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Conservation of *dishevelled* structure and function between flies and mice: isolation and characterization of *Dvl2*

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Abstract

The segment polarity gene *dishevelled* (*dsh*) of *Drosophila* is required for pattern formation of the embryonic segments and the adult imaginal discs. *dsh* encodes the earliest-acting and most specific known component of the signal transduction pathway of Wingless, an extracellular signal homologous to *Wnt1* in mice. We have previously described the isolation and characterization of the *Dvl1* mouse *dsh* homolog. We report here the isolation of a second mouse *dsh* homolog, *Dvl2*, which maps to chromosome 11. The *Dvl2* amino acid sequence is equally related to the *dsh* sequence as is that of *Dvl1*, but *Dvl2* is most similar to the *Xenopus* homolog *Xdsh*. However, unlike the other vertebrate *dsh* homologs. Like the other genes, *Dvl2* is ubiquitously expressed throughout most of embryogenesis and is expressed in many adult organs. We have developed an assay for *dsh* function in fly embryos, and show that *Dvl2* can partially rescue the segmentation defects of embryos devoid of *dsh*. Thus, *Dvl2* encodes a mammalian homolog of *dsh* which can transduce the Wingless signal.

Keywords: *dishevelled*; *Dvl2*; *Drosophila*; Mouse

1. Introduction

The *dishevelled* (*dsh*) gene in *Drosophila melanogaster* identifies a gene required for pattern formation in both the embryo and adult. Lethal alleles result in mutant phenotypes ranging from an extreme segment polarity phenotype in the ventral embryonic cuticle to severely mispatterned legs in pupae (Perrimon and Mahowald, 1987; Klingensmith et al., 1994). Such defects suggest failures in the cell interactions underlying particular patterning processes.

The molecular basis of *dsh* phenotypes is a failure by target cells to respond to the positional information conveyed by the Wingless (Wg) signal. Extensive genetic and molecular work in fly embryos has shown that *dsh* en-

codes a ubiquitously expressed, novel protein which functions cell-autonomously to transduce the Wg signal (Klingensmith et al., 1994). It is normally cytoplasmic, but becomes phosphorylated and recruited to the plasma membrane upon Wg stimulation (Yanagawa et al., 1995). *dsh* functions downstream of Wg but upstream of other known components of the Wg signalling pathway, such that Dsh relieves Zeste-white 3 (Zw3) inhibition of Armadillo (Arm) activity, leading to maintenance of *engrailed* (*en*) expression and other manifestations of proper cell fate (Noordermeer et al., 1994; Siegfried et al., 1994). While Dsh does not appear to be the Wg receptor, it is at present the earliest known component of the Wg signal transduction pathway.

All of these genes are conserved in vertebrates, and recent work in *Xenopus* suggests that the Wg signaling pathway is also conserved. Several studies have implicated *Wnt* genes, a large family of extracellular signalling molecules to which *wg* belongs, as having a role in axial

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patterning of frog embryos. Injection of mRNA transcribed from various *Wnts* into ventral vegetal blastomeres results in ectopic dorsalizing activity, leading to a duplicated body axis (Sokol et al., 1991; Moon et al., 1993). At least two of these genes are expressed at the right time and place to be part of the endogenous mechanism for axial organization (Ku and Melton, 1993; Cui et al., 1995). The *Xenopus* homolog of *dsh*, *Xdsh*, is uniformly expressed in early embryos but injection into ventral blastomeres also leads to a duplicated body axis (Sokol et al., 1995). Injection of dominant-negative forms of *GSK-3 β* , the vertebrate homolog of *zw3*, also has dorsalizing effects, and overexpression of wild type *GSK-3 β* transcript suppresses the dorsalizing activity of *Xwnt8* and *Xdsh* (He et al., 1995; Dominguez et al., 1995; Pierce and Kimelman, 1995). This is consistent with the function of *Zw3* in the *Wg* pathway, in that it must be inhibited to achieve the outcomes promoted by *Wg* and that it functions downstream of *Dsh*. Finally, injection of antibodies to β -catenin, encoded by a gene highly homologous to *arm*, leads to axis duplication too (McCrea et al., 1993). Moreover, injection of β -catenin antisense oligonucleotides into oocytes inhibits dorsal axis development, demonstrating a role for this gene in the endogenous patterning mechanism of the dorsal axis (Heasman et al., 1994). This inhibition cannot be rescued by overexpressing the dorsalizer *Xwnt8*, implying that β -catenin functions downstream of *Wnt* in the dorsalizing pathway. Taken together, the *Xenopus* experiments strongly suggest that a *Wnt* gene is involved in inducing dorsal organization, and that the *Wnt* signal is transduced by a pathway involving homologs of *dsh*, *zw3* and *arm*.

Wnt genes are also known to play important roles in mammals. Analysis of classical and targeted mouse mutants has revealed that *Wnt* genes are required for development of many structures, such as the midbrain, the kidney, and the limb (Parr and McMahon, 1994, 1995; Stark et al., 1994). Also, ectopic expression of several *Wnts* can lead to mammary tumors in mice or oncogenic transformation of mammary cell lines in culture (reviewed by Nusse and Varmus, 1992). The mammalian homolog of *Wg*, *Wnt1*, is the founding member of the family and leads to mammary tumors when misexpressed and mid-brain deletion when inactivated (Nusse and Varmus, 1992).

Little is known about how *Wnt* signals are transduced in mammals; we are interested in elucidating their signaling pathways. Despite the roles many *Wnt* genes play in many species, *wg* is the best defined in terms of the action of downstream genes. *dsh* is the earliest known component of the *wg* signal transduction pathway (Klingensmith et al., 1994; Theisen et al., 1994). The intimate and obligate association of *dsh* with *wg* signal transduction prompt us to study murine *dsh* homologs. We report here the isolation, mapping, and expression of a second murine *dsh* homolog, *Dvl2*, and show that it can partially replace

the function of *dsh* in segmentation of the fly embryo. We discuss the implications of this rescue and the importance of various conserved domains among the four identified *dsh* cognates.

2. Results

2.1. Isolation and sequence analysis of *Dvl2*

Our initial search for murine genes related to *dsh* yielded a highly homologous gene we named *Dvl1* (Sussman et al., 1994). However, in our cloning of this gene we found evidence of cross-hybridizing sequences apparently distinct from *Dvl1*. To determine whether these might represent additional genes homologous to *dsh*, we used the *Dvl1* cDNA as a probe in a low-stringency screen of a brain cDNA library. Among the weakly-hybridizing clones isolated, most appeared to represent a single locus as judged by restriction digestion and strong cross-hybridization (data not shown). The longest of these, clone 11, was sequenced and found to encode a single open reading frame of 736 codons which is flanked by an upstream Kozak consensus sequence and a downstream polyadenylation signal and tail (Genbank accession no. U24160). The translated sequence, encoding a protein with a calculated molecular weight of 78 753 Da, is homologous to both *Dvl1* and *dsh*, and thus represents a second murine *dsh* homolog. The gene encoding clone 11 is henceforth called *Dvl2*.

The sequence of the deduced amino-acid sequence of *Dvl2* is shown in Fig. 1, where it is compared to those of the other known *dsh* genes, *Drosophila dsh* (Klingensmith et al., 1994), *Xenopus Xdsh* (Sokol et al., 1995), and mouse *Dvl1* (Sussman et al., 1994). *Dvl2* has a very similar structure to the other family members, but otherwise is not related to sequences in any of several databases, except for a *discs-large* homology domain (DHR, also referred to as the GLGF motif and the PDZ domain; Cho et al., 1992; Kornau et al., 1995) also conserved in other *dsh* cognates. This domain is found in several proteins and has been suggested to be involved in transiently associating cytoplasmic proteins with the plasma membrane, possibly at cell–cell junctions (Bryant et al., 1993; Kim et al., 1995; Kornau et al., 1995). Other potential structural motifs include regions fulfilling the criteria for PEST sequences (Rogers et al., 1986), which may be associated with rapid degradation and are found in other *dsh* family members, and a potential SH3 binding polyproline domain (Ren et al., 1993), which is not conserved in the other members. Thus, like the other *dsh* genes, *Dvl2* encodes nothing suggestive a priori of its biochemical function.

