nor the PCP defects were different in *abl−/+* as compared to *abl+* backgrounds (Table 1, Figure 5 and not shown), suggesting that none of the other Tyr residues in the DEP/C-term region of Dsh is (partially) redundant with Y473.

Stable membrane recruitment of Dsh is a hallmark of its role in PCP signaling (Axelrod, 2001; Boutros et al., 2000; Rothbächer et al., 1998) and, for example, reduced in *dsh[1]* mutants or lost altogether in *fz−* mutant animals (Axelrod, 2001). We thus analyzed the subcellular localization of Dsh[Y473F] in pupal wings around 30–34 hrs APF. Strikingly, and in contrast to Dsh[WT] (Fig. 6A), Dsh[Y473F] (in the *dsh* null background) displayed hardly any membrane association in pupal wings (or elsewhere), very similar to the Dsh[1] mutant isoform (Fig. 6B–C″). This defect in membrane association is in contrast with any of the S/T-residue cluster mutants that all localized normally to the membrane (data not shown), further indicating a specific requirement of Y473 in PCP signaling.

Similar to the S/T site mutations, expression levels of Dsh[Y280F] or Dsh[Y473F] (as detected on Western blots) were very similar to wild-type DshGFP or Dsh[1], indicating that protein stability in imaginal discs was unaffected by the Dsh[Y473F] mutation (Fig. 7; also Fig. S5). However, an obvious difference between wild-type DshGFP and the Dsh[Y473F] mutant isoform was their protein migration pattern, as detected on Western blots with imaginal disc samples (Fig. 7): Dsh[Y473F] lacked the slower migrating hyperphosphorylated bands, very similar to Dsh[1] (Fig. 7; Axelrod, 2001; Klein et al., 2006). This was in contrast to Dsh[Y280F] or Dsh[Y474F], which displayed a normal Dsh banding pattern (Fig. 7 and not shown).

Dsh[K417R] disrupts PCP signaling very similarly to Dsh[1] (Dsh[K417M])

The original PCP specific and first allele of *dsh*, *dsh[1]*, a K to M transition at residue 417, lies within a Protein Kinase C (PKC) consensus site. This observation led to the hypothesis that it could affect Dsh phosphorylation as it also reduces Dsh phosphorylation in cell culture (Klein et al., 2006). However, as mentioned above, the DEP domain PKC site S/T-mutations, including the respective Dsh[T419A] or even the Dsh[DEP6A] mutant isoform, affecting all potential DEP-domain PKC sites, had no detectable effect on Dsh function. Thus, we reasoned that other types of post-translational modification such as acetylation or ubiquitination that occur on lysines may be involved. K417 is one of five lysines/arginines (K/R) within the DEP domain. A sequence alignment of Dsh of various species revealed that K417 and K465 are exclusively lysines, whereas the other three lysines and arginines appear interchangeable (Fig. 1B). Therefore, we tested whether a replacement of K with R would phenocopy the *dsh[1]* allele (Dsh[K417M]) or be fully functional, the latter indicating the importance of the positive charge. Dsh[K417R] restores viability/canonical Wg-signaling of *dsh[V26]* comparable to *dsh[1]*(Table 1 and Fig. 5B) Intriguingly, Dsh[K417R] failed to rescue PCP and revealed PCP defects very similar to *dsh[1]* in all tissues analyzed (Fig. 5D, I and not shown). Importantly, in analogy to Dsh[1] (Dsh[K417M]), subcellular localization/membrane association of Dsh[K417R] was affected as well (Fig. 6D-D″). In 34h APF pupal wings, Dsh[K417R] was barely found at membranes, very similar to the Dsh[1] protein. Further analysis suggested that Dsh[K417R] also lacked the slow migrating Dsh bands on Westerns, suggesting reduced activation (Fig. 7). Therefore, we conclude that Lysine417 in the Dsh DEP domain is essential, not merely due to a requirement of electric charges, but possibly due to covalent modification(s) required for PCP function (also Discussion).

Discussion

In vivo, Dsh becomes hyperphosphorylated upon Wnt-pathway activation and/or membrane recruitment (PCP signaling) and due to this correlation, phosphorylation has often been used as a read-out for pathway activation. Previous work has identified a region N-terminal to the PDZ domain, which is rich in Ser/Thr residues and can be phosphorylated *in vitro*, but it remained questionable whether phosphorylation in this region is functionally significant (Cong et al., 2004; Klein et al., 2006; Ossipova et al., 2005; Strutt et al., 2006). The significance of other predicted phosphorylation sites and events has not been addressed. Our data indicate that, surprisingly, all Ser/Thr residues tested, although highly conserved and phosphorylated, are dispensable for Dsh function *in vivo*. In particular, it is a striking observation that the lack of all Ser/Thr residues C-terminal to the DEP domain or within the DEP domain has no effect on Dsh function in either signaling pathway. This, taken together with previous observations that the Ser/Thr-rich region N-terminal to the PDZ domain is not essential for Dsh function (Strutt et al., 2006), strongly suggests that Ser/Thr phosphorylation of Dsh/Dvl proteins is generally (almost) non-essential, which is on its own a very surprising and unexpected finding.

