Tout-velu is a *Drosophila* homologue of the putative tumour suppressor *EXT-1* and is needed for Hh diffusion

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Hedgehog (Hh) proteins act through both short-range and long-range signalling to pattern tissues during invertebrate and vertebrate development[1]. The mechanisms allowing Hedgehog to diffuse over a long distance and to exert its long-range effects are not understood. Here we identify a new *Drosophila* gene, named *tout-velu*, that is required for diffusion of Hedgehog. Characterization of *tout-velu* shows that it encodes an integral membrane protein that belongs to the *EXT* gene family. Members of this family are involved in the human multiple exostoses syndrome, which affects bone morphogenesis[2–4]. Our results, together with the previous characterization of the role of Indian Hedgehog in bone morphogenesis[5–7], lead us to propose that the multiple exostoses syndrome is associated with abnormal diffusion of Hedgehog proteins. These results show the existence of a new conserved mechanism required for diffusion of Hedgehog.

We identified mutations in a new gene, *tout-velu* (*ttv*), which means 'all hair', in a screen for maternal-effect mutations associated with *Drosophila* segment polarity[8]. The mutant segment-polarity phenotype is reminiscent of the phenotypes of *wingless* (*wg*) or *hedgehog* (*hh*) mutant embryos. As several specific *wg*-dependent processes are not affected in embryos that lack both maternal and zygotic *ttv* gene activities, it is likely that *ttv* is required for Hh, but not for Wg, signalling (I.T. *et al.*, manuscript in preparation). We analysed the function of *ttv* in the wing imaginal disc to determine its possible role in the Hh signalling pathway.

In the wing disc, Hh is expressed in cells of the posterior compartment and diffuses into the anterior compartment[9–12]. In the anterior compartment, Hh signalling results in expression of Patched (Ptc)[13], which can be detected as far as five cells away from the anterior/posterior boundary, and in stabilization of Cubitus interruptus (Ci), which is observed as far as 8–10 cells away (Fig 1a, b; refs. 14, 15). We therefore used Ptc expression and Ci stabilization as reporters with which to examine the role of *ttv* in Hh signalling. We generated somatic mutant clones with the $ttv^{l(2)00681}$ mutation, which is either a null allele or a severe hypomorphic allele (see Methods; I.T. *et al.*, manuscript in preparation). In large anterior *ttv*