Comparison of *Dvl2* to the other *dsh* family members reveals domains of very high conservation. The major blocks of homology are near the amino terminus (CR I, 80 residues), in the central region spanning the DHR (CR

Dvl2	MAGSSAGGGG	VGETKVIYHL	DEEETPYLVK	IPVPAERITL	GDFKSVL	QR	49	Dvl2	YPGSSSMSTI	TSGSSLPDGC	EGRG.....	LSVHMDMA	SVTKAMAAP	434
Xdsh	MAETKVIYHL	DEEETPYLVK	VPVPAEDIRL	RDFKAAL	GR		39	Xdsh	YGSASMSM	TSTSTVTETE	LSHALPVPVL	PSLSVTDLA	SVKVMASPE	429
Dvl1	MAETKVIYHL	DEEETPYLVK	LPVAPERVTL	ADPKNVLSNR			30	Dvl1	YGTSPCSSAI	TRTSSSLTS	SVFGAPQLE	APLTVKSDMS	AIVRVMLPD	426
Dsh	..MDADRGG	GOETKVIYHL	DEEETPYLVK	IPVPAEQVTL	RDFKLVL	NK	46	Dsh	TSHDSIADI	AEPIKERLDQNNLE	EIVKAMTKPD	405
		ETKVIYH	DeEETPYLVK	PvP	tL	DFK vL r				Ts	S		vk M P	

CR I								CR III							
Dvl2	PAGA	KYFFK	sMDQDFGVK	EEISDDNARL	PCFNDRVSV	LVSDDTPQPE	98	Dvl2	SGLEVRDRMW	LKITTIPNAFL	GSDVVDVLYH	HVEGFPERRE	ARKYASGLLK	484	
Xdsh	GHA	KYFFK	AMDQDFGVK	EEISDDNAKL	PCFNDRVSV	LASSGSGQPD	87	Xdsh	SGLEVRDRMW	LKITTIPNAFL	GSDVVDVLYH	HVEGFQDRRE	ARKFASNLK	479	
Dvl1	PVHAYKFFFK	sMDQDFGVK	EEIPDDNAKL	PCFNDRVSV	LVLAEAGHSD		90	Dvl1	SGLEVRDRMW	LKITIPNAFL	GADVVDVLYT	HVEGFKERRE	ARKYASSMLK	476	
Dsh	QNNNYKYFFK	sMDADFGVVK	EEIADDDTIL	PCFNDRVSV	LVSADGTNS		96	Dsh	SGLEVRDRMW	LKITIPNAFL	GADAVNWVLE	NVEDVQDRRE	ARRIVSAMLK	455	
	a KyFFK	sMDQDFGVK	EEI DdNa	L	PCFNDRVSV	Lvs	g		SGLE	RDRMW	LKITIPNAF	G DvVdVly	HVEgf	RRE ARK as Lk	

Dvl2	VAPPAHE...	SRTLELVPPP	PLPPLFPERT	SGIGDSRPPS	FHPNVSSSHE	145	Dvl2	AGLIRHTVNK	ITFSEQCYYV	FGDLGG...	CESYLVNLSL	NDNDGSSGAS	531
Xdsh	SAPPAPATEV	RPEFPFVPPP	PIPPPAERT	SGIGDSRPPS	FHPNVSSSHE	137	Xdsh	AGFIRHTVNK	ITFSEQCYYI	FGDL.TG...	CENYMTNLSL	NDNDGSSGAS	525
Dvl1	AGSQGTD...	SHTLDLPP...PLERT	GGIGDSRPPS	FQPNVASSRD	129	Dvl1	HGFLRHTVNK	ITFSEQCYYV	FGDLCSN...LASL	NLNSGSSGAS	517
Dsh	DNCSE.....	LPTSCELG	MGLTRKRLQQ	QQQQHQQQQQ	130	Dsh	SNIYKHTVNK	ITFSEQCYYV	VNEERNPMLL	GRGHLHPHQL	PHGHGGHALS	505
			p Ert	Gigdsrpps	f pnv	s		g iRHTVNK	iTFSEQCYv	fgdl	sL n n	GssgAs	

Dvl2	NLEPETETES	VVSLRRDRPR	RRDSSEHAG	GHRPG.	GPS	RLERHLAGYE	193	Dvl2	DQDTLA..PL	PGATPW.PLL	PTFSYQYAP	HPYSPQPPPY	HELSSYTYGG	578
Xdsh	QLDQ..DNES	VISMRRDRVR	RRDSSEQAGV	G..RG.	VNG	RTERHLSGYE	181	Xdsh	DQDTLAPLPL	PGASPW.PLL	PTFSYQYAP	HPYSTQPPAY	HELSSYSYGM	574
Dvl1	GMDNETGTES	MVSHRRERAR	RRNRDEAART	NGHPR.	GDR	RRDLGLPF.D	176	Dvl1	DQDTLAPLPH	PSV.FW.PLG	QGYPYQYGP	PPCF..PPAY	QD.PGFSGCS	562
Dsh	QQQQHQQQQ	QQQQQVQVQ	QLAQQQQQV	LHHQKMGNP	LIQPPPLTYQ		180	Dsh	HADTESITSD	IGPLENPPY	MFYSATYNS	HGV..QPIQY	543
	es	s rr r	rr e	g	r	l		dQDTLa	p p	Fw Pl	syQY	p hpy	Pp Y	

Dvl2	SSSTLMTSEL	ESTSLGSDSE	DDTMSRFSSS	TEQSSASRL	KRHR	RRKQ	242	Dvl2	GSASSQHSEG	SRSSGSTRSD	GGAG....R	TGRPEERAE	SKS.GSGSES	622
Xdsh	SSSTLLTSEI	E.TSICDSEE	DDTMSRFSSS	TEQSSASRL	KRHR	RRKQ	229	Xdsh	GSAGSQHSEG	SRSSGSTRSD	GGRMQKDDR	SGVAGVGGGD	SKS.GSGSES	623
Dvl1	SASTVLSSEL	ESSFIDSDE	EDTMSRLSS	TEQSTSSRLV	RKHKRRKQ		226	Dvl1	GSAGSQHSEG	SKSSGSTRSSHR	TPGREERRAT	GAG.GSGSES	603
Dsh	SAS.VLSSDL	DSTSLFGTES	ELTLDR..DM	TDYSSVQLQ	VRKKPQRKQ		227	Dsh	GTAERHISGG	SSSDVLTSK	DTSASQSDIT	SVIHQANQLT	IAAHGNSKSS	593
	S St l	sel ests	ds e dt	sR sss	TeqSs	sRL rh	rRRKq	Gsa	sq	SeG	S Ssgs	rS	r	Gsgses

Dvl2	RPPRNERFSS	FSSVTDSTMS	LNIIITVTLNM	EKYNFLGISI	VQGSNERGDG	292	Dvl2	ELSSRRGSLR	RGGEFGTGD	GGPPPSRGST	GAPPNLRALP	GLHPYAPSG	672
Xdsh	RPPRLERTSS	FSSVTDSTMS	LNIIITVTLNM	EKYNFLGISI	VQGSNERGDG	279	Xdsh	EYSTRS....	SIRRVGGGE	AGPPSER.ST	SSRLPFPHP	SVHSYAAP.G	666
Dvl1	RLRQDTRASS	FSSITDSTMS	LNIIITVTLNM	ERHFLGISI	VQGSNDRGGD	276	Dvl1	DHTVPSGSGS	TGWWRPVSG	LSRGSATYNS	ASAVAP.GLP	PLHPLTKAYA	652
Dsh	RAPSMRSTSS	FSSITDSTMS	LNIIITVTLNM	EAVNFLGISI	VQGSNRGGD	277	Dsh	GSSNRGGGG	GGGGNMVND	QDVSVSNVYL	*.....		623
	R P	Ress	fss	TdSTMS	LNIIITVtlNM	E nFLGISI	VQGSN	rGDG	s	r	S	s	