In contrast, our data suggest that tyrosine phosphorylation, which previous attempts failed to identify (Willert et al., 1997b), is critical for Dsh function. In particular, Y473 within the

DEP domain is critical in PCP signaling and likely phosphorylated by Abl type kinases (Singh et al., 2010). Our data further suggest that the original mutant *dsh[1]* allele (a K417M transition) does not affect phosphorylation, but is likely to cause defects via affecting post-translational modification of Dsh on K417.

Dsh phosphorylation *in vivo*

Our mass-spectrometry approach identified several phosphorylation sites, including Tyr phosphorylation of Dsh, which has not been previously observed (Willert et al., 1997a). Importantly, several of the individual sites were confirmed to be indeed phosphorylated *in vivo* through tissue samples and phospho-specific antibodies. Nevertheless, the respective Ser/Thr residues are functionally non-essential (or possibly redundant) for Dsh function in either pathway. Due to the large number of Ser/Thr residues in Dsh/Dvl proteins (>110 in approx. 650 total residues) it is almost impossible to address phosphorylation redundancy in the whole protein and thus, we have focused mostly on the C-terminal region including the DEP domain. All mutations analyzed that contained multiple mutant residues, including all Ser/Thr within the DEP domain or lacked all C-terminal Ser/Thr residues, were fully functional in rescue assays (Wg-signaling and PCP) of a *dsh-* null mutant background. Taken together with our individual mutations in conserved residues in other regions of Dsh/ Dvl and the earlier data on the S/T cluster between the basic region and PDZ domain (Penton et al., 2002; Strutt et al., 2006), our data suggest that Ser/Thr phosphorylation of Dsh is far less important than generally assumed.

In contrast, Tyr-phosphorylation of DshY473 within the DEP domain is essential for Dsh function in PCP signaling (this study and also Singh et al., 2010). The respective Dsh[Y473F] mutation behaves like the original PCP specific *dsh[1]* alelle: (1) *dsh* null flies can be rescued to viability, (2) the mutant protein is not recruited stably to the membrane during PCP signaling *in vivo*, and (3) the mutant protein shows an overall reduced phosphorylation as assessed by gel-shift (Fig. 7; compare also to Dsh[1] analysis, e.g. Axelrod, 2001; Klein et al., 2006). Strikingly, Y473 is the only Tyr residue that is functionally required, although Dsh/ Dvl proteins contain several tyrosines that are absolutely conserved across all species. For example, Y280 within the PDZ domain, which, although phosphorylated *in vivo* and conserved in all Dsh/Dvls, appears dispensable in functional rescue assays.

Overall, our data suggest that most phosphorylation target residues, although phosphorylated *in vivo*, are not essential for Dsh function with the notable exception is Y473, which behaves like a PCP specific *dsh* allele when mutated.

The Dsh C-terminal region

A striking observation is the lack of a requirement of the C-terminal region of Dsh (C-terminal to the DEP domain). Not only are all potential phosphorylation sites, S/T-sites and tyrosines, dispensable (Table 1), but even a complete deletion of the region C-terminal to residue 504 has no apparent functional consequences: The respective DshΔCterm isoform fully rescued all aspects of Dsh function. This is surprising as the equivalent C-terminal region of *Xenopus* Dsh has been suggested to be sufficient to localize GFP to the basal body of cilia and to interfere with their polarity when overexpressed (Park et al., 2008). Although patches of Ser/Thr residues are conserved within this C-terminal region (Fig. 1C and 2B), the sequence alignment of vertebrate and *Drosophila* Dsh proteins show overall less conservation in this protein region. The vertebrate Dvl proteins are highly conserved among themselves in this region, however. Therefore, it is likely that vertebrate Dsh/Dvls share functional requirements within these sequences that are not conserved between *Drosophila* and vertebrates and might lie outside the canonical Wnt-pathway and PCP signaling.