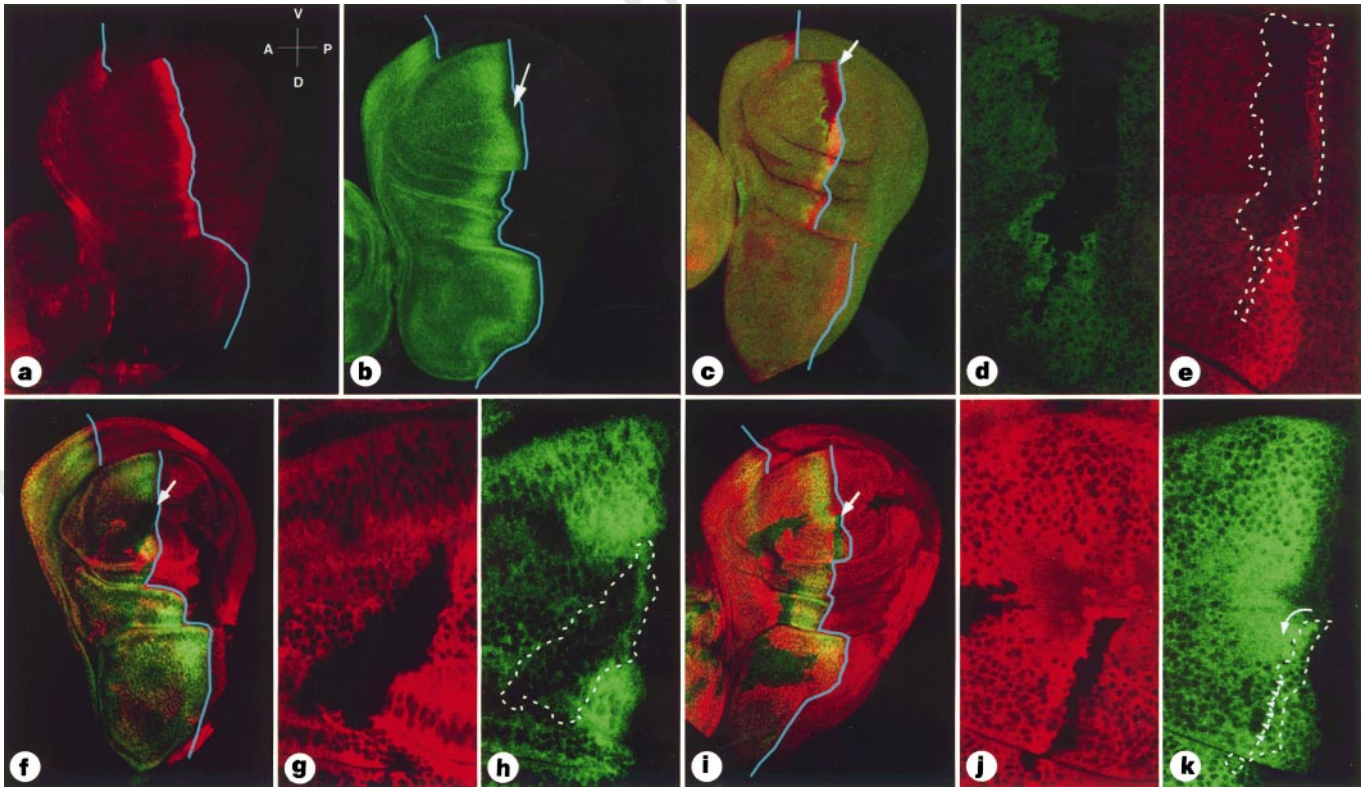

Figure 1 *ttv* affects Hh signalling. All imaginal discs are orientated as in **a**. A, anterior; P, posterior; V, ventral; D, dorsal. Blue lines indicate the A/P boundary. Dashed lines indicate the limit of the relevant clones as determined by the absence of β-galactosidase staining (see Methods). **a**, Wild-type Ptc expression (red)[25,26]. **b**, Wild-type Ci expression (green) is detected with antibody 2A1 which has a higher affinity for the full-length Ci gene product[15]. Ci is only expressed in the anterior compartment. The posterior limit of the domain of Ci stabilization does not correspond with the anterior/posterior boundary (see arrow)[14]. **c–i**, *ttv* mutant clones. **c**, **f**, **i** Full discs; the relevant *ttv* mutant clones are indicated by an arrow. **d**, **e**, **g**, **h**, **j**, **k**, Magnifications of the relevant clones. **c–e**, Ptc expression in red (**c**, **e**) and β-galactosidase expression in green (**c**, **d**). **d**, **e**, In a *ttv* clone, Ptc expression is only weakly induced, in one row of cells adjacent to the anterior/posterior boundary. **f–k**, Ci staining (green) (**f**, **h**, **i**, **k**) and β-Gal staining (red) (**f**, **g**, **i**, **j**). **g**, **h**, Ci stabilization is induced only at the posterior edge of the clone. **j**, **k**, Wild-type cells anterior to a *ttv* clone do not stabilize Ci, although they are in a domain that is competent to respond to Hh (arrowheads); thus, the non-cell-autonomous effect is directional (see also **h**). Within the clone, the distance between the domain of Ci stabilization and the A/P boundary is reduced, indicating that the level of Hh signalling is reduced[14]. We interpret the stabilization of Ci in the ventral cells of the *ttv* clone as being due to the diffusion of Hh from ventral wild-type cells (see arrow).

mutant clones adjacent to the anterior/posterior border, Hh signalling is impaired, as indicated by the lack of Ptc staining in most of the clone (Fig. 1c, e). However, Ptc expression still occurs at the posterior edge of the clone next to wild-type cells (Fig. 1e). The level of signalling in these cells is nevertheless diminished, as shown by a reduction in their levels of Ptc staining compared with wild-type cells (Fig. 1e).