CR II													
Dvl2	GIYIGSIMKG	GAVAADGRIE	PGDMLLQVND	MNFENMSNDD	AVRVLRDIVH	342	Dvl2	MALPVNEMMV	VMMPPPPPPV	STAVQPPGAP	P.....VRD	LGSVPPDELTA	716
Xdsh	GIYIGSIMKG	GAVAADGRIE	PGDMLLQVND	INFENMSNDD	AVRVLRDIVH	329	Xdsh	VFLSVNEMML	MMPPPPPPV	PGVCPPNSVV	PPGAPPLVRD	LASVPPDELTA	716
Dvl1	GIYIGSIMKG	GAVAADGRIE	PGDMLLQVND	VNFENMSNDD	AVRVLREIVV	326	Dvl1	VV.....G	GPPGGPPVRE	LAAVPPDELTA	675
Dsh	GIYIGSIMKG	GAVALDGRIE	PGDMILQVND	VNFENMND	AVRVLREIVV	327	Dsh	vr 1	vppelt

Dvl2	KPGPIVLTVA	KCWGPPSQAY	FTLPRNEPIQ	PIDPAAWVSH	SAALTGAPPA	392	Dvl2	SRQSFHMAMG	NPSEFFVDVM	* 736			
Xdsh	KPGPIVLTVA	KCWDPSPQGY	FTLPRNEPIH	PIDPAAWVSH	SAALSGSFPV	379	Xdsh	TRQSFHMAMG	NPSEFFVDVM	* 736			
Dvl1	QTGPISLTVV	KCWDPPTPRSY	FTIPRDPVPR	PIDPAAWVSH	TAALTGALPR	376	Dvl1	SRQSPQKAMG	NPCEFFVDIM	* 695			
Dsh	KPGPIKLVVA	KCWDPNPKGY	FTIPRTEPVR	PIDPGAUAH	TQAL.....	371	Dsh			
	KpGPI	LtVA	KCWp	P Y	FT PR	eP	PIDPA	AwVsh	aAL
										rqsf	amg	np	effvdim

Fig. 1. Multiple sequence alignment of *Dvl2* with the other known *dsh* homologs. Deduced amino acid sequences for murine *Dvl2* and *Dvl1*, *Xenopus* *Xdsh*, and *Drosophila* *dsh* are aligned to show their conservation. The fifth line displays a consensus residue for each position in a capital letter for absolutely conserved residues and in lower case for those conserved in three of the four homologs. Numbers to the right of each line refer to the position in the sequence of the corresponding gene. There are three principle regions of very high conservation in all four family members, which are indicated by lines above the conserved regions. These include one near the amino terminus spanning 80 residues, designated CR I, a large central domain of 138 amino acids (CR II) which includes the discs-large homology region (DHR), and a stretch of 72 residues in the carboxyl half (CR III). In addition, *Xdsh* and *Dvl1* share an extension of the carboxyl end which is also highly conserved. The *dsh* gene encodes an *opa* repeat (Wharton et al., 1985), the polyglutamine tract between the CR I and CR II, which is not conserved at the amino acid level but which is replaced by similarly hydrophilic domains of similar length in the other proteins. Preceding CR II is a highly basic stretch present in each protein (dotted line above the sequences). Each protein also has proline-rich stretches falling outside the conserved regions, which fulfill the criteria for PEST sequences (Rogers et al., 1986). Such sequences are associated with rapid degradation of the proteins encoding them. Biochemical analysis of *Dsh* shows that it is phosphorylated on serine and/or threonine residues in response to Wg signaling (Yanagawa et al., 1995), and there are 37 completely conserved serine and threonine residues in these proteins. One of these (*) occurs in a region which, when deleted, eliminates Wg-mediated phosphorylation of *Dsh*, and so is a candidate for this phosphorylation event. This residue is conserved as a serine vertebrate member, and the flanking residues are completely conserved, suggesting the residue is recognizable by the same potential serine/threonine kinase.

Table 1
dsh homology identity and similarity

	Dvl2	Dvl1	Xdsh	dsh	
Dvl2		80	85	62	SIMILARITY
Dvl1	64		74	63	
Xdsh	76	60		62	
dsh	47	48	46		
	IDENTITY				

Translated open reading frames from sequences of *Dvl2*, *Xdsh*, *Dvl1*, and *dsh* are compared. Percent identity is indicated to the left of the diagonal line, and similarity to the right.

II, about 150 residues) and in the carboxyl half (CR III, 70 residues). All four genes also share a cluster of basic residues just before CR II. Table 1 presents the degree of identity and similarity among *dsh* genes. *Dvl2* is most closely related to *Xdsh*, with 76% identity overall and blocks of very high conservation; for example, from residues 216 to 411 these genes are 97% identical. The three vertebrate genes are equally related to fly *dsh*; by structural criteria we cannot determine whether either *Dvl* is a true mouse *dsh* ortholog.

2.2. *Dvl2* maps to chromosome 11

We used single-strand conformation polymorphism (SSCP) analysis of recombinant inbred mouse strains (Beier et al., 1992) to map *Dvl2*. Primers corresponding to an intron of *Dvl2* were analyzed and found to identify an SSCP between mouse species (see Section 4). The BSS interspecific backcross was genotyped and the strain distribution pattern analyzed using the Map Manager program. *Dvl2* was found to map to chromosome 11 with a LOD likelihood score of 19.4 (Fig. 2). A single recombinant with D11Mit4 was identified in 73 mice, and six recombinants with *Csfgm*. The recombination frequencies (expressed as genetic distance in centimorgans \pm SE) are *Csfgm*–(8.22 \pm 3.21)–*Dvl2*–(1.37 \pm 1.36)–D11Mit4. The *Dvl2* locus is flanked by the genes *Csfgm*, which has been mapped to 5q21–q32 in humans (Huebner et al., 1985), and *Fert*, which has been mapped to 5q21 (Morris et al., 1990). The genes *Nos2* and *Pitpn*, which are non-recombinant with *Fert*, have been mapped to human chromosome 17. Since sub-chromosomal linkage relationships are generally conserved between mouse and man, and *Dvl2* maps proximal to *Fert*, we would have expected the human homolog of *Dvl2* to map to chromosome 5q. However, in an analysis of a rodent/human somatic cell panel, *Dvl2* has been found to map to human chromosome 17 (Greco et al., 1996).

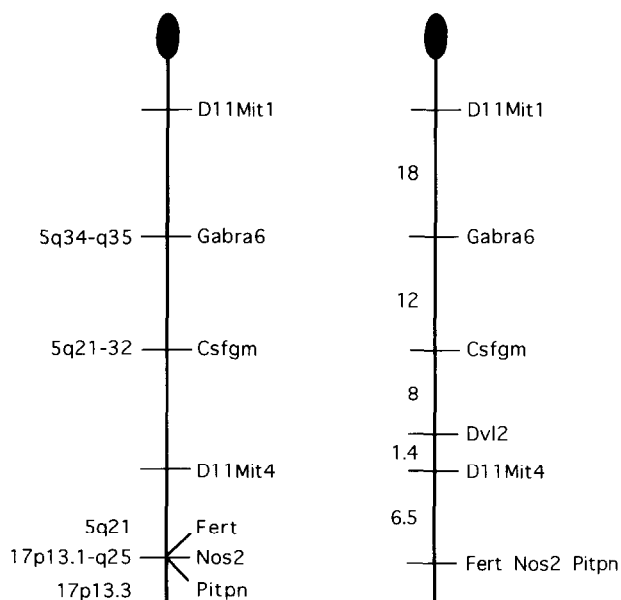


Fig. 2. Map position of *Dvl2* on mouse chromosome 11. Data from consensus map for chromosome 11 is on the left, with chromosome positions of human homologs shown. Data for microsatellite markers and *Dvl2* in the BSS cross is on the right, with genetic distance shown in cM.