The nature of the *dsh¹* allele

The original allele of Dsh, *dsh¹*, a Lys-to-Met transition at position 417 within the DEP domain, is a PCP specific allele (Axelrod et al., 1998; Boutros et al., 1998). As this Lysine is located within a potential PKC consensus site, it has been speculated that it could affect phosphorylation of the nearby serine (Boutros and Mlodzik, 1999; Wallingford and Habas, 2005). We demonstrate that this is not the case and that potential PKC phosphorylation within the DEP domain is not essential for Dsh function. However, our data indicate that it is the Lysine itself that is essential and a mutation to the related Arginine residue (which is similarly charged and often can substitute for Lys) also causes the same defects as the original DshK417M mutation. These data suggest that it is a post-translational modification such as ubiquitination or acetylation of Lys417, as it does not appear to be of structural importance (Wong et al., 2000). This notion is consistent with recent work in mammalian cell culture (Ganner et al., 2009), although we cannot exclude a direct involvement of Lys417.

In summary, our analyses have revealed that many phosphorylation events on Dsh are functionally dispensable *in vivo*, even when they occur on highly conserved residues. This is in stark contrast to many previous assumptions and suggestions and thus an important observation. In addition, we have identified that Dsh is phosphorylated on Tyr residues, which has not been observed earlier. Consistent with recent data on an Abl requirement in PCP (Singh et al., 2010), Tyr-phosphoryation events on at least one site, DshY473, are critical for Dsh function in PCP signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- Although Dsh is a key cytoplasmic component of both Wnt/Fz-pathways, regulation of its signaling specificity is not well understood.

- Dsh is phosphorylated, but the functional significance of its phosphorylation remains unclear.

- We have systematically investigated the phosphorylation of Dsh by combining mass-spectrometry analyses, biochemical studies, and *in vivo* genetic methods in *Drosophila*.

- Our approaches identified multiple phospho-residues of Dsh *in vivo*. Our data define three novel and unexpected conclusions:

- (1) Strikingly and in contrast to common assumptions, all conserved serines/threonines are non-essential for Dsh function in either pathway;

- (2) phosphorylation of conserved Tyrosine473 in the DEP domain is critical for PCP-signaling - Dsh^{Y473F} behaves like a PCP-specific allele;

- (3) defects associated with the PCP specific *dsh^1* allele, Dsh^{K417M}, located within a putative Protein Kinase C consensus site, are likely due to a post-translational modification requirement of Lys417, rather than phosphorylation nearby.

- our data indicate that while many Ser/Thr and Tyr residues are indeed phosphorylated *in vivo*, strikingly most of these phosphorylation events are not critical for Dsh function with the exception of DshY473.
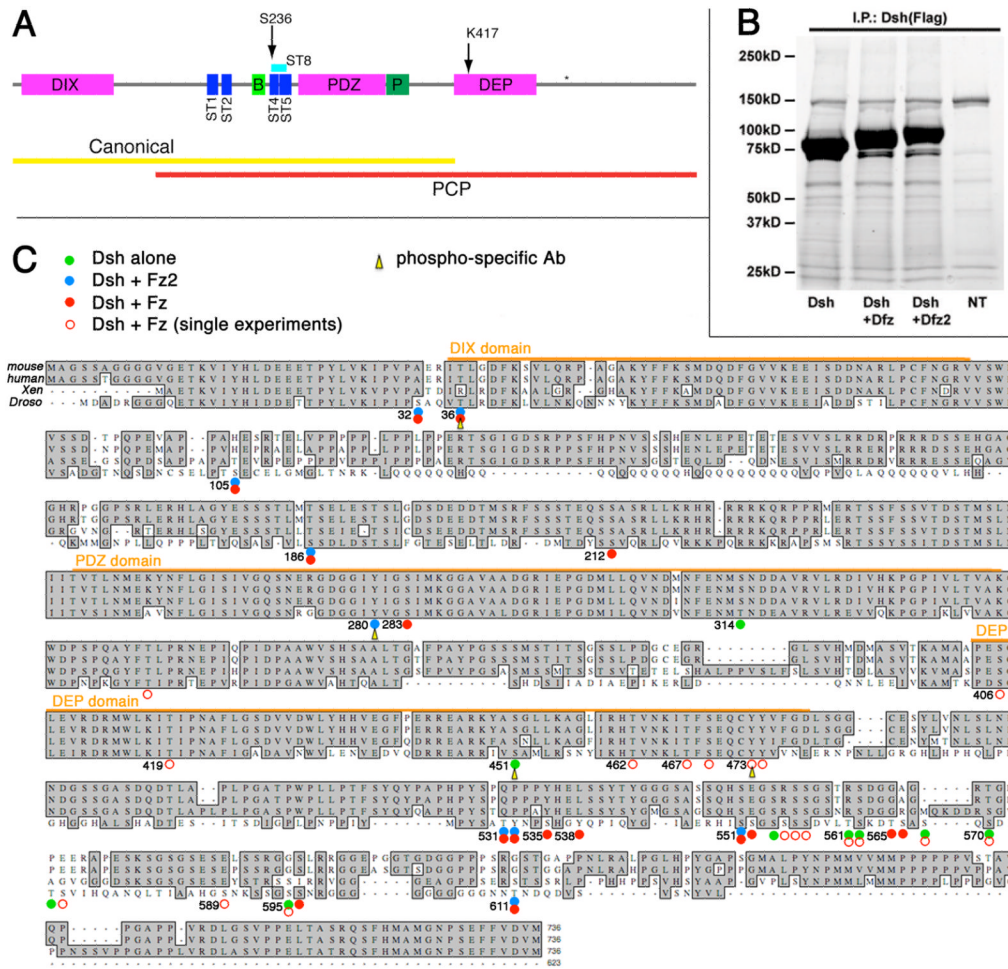
Figure 1. Identification of phosphorylated residues of Dsh