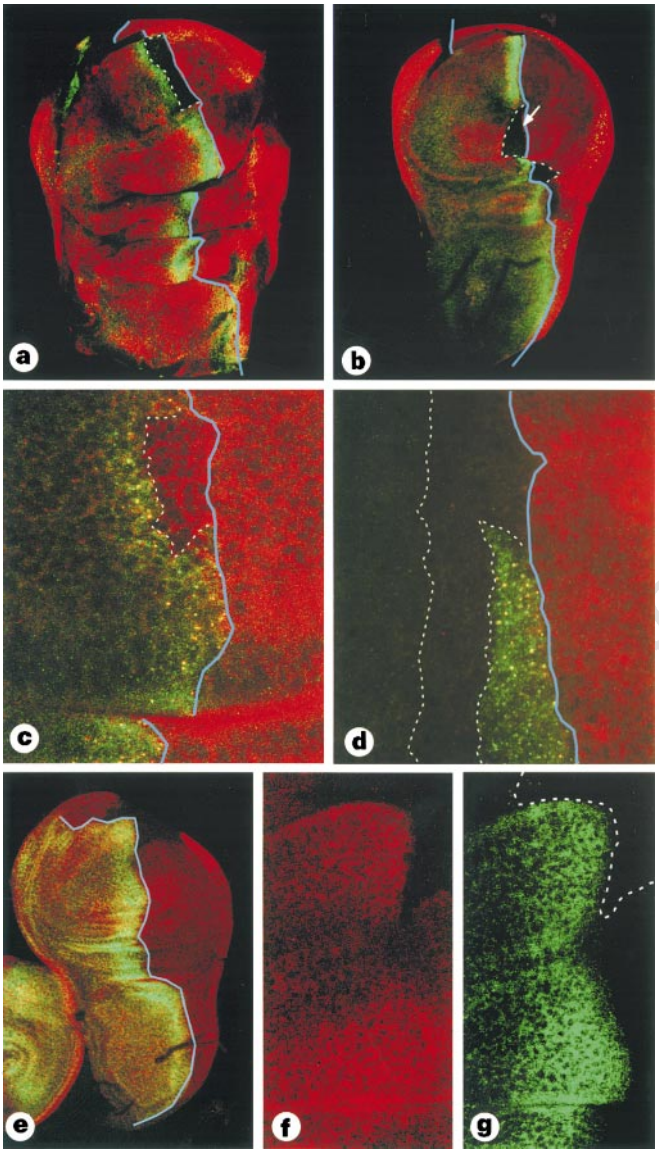

Figure 2 *ttv* is required for Hh diffusion. Blue lines indicate anterior/posterior boundary. Dashed lines indicate clone limits. Orientation is as in Fig. 1a. **a**, **b**, Overlays of Ptc (green) and β-galactosidase (red) stainings. **a**, A *ptc* mutant clone at the anterior/posterior boundary causes ectopic expression of Ptc in wild-type cells anterior to the clone (see also ref. 12). **b**, Two independent *ptc ttv* mutant clones in the anterior (see arrow) and posterior compartments. In an anterior *ptc ttv* clone, *ptc* expression is not induced anterior to the clone. In a posterior *ptc ttv* clone, Hh diffusion is not affected as *ptc* expression is induced. **c**, **d**, Overlays of Ptc (green) and Hh (red) staining[27]. **c**, The *ptc* clone is in the anterior compartment. Hh staining is detected in, and anterior to, the *ptc* clone. **d**, Hh staining cannot be detected in a *ptc ttv* clone located in the anterior compartment. Clone boundaries are identified by β-galactosidase staining (not shown). **e–g**, Ci (green) (**e**, **g**) and β-galactosidase (red) staining (**e**, **f**). **e**, Full disc with a posterior clone. **f**, **g**, Magnification of the clone. Loss of *ttv* activity in the posterior compartment does not affect the diffusion of Hh in the anterior compartment, as Ci is stabilized. We consistently see a reduction of the domain of Ci stabilization. Hh signalling probably depends on the cumulative expression of Hh from several rows of cells adjacent to the anterior/posterior boundary. In a posterior *ttv* clone, only Hh produced by the row of cells adjacent to the anterior/posterior boundary can diffuse into the anterior compartment and contribute to signalling.