2.3. Expression of *Dvl2* in adults and embryos

Our cloning of *Dvl2* revealed it to be a fairly abundant transcript in adult brain, as judged by the number of independent clones recovered (data not shown). We examined the expression of *Dvl2* in a wide variety of adult tissues by RNase protection. As shown in Fig. 3, transcripts are particularly abundant in testis, and also occur at significant levels in spleen, lung, heart, muscle, brain and breast. These tissues derive from all three germ-layers, revealing that *Dvl2* expression is not specific to any particular primary lineage. However, there are some tissues in which little if any expression can be detected, including kidney, liver, thymus and small intestine. Thus, at least some endodermal and mesodermal tissues do not express *Dvl2*. In short, we conclude that *Dvl2* expression in adults is widespread and occurs at various levels, but is not ubiquitous.

We included RNA from various mid- to late embryonic stages in our RNase protection assays and observed abundant expression of *Dvl2* in each case (Fig. 3, and data not shown). We therefore surveyed expression throughout embryogenesis, via reverse transcription (RT) of polyadenylated RNA followed by the polymerase chain reaction (PCR) with *Dvl2*-specific primers. We find that *Dvl2* is expressed at all stages tested, ranging from early gastrulation through late embryogenesis (data not shown).

2.4. *Dvl2* is ubiquitously expressed during gastrulation and organogenesis

We used whole-mount in situ hybridization to examine the spatial and temporal pattern of *Dvl2* expression in postimplantation embryos. From 7.0 days post coitum (dpc), when gastrulation begins, through to 10.5 dpc, by which time all major organs have begun to form, *Dvl2* appears to be ubiquitously expressed at moderate levels. Fig. 4A–C shows *Dvl2* expression in whole-mount embryos from headfold to 25-somite stages, during which time most of the body plan is established. To ascertain whether *Dvl2* is expressed in all cell types, we sectioned some of these embryos. As shown in Fig. 4D,E, *Dvl2* is expressed in all tissue types; we have not detected cells in such sections which appear to lack expression. We have examined later embryos for *Dvl2* expression by RT-PCR and/or in situ hybridization of dissected pieces of embryos, and find that *Dvl2* expression appears to be uniform in later embryos as well (data not shown). Thus, the expression of *Dvl2* is like that of the other *dsh* family members in that it is unrestricted during embryogenesis.

2.5. *Dvl2* can substitute for *dsh* in *Drosophila* embryo segmentation

Although *Dvl2* encodes a gene product with similar

sequence and expression to *dsh*, it does not necessarily encode a product with conserved function. To determine whether *Dvl2* is a functional homolog of *dsh*, we tested the ability of *Dvl2* to substitute for *dsh* during segmentation of the *Drosophila* embryo. During segmentation, Dsh serves to transduce a signal mediated by the Wg protein.

The fly embryo is segmented such that each metamere is delimited by deep segmental furrows, with the ventral epidermal cells at the anterior of each segment secreting a belt of hook-like denticles in orderly rows, whereas the posterior cells make plain or 'naked' cuticle (Fig. 5A). The ventral cuticles of embryos lacking both maternal and zygotic *dsh* transcripts display an extreme segment polarity phenotype, with the naked posterior of each segment deleted and replaced with denticles, such that the entire region is covered with a lawn of denticles (Perrimon and Mahowald, 1987) (Fig. 5B). Only subtle evidence of segmentation remains. Moreover, these embryos are much reduced in size, lack all terminal structures (Fig. 5F), and do not hatch.

We reasoned that because paternally supplied *dsh* expression fully rescues embryos lacking all maternal *dsh* (Perrimon and Mahowald, 1987), we might be able to rescue *dsh* embryos by injecting synthetic *dsh* mRNA into pre-gastrulation embryos devoid of maternal and zygotic *dsh*. To distinguish potential rescued embryos from their wild type siblings, the *dsh* mutant embryos were marked with a genetically linked mutation called *shavenbaby*

(*svb*), which reduces the size and number of denticles (Gergen and Wieschaus, 1986).

We first demonstrated that full length *dsh* mRNA effects a complete rescue of the embryonic *dsh* mutant phenotype (Fig. 5C). After injection of *dsh* mRNA, segmentation is restored, terminal structures such as the head skeleton (Fig. 5G) and the filzkörper are rescued, and the embryos hatch. We then injected full-length *Dvl2* mRNA to test its capacity to rescue the *dsh* phenotype. These injected embryos are shown in Fig. 5D,H–J. The segment polarity phenotype was substantially rescued by *Dvl2*, with restoration of naked cuticle, denticle organization and segmental furrows, as well as development of filzkörper at the posterior termini. However, the rescue was incomplete, since the embryos were still reduced in size, developed abnormal head skeletons (Fig. 5H), and failed to hatch. Greater than 95% of all *dsh* mutant embryos injected with *Dvl2* mRNA showed a significant degree of rescue. The amounts of *dsh* and *Dvl2* injected were approximately equal, and no change in the degree of rescue by either transcript was seen over a three- to five-fold range in concentration (data not shown).

In summary, we have described an *in vivo* assay for *dsh* function which has allowed us to test whether *Dvl2* is functionally conserved. We found that the segment polarity phenotype of *dsh* mutant *Drosophila* embryos was substantially rescued by *Dvl2*. Normal segmentation was largely restored by *Dvl2* function, while restoration of the head skeleton was only partially rescued.