(**A**) Schematic of Dsh with the DIX, PDZ, and DEP domains indicated in purple. Light and dark green domain correspond to the Basic and Proline rich regions, respectively. Dark blue boxes represent the Serine/Threonine clusters that were mutated in Penton et al. (2002). ST8 (light blue box) corresponds to the 8 S/T residues that were mutated in Strutt et al. 2006 and have been identified as potential Par1 sites (Ossipova et al 2005). S236 corresponds to the CK1ε site in Klein et al. 2006. K417 and (*) depict the position of the *dsh[1]* mutation and the BlpI site used to engineer the C-terminal mutations, respectively. Note that re-sequencing of the ST5 region of the mutant constructs from Penton et al. (2002) showed that T252 is not mutated in the ST5 containing constructs. The defined functional requirements for the two Wnt-signaling pathways (Boutros et al. 1998; 1999; Wallingford and Habas, 2005) are indicated as yellow (canonical pathway) and red (PCP signaling) bars below the scheme.
(**B**) Purification of transfected Dsh protein from HEK293T cells. Protein staining (Simply Blue, Invitrogen) of SDS-PAGE gel is shown. 3xFlag-tagged *Drosophila* Dsh was transfected alone or co-transfected with either Dfz or Dfz2 as indicated. Cell lysates were immunoprecipitated with anti-Flag antibodies. The respective products were analyzed in 4–15% gradient SDS-PAGE gel (Biorad; BSA was used to estimate protein concentration). NT lane: untransfected control. The upper bands/region (hyperphosphorylated Dsh) were cut out, eluted and subjected to mass-spectrometry (MS) studies (see Methods).
(**C**) Phosphorylated residues of *Drosophila* Dsh as identified by mass-spectrometry, *Drosophila* Dsh sequence is aligned with mouse Dvl2, human Dvl2, and *Xenopus* Dsh.

Marked residues were detected as phosphorylated in independent transfections and MS experiments. The results shown reflect three independent experimental analyses; phosphorylation events detected only in one of the three analyses are shown with open circles instead of dots. Green dots: Dsh transfected alone (naïve state); blue dots: Dsh co-tranfected with Fz2 ("canonical" signaling state); red dots: Dsh co-transfected with Fz ("PCP" state); the respective signaling states were assigned based on observations of Dsh membrane recruitment (T.J. Klein and MM, unpublished) and activation of the Top-flash Wnt-signaling reporter assay. Orange color bars indicate the extent of the three conserved domains: DIX (residue 35–83), PDZ (252–338) and DEP (404–478). Yellow arrowheads indicate residues that were chosen for anti-phospho-residue antiserum generation. All four phospho-residues were confirmed by *in vivo* analyses.
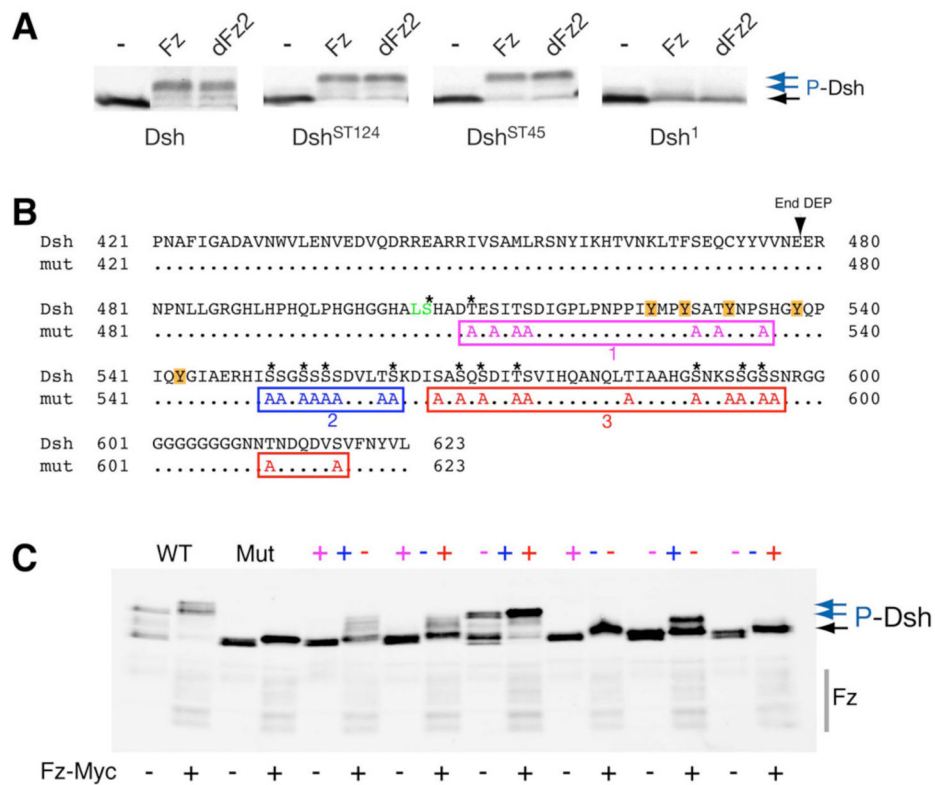
Figure 2. Dsh mobility shift assays