A similar result was observed when Ci levels were used as a reporter for Hh signalling (Fig. 1f, h). In addition, the large region of Ci stabilization allowed us to determine a non-cell-autonomous effect of *ttv* mutant clones. When *ttv* mutant clones of only a few cells wide are located along the anterior/posterior boundary, wild-type cells anterior to the *ttv* mutant clone do not respond to the Hh signal, as shown by Ci staining (Fig. 1i, k). As these cells are located within a domain to which Hh is normally able to diffuse, and in which Hh can normally induce the stabilization of Ci, we conclude that *ttv* mutant clones have a directional, non-cell-autonomous effect on wild-type cells located anteriorly. As the domain of Ci stabilization is under direct control of Hh signalling[14,15], we interpret this result as an inability of Hh to reach wild-type cells located anteriorly to *ttv* mutant clones. We then investigated whether diffusion of Hh is impaired in the absence of *ttv*.

In a *ptc* mutant clone, Hh diffusion is observed as an ectopic induction of Ptc in wild-type cells localized anteriorly to a clone of *ptc* mutant cells (Fig. 2a; ref. 12). To determine whether Hh diffusion is modified by *ttv* in the *ptc* mutant clones, we generated clones with mutations in both *ptc* and *ttv* and analysed the expression of Ptc. In these double mutant clones, Ptc expression is not induced in wild-type cells anterior to the clone (Fig. 2b). To assess Hh diffusion in the *ptc ttv* mutant clones directly, we compared the distribution of Hh in *ptc* and *ptc ttv* clones. In *ptc* mutant cells, we detected Hh protein as diffuse membrane staining. When Hh reaches wild-type cells beyond a *ptc* clone, it can be seen in a punctate staining pattern that, for the most part, coincides with the punctate Ptc staining that reflects Ptc localization in vesicles (Fig. 2c). In *ptc ttv* double mutant clones, there is no Hh staining in mutant cells (Fig. 2d). On the basis of this result and the directional non-cell-autonomous effect of *ttv* mutant clones, we propose that Hh cannot diffuse in the absence of *ttv* activity.

In order to diffuse, Hh must move from the sending to the receiving cell; therefore, *ttv* could function in the sending cell and/or the receiving cell. In the anterior cell, the sending of Hh depends on its reception from the previous cells; thus it is difficult to determine where Ttv functions by analysing *ttv* mutant clones in the anterior compartment. We therefore generated mutant clones in the posterior compartment next to the anterior/posterior boundary and analysed their effects on Ci stabilization. We found that *ttv* mutant clones in the posterior compartment do not affect the diffusion of Hh (Fig. 2b, e, g). Thus, we propose that *ttv* functions in the receiving cells for the movement of Hh from sending to receiving cells. This observation also indicates that *ttv* is not required for Hh production. In a *ttv* clone located in the anterior compartment, Hh can still signal, although less efficiently, to the first receiving cells. We suggest that this weakened signalling activity is mediated by Hh present on the membrane of the Hh-sending cells and that efficient Hh signalling, even to adjacent cells, requires diffusion of Hh.

To clone the *ttv* gene, we isolated the genomic region surrounding the inserted P-element, $ttv^{l(2)00681}$ (Fig. 3a), and identified two genes in the vicinity of the P-element insertion site. One gene is *lamin C*, which is unlikely to correspond to *ttv* as it is not expressed maternally[16]. Sequencing of the putative 3.8-kilobase (kb) complementary DNA encoding *ttv* shows that Ttv is a protein of 760 amino acids that is similar to the human multiple exostoses (EXT-1) protein[2] (Fig. 3b). The 3.8-kb cDNA identifies a maternally expressed transcript on an RNA blot. A transgene of this transcript under the control of a heat-shock protein 70 gene (*hsp70*) promoter rescues *ttv* homozygous flies to viability (data not shown).