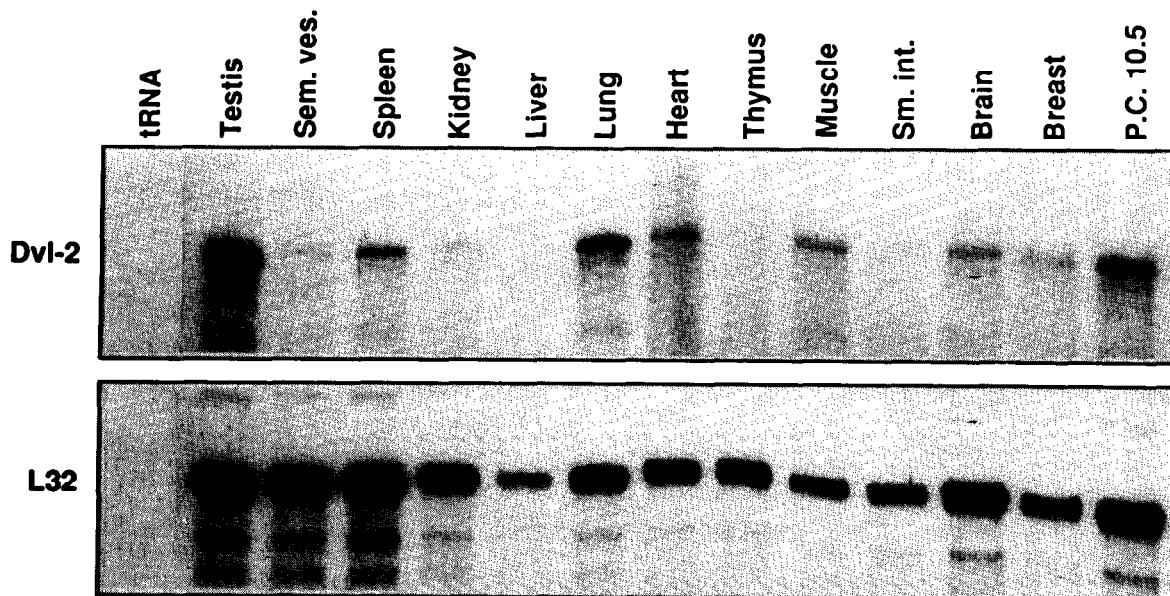


Fig. 3. Analysis of *Dvl 2* expression by RNase protection. Total RNA (20 μ g) was analyzed by ribonuclease protection using a riboprobe specific for *Dvl2* (see Section 4). The undigested probe (not shown) is 510 bp in length and protects a 430 bp fragment. Included in each assay is an internal standard riboprobe that protects 83 bases of ribosomal L32 protein transcript. A tRNA control is included to reveal possible background fragments from incomplete digestion or probe artifact. The adult tissue or developmental stage (in dpc) of each sample is indicated above the corresponding lane. Samples from various other embryonic stages were also analyzed by RNase protection and/or RT-PCR, and in each case expression of *Dvl2* was detected.

3. Discussion

The *Drosophila* segment polarity gene *dsh* functions in the signal transduction of Wg, the genetic paradigm of the widely conserved *Wnt* family of extracellular signaling proteins (reviewed by Klingensmith and Nusse, 1994). In this report we have described our analysis of *Dvl2*, a second mammalian relative of *dsh* residing on chromosome 11. We find that it is a widely expressed, highly conserved gene, and as such is similar to other members of the *dsh* family. We have devised an assay to test whether *dsh* homologs are functionally conserved, and find that *Dvl2* can rescue the segmentation defects of *Drosophila* embryos lacking *dsh*. Our data, together with previously reported structure/function studies of *dsh*, allow us to

consider the functional significance of many of the conserved domains among *dsh* cognates. Further, rescue of the *dsh* defect by *Dvl2* has implications about unknown members of the *Wnt* signal transduction pathway. We will consider these issues after commenting on our expression studies.

We have analyzed expression of *Dvl2* by RNase protection, RT-PCR, and whole-mount in situ hybridization. Transcripts occur in many organs of the adult but are undetectable in others, and their distribution does not seem to correlate with any particular organ type or embryonic origin. In embryos undergoing gastrulation and organogenesis, however, *Dvl2* appears to be uniformly expressed. Expression occurs throughout embryogenesis from at least the initiation of body pattern formation

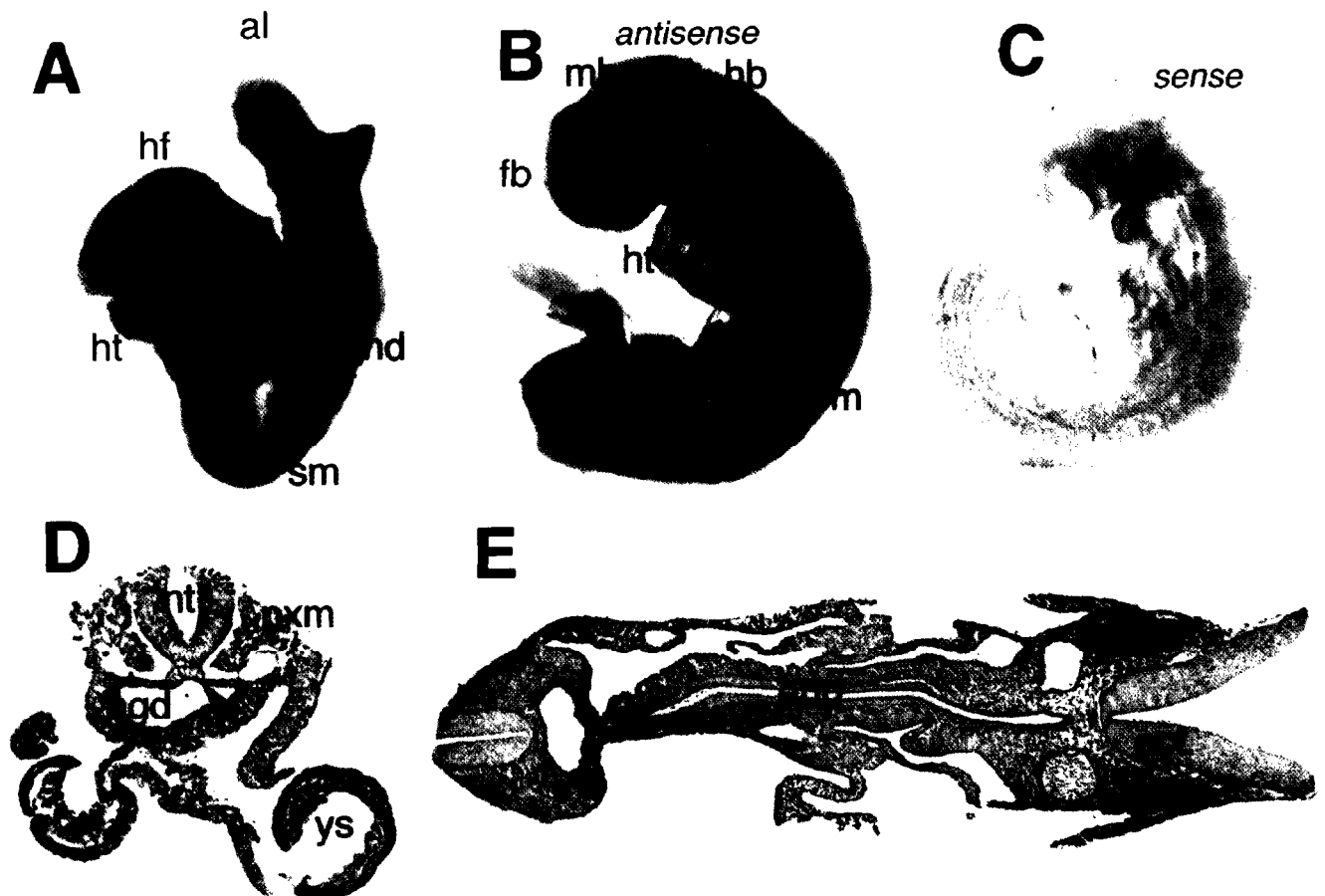


Fig. 4. Whole-mount in situ hybridization of *Dvl2* in embryogenesis. Embryos were hybridized with sense (C) or antisense (A,B,D,E) *Dvl2* RNA probes labeled with digoxigenin, and processed for color development to reveal sites of expression (no counterstain was used). (A) A headfold stage embryo (8.0 dpc) with four pairs of somites; anterior to the left. Transcript is expressed ubiquitously in all major body regions, including the headfolds (hf), primitive heart tube (ht), the somites (sm) and presomitic mesoderm, the node (nd) and primitive streak. Expression is lower in the allantois (al). Differences in intensity appear to be due mainly to differences in the thickness of the various regions, although there may be somewhat higher levels of expression in the neuroectoderm. (B) An 9.0 dpc embryo (after turning). Expression is uniform and seen in forebrain (fb), midbrain (mb), hindbrain (hb), somites, and heart (ht). (C) Sense control of a 9.0 dpc embryo (after turning). (D) Transverse sections, 8.5 dpc embryo, dorsal uppermost. *Dvl2* is expressed at a uniform intensity throughout the yolk sac (ys) and the embryo proper. Transcripts occur in ectodermal structures such as the neural tube (nt), mesodermal tissues like the paraxial mesoderm (pxm) and the notochordal plate (arrow), and in the endoderm as in the hindgut diverticulum (hgd). (E) Transverse section through the trunk region of a 9.5 dpc embryo, with anterior to the right. Transcript is expressed at an essentially uniform level throughout the ectoderm (ect), mesoderm (mes) and endoderm (end).