(**A**) Gel-shift assays show that Dsh^ST124 and Dsh^ST45 phosphorylation (blue arrows on right) can be induced by co-transfections of Fz or dFz2 in S2 cells. As previously shown *in vivo* (Axelrod 2001), *dsh^1* barely shows a gel-shift under these conditions. (**C, D**) The C-terminal S/T residues affect the Dsh gel-shift. (**B**) Sequence of the C-terminus of Dsh with the end of the DEP domain indicated. To assess the contribution of the 29 S/T residues of the C-tail to the phosphorylation induced gel-shift, we mutated 28 of them in three clusters (#1: purple, #2: blue, and #3: red in color code; note that the first serine is part of the BlpI site (green) and was not mutated for cloning reasons). Asterisks indicate conserved S/Ts. The region around cluster #1 also contained 5 Tyrosines (marked by orange boxes). (**C**) Myc-Fz induced Dsh gel-shift assay in 293T cells. In contrast to wild-type Dsh, a Dsh with a fully mutant C-terminus is not shifted (Mut; blue arrows indicate phosphorylated forms of Dsh; note the strong shift due to endogenous Dsh activation of *WT*-Dsh in the 293T cells used). Analysis of mutations of individual clusters shows that the second and third clusters have bigger effects on the gel-shift than the first cluster. Combinations of the clusters, however, indicate that the first cluster also contributes to Dsh phosphorylation as assessed by gel-shifts. Grey bar on the right shows migration of Myc-Fz.
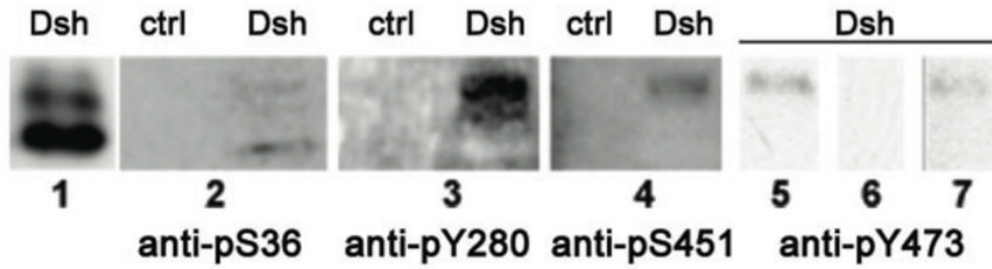
Figure 3. Phosphorylation of Dsh protein *in vivo*
Immunoprecipitated Dsh protein from late larval and pupal stages was analyzed with phospho-residue specific antibodies (see Methods for details).
Purified Dsh protein from dsh-Dsh3xFlag, dsh[V26] flies were probed with the following antibodies (all control lanes are BSA): 1: anti-Dsh; 2: anti-phosphoThr36; 3: anti-phosphoTyr280; 4: anti-phosphoSer451; 5–7: anti-phosphoTyr473 with phosphorylated antigen peptide as competitor (lane 6) and non-phosphorylated antigen peptide as competitor (lane 7).
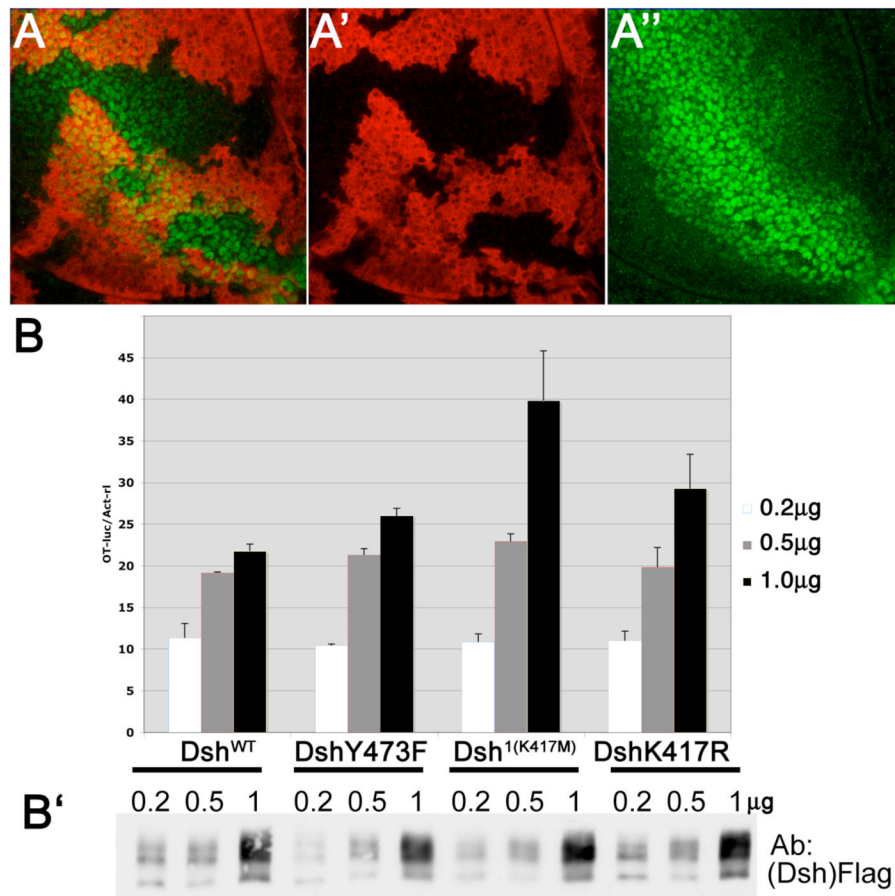
Figure 4. Canonical Wg-signaling activity of Dsh[1], DshY473F, and DshK417R mutants
(A) *dsh-DshY473F* (in a *dsh-* background) fully rescues the expression of Wg-signaling target genes during wing disc development. Example shown is Distalless (Dll). Senseless/ Sens was also tested and rescued (not shown).