Ttv is 56% and 25% identical to the human EXT-1 and EXT-2 proteins, respectively. EXT-1 and EXT-2 probably form a new family of conserved molecules, as mouse and *Caenorhabditis elegans* homologues have been identified[17]. However, the cellular

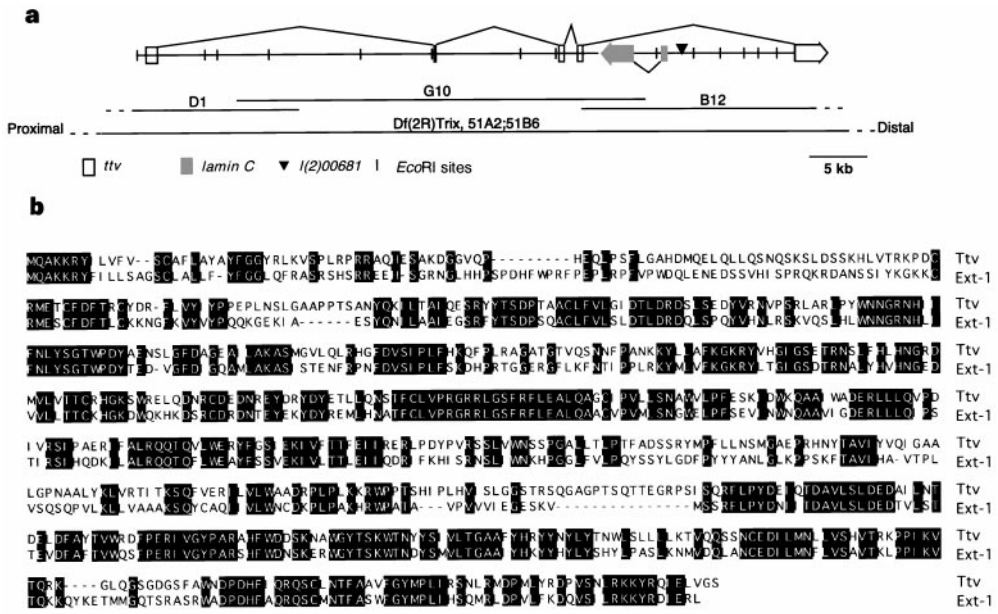

Figure 3 Cloning of the *ttv* locus. **a**, Genomic map of the *ttv* locus. The P-element, $ttv^{l(2)00681}$ (black triangle), maps to the 51A polytene chromosome band which is deleted in *Df(2R)Trix*. The P-element is inserted into the sixth intron of the *ttv* gene, which also includes the *lamin C* gene. Only introns of more than 1 kb in length are shown. D1, G10 and B12 are three overlapping cosmids that were previously mapped to the region[28]. **b**, Sequence comparison between the Ttv and human EXT-1 proteins. Black boxes indicate amino acids that are identical in EXT-1 and Ttv. The overall identity between Ext-1 and Ttv is 56%.

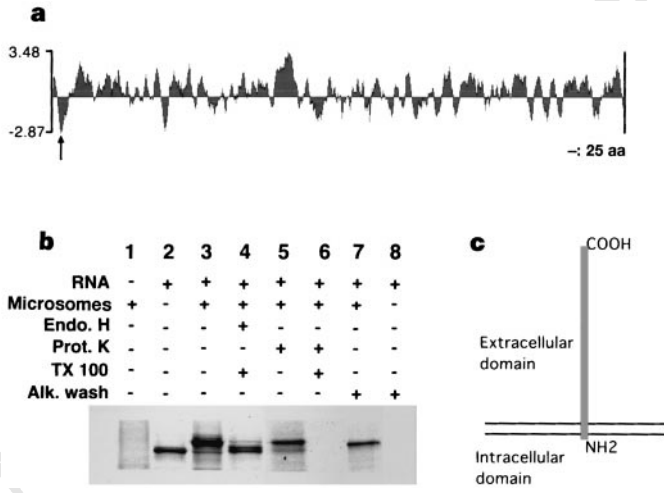

Figure 4 Ttv is a type II integral membrane protein. **a**, The Ttv Kyte–Doolittle hydrophobicity plot shows a hydrophobic stretch at the N terminus of the protein (amino acids (aa) 7–24, see arrow). **b**, *In vitro* translation and microsome assays. *In vitro* transcribed *ttv* cDNA is translated in the presence of rabbit reticulocyte extract (lane 2). When the same extract is supplemented with dog pancreatic microsomes, a mobility shift is detected (lane 3). This shift can be abolished by treatment with endoglycosidase H (lane 4). The full-length Ttv protein enter microsomes: Ttv is protected from digestion by proteinase K in the presence of intact microsomes (lanes 5, 6). Ttv remains associated with the membrane fraction after alkaline wash (lanes 7, 8), indicating that Ttv is a membrane protein. **c**, Proposed structure of the Ttv protein. The cytoplasmic tail is only six amino acids in length.