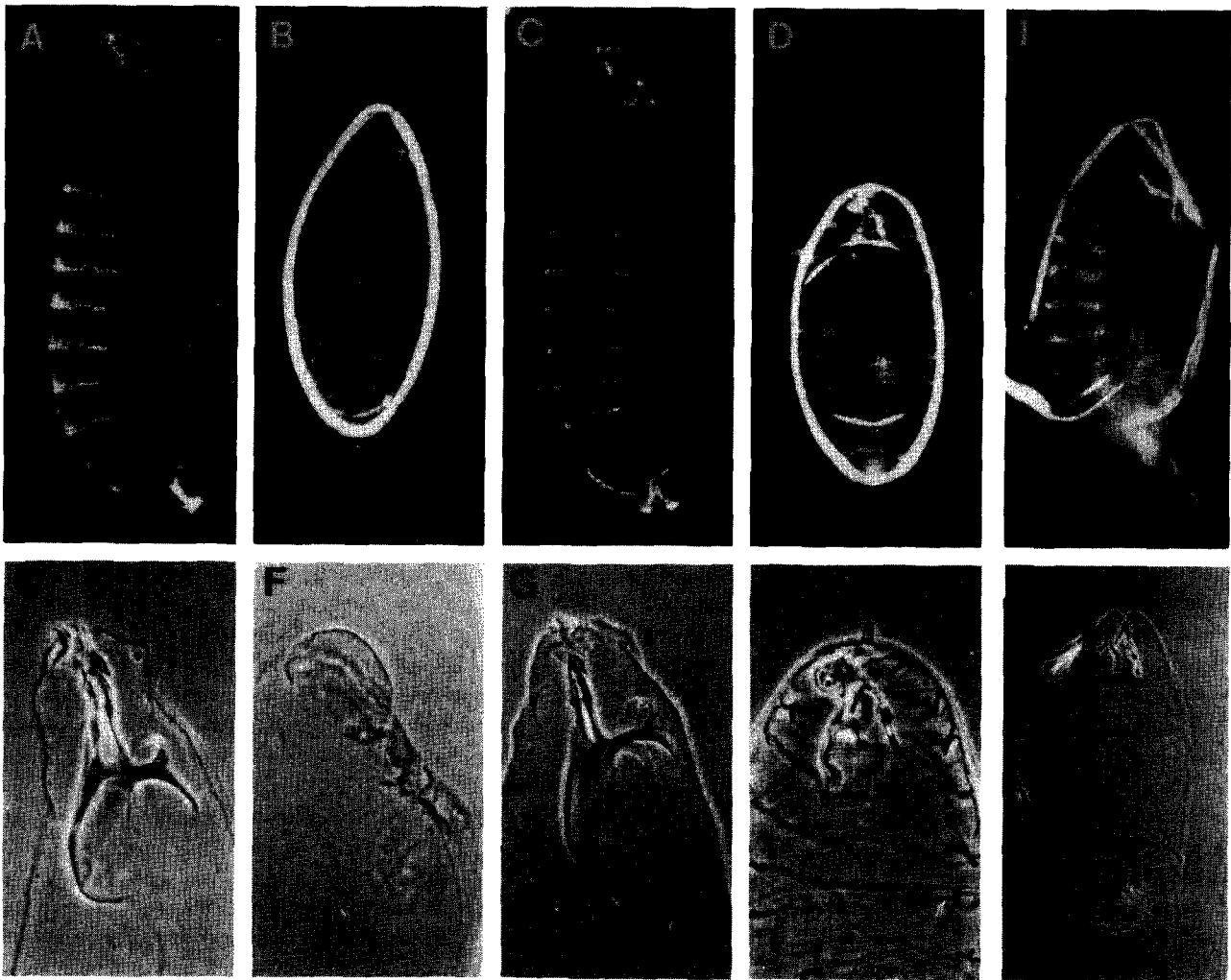


Fig. 5. *Dvl2* mRNA partially rescues the phenotype of *dsh* mutant *Drosophila* embryos. Cuticle preparations of embryos viewed under dark field (A–D,I) and at higher power under phase contrast (E–H,J); ventral and ventrolateral views with anterior uppermost. (A) A wild type embryo. The ventral cuticle bears belts of denticles separated by regions of naked cuticle. The head skeleton is visible at the anterior, and the filzkörper at the posterior. (B) *svb, dsh/Y* embryo derived from a *dsh* germline clone (GLC). In embryos devoid of either maternal or zygotic *dsh* activity, embryos exhibit an extreme segment polarity phenotype in which naked cuticle and segmental furrows are replaced by a lawn of denticles. The denticles are marked by the linked genetic locus *svb*, which reduces the size and number of denticles. The head skeleton and filzkörper fail to develop and the *dsh* mutant embryos do not hatch. (C) *dsh* GLC-derived *svb, dsh/Y* embryo injected with *dsh* RNA. All aspects of the *dsh* mutant phenotype were rescued. The unambiguous identity of this embryo as a *dsh* mutant is indicated by the *svb* marker. (D) *dsh* GLC-derived *svb, dsh/Y* embryo injected with *Dvl2* RNA. The segment polarity phenotype was substantially rescued, as revealed by restored denticle belts and segmental furrows. However, terminal structures were only partially rescued and the embryo did not hatch. (E) Detail of the head skeleton in a wild type embryo. (F) *dsh* mutant embryo, completely lacking head-skeletal elements. (G) *dsh*-injected *dsh* mutant embryo, showing fully rescued head skeleton. (H) *Dvl2*-injected *dsh* mutant embryo, with partially rescued head skeleton. (I) *Dvl2*-injected *dsh* mutant embryo, partially out of vitelline membrane, showing significant rescue of the segment polarity phenotype. (J) *Dvl2*-injected *dsh* mutant embryo, fully out of vitelline membrane, showing partially rescued head skeleton. The cuticle preparation procedure for injected embryos is not optimal for obtaining high quality samples that are out of the vitelline membrane; however, representative samples are shown.

(6.5 dpc). Expression of *Dvl2* is very similar to that of *Dvl1*, although there are a few organs of the adult which express only one of the two, and at 7.5 dpc the anterior primitive streak expresses little if any *Dvl1* (Sussman et al., 1994). This similarity in temporal and spatial expression patterns along with their high homology raises the possibility that these genes might have redundant functions. Potentially, phenotypes of each mutant gene might

only be seen in tissues expressing that gene uniquely, as was the case for the murine homologs of the *engrailed* segment polarity gene (Joyner et al., 1991; Wurst et al., 1994; Hanks et al., 1995).

In embryos, *Dvl2*, *Dvl1* (Sussman et al., 1994), *Xenopus Xdsh* (Sokol et al., 1995), and *Drosophila dsh* (Klingensmith et al., 1994; Yanagawa et al., 1995) are all ubiquitously expressed. We suggest that this is to be ex-

pected if these genes are functionally conserved in *Wnt* signal transduction. Although Dsh protein is in all cells of the ventral embryonic ectoderm, it is required in this tissue only for Wg signaling (Klingensmith et al., 1994; Noordermeer et al., 1994; Siegfried et al., 1994), and Wg protein is expressed in narrow stripes (van den Heuvel et al., 1989). Interestingly, all ventral epidermal cells can respond to Wg (Noordermeer et al., 1992), so the Wg signal transduction pathway should be ubiquitous. Indeed, the other known members of the pathway, *zw3* and *arm*, are also ubiquitously expressed (Riggleman et al., 1990; Siegfried et al., 1990). Furthermore, these proteins are all present well before the Wg signal. In short, analysis of Wg signaling in the fly shows that Wg pathway components are expressed ubiquitously and prior to any Wg signal.