3rd instar larval wing disc stained for the clonal marker *lacZ* (red, marks wild-type regions) and Distalless (Dll; green), expressed along the dorso-ventral margin flanking the Wg expression stripe and weaker throught the wing blade anlage. Dll is a direct target of Wg. *dsh*[V26] clones loose Dll expression (not shown). The presence of DshY473F fully rescued the mutant *dsh*[V26] effect. Dll expression showed no difference between mutant and wild-type tissue. (**A′**) and (**A″**) show single channels for lacZ and Dll, respectively.

(B-B′) Dsh mutants do not affect expression levels of Top-flash reporter. Dsh mutant expression constructs were transfected with Top-flash luciferase reporter (and SV40-renilla luciferase reporter as control) in HEK293T cells. The ratios of Top-flash vs. renilla were normalized against empty vector control at three doses of transfection as indicated. Note that DshY473 showed no significant reduction as compared to wild-type Dsh (Dsh[WT]); the same was the case with Dsh[1](K417M) and DshK417R mutants. (**B″**): Western-blot against Dsh to visualize similar protein levels.
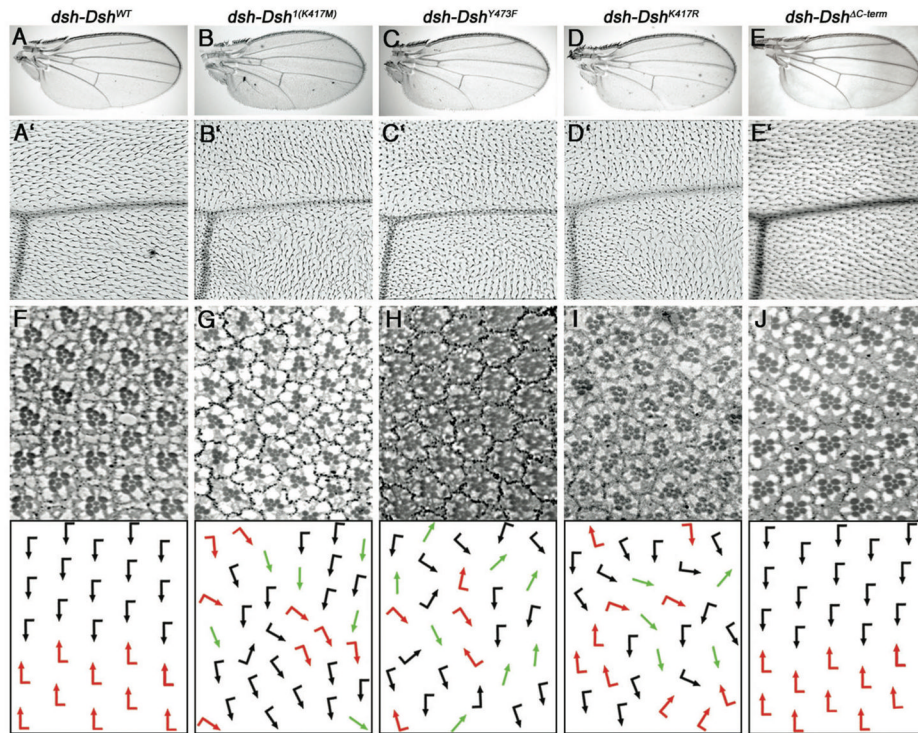
Figure 5. Functional requirements of specific Dsh residues *in vivo*
All rescue constructs were expressed under the control of the endogenous *dsh* promoter in a *dsh^{V26}* (null), *f⁻* background; the only Dsh protein present is provided by the transgenic lines (note that all genotypes are *forked* [*f*] to control for *dsh^{V26}*, giving the cellular hairs a wavy appearance). For all constructs, several independent lines behaved identically and displayed very similar expression levels, as expected (see Suppl. Fig. S5).