compartment in which EXT molecules could function has remained unknown[2,18]. Analysis of the Ttv sequence shows that there is a hydrophobic stretch at the amino terminus of the Ttv protein, indicating that Ttv might be a transmembrane protein (Fig. 4a). We used an *in vitro* translation assay to show that Ttv is indeed a membrane protein (Fig. 4b). *In vitro*-transcribed *ttv* messenger RNA is translated into a putative protein with a relative molecular mass (M_r) of ~80K. In the presence of microsomes, the protein is glycosylated and fully protected from degradation by proteinase K, indicating that the protein is imported into microsomes. Furthermore, the Ttv protein remains associated with the membrane fraction after an alkaline wash; it is therefore a membrane-associated protein[19]. As the sequence surrounding the signal sequence does not closely match consensus cleavage sequences[20], we propose that the signal sequence is not cleaved and instead acts as an anchor region. We conclude that Ttv is a type II integral membrane protein (Fig. 4c).

As the active form of Hh is tethered to the membrane by a cholesterol moiety[21,22], it is not known how Hh can diffuse from one cell to another. The movement of Hh does not depend on Ptc and Smoothened[12]. As Ttv is needed in the receiving cell to allow Hh diffusion, we suggest that Ttv does not function in the dissociation of Hh from the membrane of the sending cells and is probably to be required to allow reassociation or maintenance of Hh on the surface of the receiving cells. The multiple exostoses syndrome is a dominantly inherited disease that is characterized by short stature, limb length inequalities, bone deformities and the presence of bone outgrowths, called exostoses, at the ends of long bones[4]. The loss of any of the *EXT-1*, *-2* or *-3* loci is responsible for this syndrome[4]. The *EXT-1* and *EXT-2* genes have been cloned and may be tumour-suppressor genes[2–4]. During bone morphogenesis one of the established roles of Indian Hh is to limit the rate of chondrocyte differentiation[5–7]. On the basis of the similarity between EXT-1 and Ttv, we propose that some aspects of the multiple exostoses syndrome are due to defects in diffusion and efficient signalling of Indian Hh. The characterization of Ttv is an important step toward the understanding of a cellular mechanism that allows Hh to diffuse and to exert its patterning activity. □

Methods

Somatic clonal analyses. The $ttv^{l(2)00681}$ allele behaves as a genetic null allele: the phenotype of embryos derived from $ttv^{l(2)00681}$ germline clones (GLCs)[8] crossed with $ttv^{l(2)00681}$/*Cyo* GLCs is identical to the phenotype of embryos derived from $ttv^{l(2)00681}$ GLCs crossed with *Df(2R)Trix/Cyo* GLCs. Somatic clones were induced using the FRT/FLP-mediated recombination system[23]. Mutant clones were identified by the loss of β-galactosidase staining, except