In *Xenopus*, genes in the *Wnt* response pathway should be maternally expressed (Sokol et al., 1991; Ku and Melton, 1993), as indeed are *Xdsh* (Sokol et al., 1995), *Xgsk3* (He et al., 1995; Pierce and Kimelman, 1995), and *β -catenin* (DeMarais and Moon, 1992); moreover, all three genes are expressed ubiquitously in oocytes and embryos through the end of neurulation. As detailed in Section 1, these genes appear to be functioning in a *Wnt* signaling pathway. While *Xwnt1* is not expressed at the right time or place (Moon et al., 1993), *Xwnt-8b* is maternally expressed and mimics the activity of *Wnt-1* in ectopic expression assays (Cui et al., 1995). These results suggest that the Wg pathway described in flies is not limited to Wg/*Wnt1* signal transduction. If mouse homologues of these genes are also involved in *Wnt* signal transduction, they should be expressed before their ligands, and many *Wnts* seem to act in gastrulation and organogenesis (reviewed by Parr and McMahon, 1994). As mentioned, *Dvl* genes are expressed early and uniformly; their functions are currently being addressed by targeted mutagenesis. Mouse *β -catenin* is expressed ubiquitously in early embryos and homozygous mutants die at the beginning of gastrulation, with defective embryonic ectoderm and a lack of mesoderm formation (Haegel et al., 1995). It is unclear whether this phenotype represents a failure in cell adhesion, cell signalling, or both. Information about the expression of mouse *GSK3 β* during gastrulation and organogenesis has not been reported. In short, data available to date are consistent with the idea that the Wg signaling pathway is conserved in vertebrates and, at least in frogs, seems to play a role in embryonic development.

The exact molecular mode of action of Dsh in the Wg signal transduction pathway remains unknown, and the deduced protein structure is devoid of established functional domains (Klingensmith et al., 1994; Theisen et al., 1994). However, our comparison of the *Dvl2* amino acid sequence to those of the other *dsh* family members provides some clues as to the importance of different parts of the protein. The high conservation of three regions (CR I–

III) and the other conserved structural features of the *dsh* family members clearly indicate crucial roles for these domains in the proteins' functions, but this does not necessarily mean each of these conserved features is required for Wg signaling. Yanagawa et al. (1995) have studied *dsh* in cultured *Drosophila* cells, finding that transfected *dsh* leads to the same elevation of Arm protein levels as secreted Wg. By making deletions in the gene and measuring the consequent effects on Arm levels, Yanagawa et al. (1995) have performed a structure/function analysis of Dsh. The last third of the protein is not necessary to elevate Arm, and thus CR III is dispensable for at least this aspect of Wg signalling. A deletion of the conserved basic region is also inconsequential. Conversely, the amino-terminal domain of conservation is required. The large, very highly conserved central portion (CR II) is necessary but not sufficient, implying a crucial role for CR I as well. Within CR II a 50-residue deletion removing most of the DHR abolishes Arm elevation. The DHR deletion also abolished the Wg-dependent serine/threonine phosphorylation of Dsh. This suggests that the phosphorylated residue(s) lie in this region (amino acids 286–336 of Dsh). Only one threonine and no serine residue occurs therein and it is conserved as a serine in the other three family members, making it a good candidate for a residue important in Wg signal transduction and a target for mutational analysis.

The mRNA injection assay for rescue of the *dsh* segment polarity phenotype described in this paper provides a stringent assay for *dsh* function. We scored the injected RNAs for their ability to trigger all the cellular responses needed for intrasegmental patterning which are lacking in *dsh* mutant embryos. Because the function of *dsh* in this process appears to be entirely to mediate Wg signaling, this is an assay for *dsh*-mediated Wg signal transduction. We demonstrate that injection of wild type *dsh* transcripts fully rescues embryos devoid of endogenous *dsh*, opening the door for future studies in which transcripts bearing specific mutations can be assayed. Here we have used the assay to test the ability of murine *Dvl2* transcripts to rescue *dsh* mutants. We find that *Dvl2* can significantly rescue the segment polarity phenotype of *dsh* mutants, and thus can function as a component of the Wg signaling pathway.

The *Dvl2* rescue of the *dsh* segmentation phenotype reveals some residues that are not required for the function of Dsh in Wg signaling. Those residues which are not similar or identical between Dsh and *Dvl2*, 38% of the total, are unnecessary at the primary sequence level for Wg signal transduction. However, about a quarter of these are in the hydrophilic region which corresponds to the Opa repeat of Dsh, and may fulfill an important structural requirement for *dsh* function. Interestingly, we were unable to demonstrate rescue of *dsh* mutant embryos with *Dvl1* mRNA (data not shown). Assuming that similar amounts of *Dvl1* protein were made, this suggests that

Dvl1 is more highly diverged from *dsh* than is *Dvl2*. The *Dvl2* rescue also emphasizes that the carboxyl-terminal extension common to *Dvl1* and *Xdsh* is not necessary for Wg/*Wnt1* signal transduction, and may represent some other function of these proteins relative to Dsh. However, *dsh* transcripts result in the same axial duplication as *Xdsh* and *Wnts* in *Xenopus* injection experiments, and activate transcription of a *Wnt*-responsive reporter gene (Rothbacher et al., 1995). Thus, this carboxyl domain seems not to be necessary for *Wnt* signaling in *Xenopus* either, perhaps representing an additional role for these proteins unrelated to *Wnts*.

There are a few other cross-species experiments which also support the idea that the functions of individual Wg signaling pathway components are conserved in their vertebrate homologs. *wg* transfection can transform mouse mammary cells in culture in the same manner as *Wnt-1* (Ramakrishna and Brown, 1993). Injection of *wg* (Chakabarti et al., 1993) or *dsh* (Rothbacher et al., 1995) RNA into *Xenopus* embryos results in axial inductions very similar to those of *Xwnts* (Sokol et al., 1991) or *Xdsh* (Sokol et al., 1995). Expression of mammalian *GSK3 β* can rescue the *zw3* segment polarity defect (Siegfried et al., 1992). Thus, it would appear that the Wg pathway as a whole and the molecular functions of its components are conserved across a wide phylogenetic distance.

The structural and functional conservation of *dsh* and the rest of the Wg signaling pathway has important implications for the search for missing members of the pathway in vertebrates. Because *Dvl2* can substitute for *dsh* in Wg signal transduction, it must be able to interact with critical proteins upstream and downstream of *dsh* in the pathway. *dsh* is the earliest-acting known component of the Wg signaling pathway and is phosphorylated and recruited to the plasma membrane upon Wg stimulation of target cells (Noordermeer et al., 1994; Siegfried et al., 1994; Yanagawa et al., 1995). Thus, it is very likely that Dsh interacts with a protein kinase which is part of the unknown Wg receptor or closely associated with it. Because *wg* is about 54% identical to *Wnt-1* (Rijsewijk et al., 1987) and *dsh* is 47% identical to *Dvl2*, and the two genes of each type are largely interchangeable, the proteins which function between the Wnt signal and the Dsh transducer also should be highly conserved between mouse and fly. Our results provide useful information for the design of genetic and biochemical experiments to identify such genes.