(**A–E′**) Wings of rescued flies of indicated genotype are shown; low magnification (A–E) highlight normal Wg-signaling: development with of normal sized wing and normal wing margin development demonstrating correct canonical Wnt signaling. High magnification (panels A′-E′) show PCP arrangements of the respective wings. The high magnification pictures show a region near the posterior cross vein and L4. Note PCP defects in the genotypes *dsh-Dsh^1*, *dsh-Dsh^{Y473F}*, *dsh-Dsh^{K417R}*. (**F–J**) Adult eye sections of the respective genotypes with schematic presentation in lower panels; black and red arrows represent ommatidia with chiral arrangements, which are normally arranged in a mirror-image across the dorso-ventral midline (see panel F for wild-type); green arrows represent symmetric ommatidia. Note that in G–I, the mirror-image arrangement is lost and a random distribution of the two chiral forms and symmetrical ommatidia is detected, reflecting typical PCP defects.
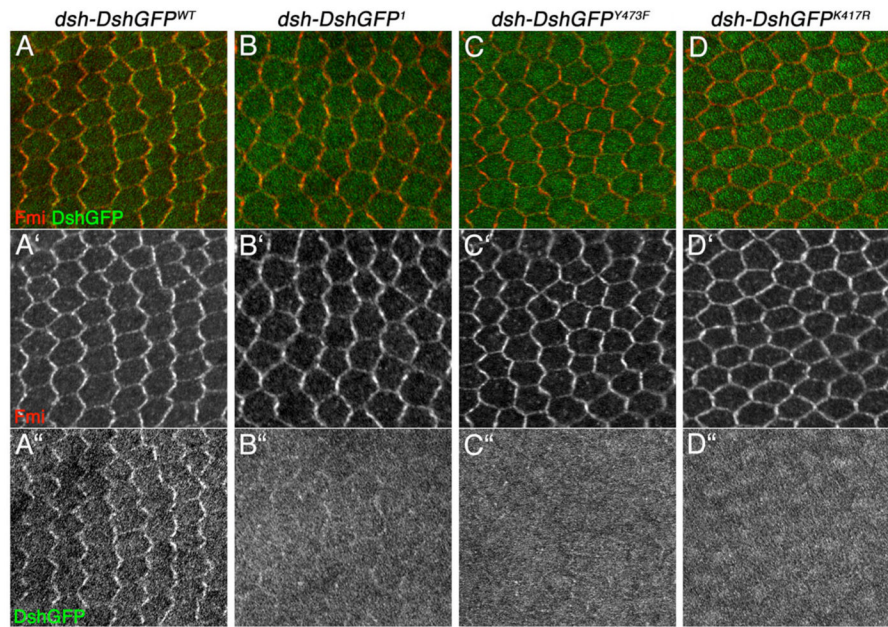
Figure 6. Localization of mutated Dsh isoforms in pupal wings during PCP signaling. Confocal images of DshGFP (green) and Fmi (red) staining in pupal wings at the 30 to 34hrs APF stage are shown. The respective genotypes are indicated above