4. Experimental procedures

4.1. Isolation of *Dvl2*

A probe consisting of a 719 bp Bgl II-Pst I fragment of mouse *Dvl1* cDNA (Sussman et al., 1994) was used to screen a mouse brain cDNA λ Zap library (purchased from Stratagene, Inc.). The probe was labeled with α -

[³²P]dCTP using the random-priming method (Boehringer Mannheim). Libraries were plated in top agarose at a density of $3\text{--}5 \times 10^5$ PFU per 24 cm \times 24 cm plate. Plaques were transferred to duplicate membranes of Gene Screen Plus (Dupont/NEN), processed and hybridized as described (Sussman et al., 1994). Hybridization was performed for 12 h at 42°C. Low stringency wash of the membranes was performed using $1 \times$ SSC/0.1% SDS at room temperature for 30 min (one change of wash) and $1 \times$ SSC/0.1% SDS for 60 min (one change of wash) at 65°C. Basic techniques for handling nucleic acid were performed as described (Ausubel et al., 1987).

4.2. DNA sequencing and analysis

DNA sequencing (dideoxy method) was performed using both [³⁵S]dATP manual (United States Biochemical Sequenase Version 2.0) and automated (Applied Biosystems model 370A Sequencer) techniques. Sequencing of both strands was accomplished by generating subclones utilizing the Promega Erase-a-Base System. Regions that were missed or ambiguous were sequenced utilizing oligonucleotide primers generated on an Applied Biosystems model 391 DNA Synthesizer.

Sequence similarity searching of the Genbank data base and structural analysis was done using software (IFIND, BLAST, and QUEST) from Intelligenetics, Inc. Pairwise comparisons of sequences used the GAP program and multiple alignments used the PILEUP program of the Genetics Computer Group (Devereux et al., 1984).

4.3. Mapping

Mapping of *Dvl2* was performed by single-strand conformation polymorphism (SSCP) analysis of recombinant mouse lines as described (Beier et al., 1992). The primer pair used for the analysis was designed to amplify a region corresponding to intron 9 of *Dvl2* (Yang and Sussman, unpublished). Briefly, oligonucleotides were radiolabeled with [³²P]ATP using polynucleotide kinase and used to amplify (anneal at 55°C for 1 min, extend at 72°C for 2 min, and denature at 94°C for 1 min, for 40 cycles, with a final extension at 72°C) genomic DNAs from a series of mouse strains. A 2 μ l aliquot of the amplified reaction was added to 8.5 μ l of stop solution (United States Biochemical Corp.), denatured at 94°C for 5 min and immediately placed on ice. A 2 μ l aliquot was resolved by electrophoresis in $0.5 \times$ TBE buffer (2–3 h, 40 W in a 4°C cold room) on a 6% non-denaturing acrylamide sequencing gel. The primers, Y44 forward (5' GAGACATCGTGCACAAGC 3') and Y45 reverse (5' GATGGACTGAACTCAG 3') identified a polymorphism between the C57BL/6J and *M. spretus* strains and were used to analyze DNA prepared from the BSS backcross (Rowe et al., 1994). The strain distribution pattern

was analyzed using the Map Manager Program (Manley, 1993).

4.4. RNA expression analysis

For ribonuclease (RNase) protection assays, total RNA was isolated according to the procedure of Chirgwin et al. (1979). RNase protection assays were performed according to the procedure described by Melton et al. (1984). The probe used was a 430 bp EcoRI-Bgl II fragment from clone 11, subcloned into pBluescript KS (Stratagene), digested with Xho I, and transcribed with T7 polymerase. The ribosomal protein L32 riboprobe, used as an internal standard, was a generous gift of Dr. Michael M. Shen.

The substrate for RT-PCR was poly(A)⁺ RNA, isolated from whole embryos or adult brains via the 'Quickprep micro' mRNA purification kit (Pharmacia). RNA (200 ng) was reverse transcribed with 10 U/ μ l MMLV reverse transcriptase (Gibco-BRL), primed by 5 μ M random hexanucleotides (Boehringer Mannheim). Reaction conditions were as recommended by the enzyme manufacturer. PCR amplification used primer pair DJS106/107 (DJS106: 5' CTGATGGCTGGAGCTCAC 5'; DJS107: 5' GTAGAAGGCACTCATGTG 3'), which amplify a 145 bp product from the 3' untranslated region of *Dvl2*. Amplification of 1/10 of each cDNA reaction in the presence of 1.5 mM MgCl₂ was performed by Taq polymerase from Sangon according to manufacturer's recommendations on a PTC-100 thermocycler (MJ Research). Water was used as a negative control and 1.0 ng clone 11 cDNA as a positive control for the amplification reaction. The cycling parameters were as follows: 94°C 30 s, 60°C 40 s, 72°C 30 s, repeated 32 times, followed by a final extension of 5 min. at 72°C. Amplification products were electrophoresed on a 3% Nuseive, 1% agarose (FMC) gel.

4.5. In situ hybridization

Whole-mount in situ hybridization followed the protocol of Conlon and Rossant (1992), except that embryos were fixed overnight at 4°C, and bleaching was limited to 30–60 min. Embryos were derived from crosses between CD1 adults (Charles River, Quebec). The following *Dvl2* probes were used: pKS-11S/H (digested with XbaI and transcribed using T3 polymerase), a 433 bp SmaI/HindIII insert from the 3' non-coding region of clone 11; and E/B (digested with EcoRI and transcribed using T7 polymerase), a 430 bp EcoRI/BglIII insert encompassing the 5' non-coding region of clone 11. In addition to *Dvl2* sense and antisense RNA probes, antisense probes for several spatially restricted messages were also used as positive controls (data not shown). Embryos to be sectioned after color development were post-fixed in 4% paraformaldehyde, 0.2% glutaraldehyde for 30 min. Sections of 8 μ m were cut on a Leitz Jung Biocut microtome. Photomicrography used a Leica M10 stereomicroscope

for whole-mounts and a Leitz DMRXE for sections with Kodak Royalgold 100 film, and image processing of scanned slides (Nikon) used Adobe Photoshop software. Only the brightness of the images was varied to create even backgrounds.

4.6. *Drosophila dsh* mutant rescue by mRNA injection

Full-length cDNAs encoding *dsh* (Klingensmith et al, 1994) or *Dvl2* were subcloned into a vector that allows synthesis of poly(A)⁺ mRNA with a 5' globin ribosome binding site using SP6 polymerase (R. Lehmann, personal communication). mRNAs were synthesized and injected into embryos as described (Driever et al., 1990) to test the ability of the mRNAs to rescue the *dsh* mutant phenotype.

Since *dsh* is expressed maternally, embryos used for injection must have no maternal or zygotic expression of *dsh*. The embryos were generated using the FLP-DFS technique (Chou and Perrimon, 1992). Females of the genotype *svb dsh^{v26} FRT/ DFS FRT; hs-flipase* (where *svb* served as a marker producing a cuticle phenotype (Gergen and Wieschaus, 1986), *FRT* is the target for mitotic recombination by *flipase*, *DFS* is a dominant female sterile gene, and *dsh^{v26}* is a null allele), were used to generate animals with mitotic germ line clones induced by heat shock activation of the *flipase*. When crossed to wild type males, two kinds of embryos were generated. (1) Males of the genotype *svb dsh^{v26}/Y* bear no maternal *dsh* transcript, are identified by the *svb* marker, and develop the *dsh^{v26}* mutant phenotype with 100% penetrance (unless rescued by injected mRNA). (2) Females of the genotype *svb dsh^{v26}/++* do not show the *svb* phenotype, and develop wild type cuticles, since the *dsh* maternal effect is paternally rescuable. To assess rescue, only the male embryos, identified by the *svb* phenotype, were scored.

For mRNA injection, embryos derived from the *svb dsh* germline clone females crossed to wild type males were collected and injected before blastoderm formation. Transcript concentrations varied from approximately 100 to 500 ng/ μ l. They were then allowed to develop until hatching was expected to occur, at which time cuticle preparations were made to analyze the phenotypes (Wieschaus and Nusslein-Volhard, 1986). The procedure for embryo preparation does not allow for effective removal of the vitelline membrane. Thus, the embryos obtained out of the membrane are of somewhat poor quality.

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