A′-D′ display anti-Fmi monochrome images (highlighting membranes and the PCP complexes localized there), and A″-D″ show monochromes of DshGFP (A–D are the merged channels). Note that in B/B″, C/C″, and D/D″ the membrane levels of DshGFP are reduced or even absent, although the overall signal and protein levels are not affected (see also Suppl. Figure S5).
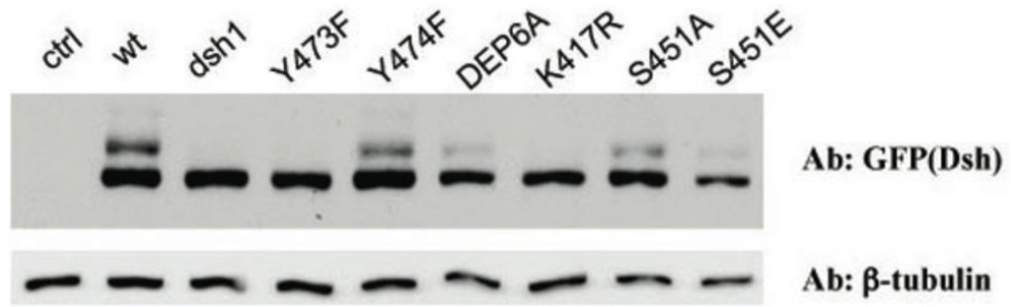
Figure 7.
Migration behavior and phophorylation status of mutant DshGFP isoforms as detected by Western blots of late 3rd instar/early pupal tissues. The respective Dsh isoforms are indicated above the gel and were detected with an anti-GFP antibody ("ctrl" lane has no DshGFP protein present as control). All lines are expressed from the endogenous *dsh*-expression cassette and exhibit very similar protein levels (also Suppl. Figure S5). Note that Dsh[1], Dsh[Y473F] and Dsh[K417R] show markedly reduced gel shift, indicative of reduced Dsh phosphoprylation and correlating with their defective behavior in PCP signaling.

Table 1

Rescue analysis of dsh^{V26} by respective Dsh phosphorylation mutants.

Protein mutant (all in dsh^{V26} null background)	viability	PCP aspect	Domain location
dsh^{V26}	--	**defective** (in clones)	
dsh-DshGFP	+++	wild type	
dsh-Dsh3xFlag	+++	wild type	
$dsh^1(K417M)$	$_+$*	**defective**	DEP
Y24F	+++	wild type	DIX
T36A	+++	wild type	DIX
T252A	+++	wild type	PDZ
S254A	+++	wild type	PDZ
S266A	+++	wild type	PDZ
S271A	+++	wild type	PDZ
Y280F	+++	wild type	PDZ
S283A	+++	wild type	PDZ
T314A	+++	wild type	PDZ
T349A	+++	wild type	proline rich
S406A	+++	wild type	DEP
T419A	+++	wild type	DEP
S451A	+++	wild type	DEP
T462A	+++	wild type	DEP
T467A	+++	wild type	DEP
S469A	+++	wild type	DEP
Y473F	$_+$*	**defective**	DEP
Y473F in abl−/+	$_+$*	**defective**	DEP
Y474F	+++	wild type	DEP
S406E	+++	wild type	DEP
T419E	+++	wild type	DEP
S451E	+++	wild type	DEP
DEP6A	+++	wild type	DEP
K417R	$_+$*	**defective**	DEP
C-term#2	+++	wild type	C-terminus
C-term#1-2	+++	wild type	C-terminus
C-term#1-3	+++	wild type	C-terminus
C-term#2-3	+++	wild type	C-terminus
C-term5Y-F	+++	wild type	C-terminus
DshΔC	+++	wild type	C-terminus

The table indicates the location of the mutation (domain that is affected), capability to rescue flies to viability, and PCP features. All data are based on rescue of the following genotype: dsh^{V26}, *f-/Y; dsh-DshGFP**/+*, multiple lines were analyzed for all constructs.

PCP aspects were recorded based on wing, thorax, and eye phenotypes (see also Figure 4).

*note that the dsh^1 allele and the other two DEP domain mutants that did rescue to viability with PCP defects showed reduced viability.