in Fig. 2c where the clone was identified by loss of Ptc staining. Relevant genotypes: for *ttv* clones: *hsFLP*; $FRT^{G13}ttv^{l(2)00681}/FRT^{G13}$ *arm-LacZ*, for *ptc* clones: *hsFLP*; $FRT^{42D}ptc^{IIW}/FRT^{42D}$*arm-lacZ* ($ptc^{IIW}$ is a protein-null allele[24]); and for *ptc ttv* double mutant clones: *hsFLP*;$FRT^{42D}ptc^{IW}$ $ttv^{l(2)00681}/FRT^{42D}$ *arm-LacZ* (ref. 1). First-instar larvae were heat-shocked for 1 h at 37 °C. Third-instar larval imaginal discs fixed for 20 min in 4% formaldehyde in PBS with 0.1% Tween20 (PBT). Antibodies were diluted in PBT: rabbit anti-β-galactosidase (Cappel), 1:4,000 dilution; rat anti-β-galactosidase, 1:100; mouse anti-Ptc, 1:100; rabbit anti-Hh, 1:2,000 (TSA amplification was used to enhance the signal; NEN); rat anti-Ci, 1:5. Secondary antibodies coupled to fluorescent isothiocyanate, Texas Red or Cy5 (Jackson) were used at 1:200 dilution. Secondary antibodies coupled to horseradish peroxidase for TSA amplification (Vector) were used at 1:2,000 dilution. Stainings were visualized using a Leica TCS/NT confocal microscope.

Rescue construct. A *Spe*I, *Not*I fragment of the *ttv* gene was cloned downstream of the *hsp70* promoter of the pCasper-hsp vector and used to transform the *y w* strain using the helper plasmid pp25.1. One insertion on the third chromosome rescued *ttv* homozygous mutants to viability without heat shock. $ttv^{l(2)00681}$ and two other alleles, $ttv^{l(2)k066109}$ and $ttv^{l(2)02055}$, were tested.

***In vitro* translation assay.** *ttv* RNA was produced by *in vitro* transcription using the SP6 RNA machine kit (Ambion); rabbit reticulocyte lysates, microsomes and endoglycosidase H were purchased from Boehringer. *In vitro* translation assays in the presence of 10 μCi ^{35}S-labelled methionine, proteinase K digestion (0.1 mg ml^{-1} per reaction) and endoglycosidase H treatments (2 μU per reaction) were performed according to manufacturers' protocols. The samples were analysed by SDS–PAGE. The alkaline wash was performed as described[19].

Sequence analysis. The hydrophobic plot was generated using Protean software. Analysis of the peptide cleavage site was done on the PSORT II web server (http://psort.nibb.ac.jp:80/form2.html) using an algorithm derived from ref. 20.

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A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity

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cRaf-1 is a mitogen-activated protein kinase that is the main effector recruited by GTP-bound Ras in order to activate the MAP kinase pathway[1]. Inactive Raf is found in the cytosol in a complex with Hsp90, Hsp50 (Cdc37)[2,3] and the 14-3-3 proteins[4]. GTP-bound Ras binds Raf and is necessary but not sufficient for the stable activation of Raf that occurs in response to serum, epidermal growth factor, platelet-derived growth factor or insulin[5–8]. These agents cause a two- to threefold increase in overall phosphorylation of Raf on serine/threonine residues[8,9], and treatment of cRaf-1 with protein (serine/threonine) phosphatases can deactivate it, at least partially[10]. The role of 14-3-3 proteins in the regulation of Raf's kinase activity is uncertain[4,11] and is investigated here. Active Raf can be almost completely deactivated *in vitro* by displacement of 14-3-3 using synthetic phosphopeptides. Deactivation can be substantially reversed by addition of purified recombinant bacterial 14-3-3; however, Raf must have been previously activated *in vivo* to be reactivated by 14-3-3 *in vitro*. The ability of 14-3-3 to support Raf activity is dependent on phosphorylation of serine residues on Raf and on the integrity of the 14-3-3 dimer; mutant monomeric forms of 14-3-3, although able to bind Raf *in vivo*, do not enable Raf to be activated *in vivo* or restore Raf activity after displacement of 14-3-3 *in vitro*. The 14-3-3 protein is not required to induce dimerization of Raf. We propose that dimeric 14-3-3 is needed both to maintain Raf in an inactive state in the absence of GTP-bound Ras and to stabilize an active conformation of Raf produced during activation *in vivo*.

Table 1 Interaction of Raf variants with 14-3-3ζ, Ras and MEK-1 in a yeast two-hybrid assay

	Colour produced in an assay with		
	14-3-3ζ	MEK-1	Ras
Raf (WT)	Blue	Blue	Blue
Raf (S259A)	Blue	Blue	Blue
Raf (S259A/S621A)	White	Blue	Blue

A blue colour in the two-hybrid assay indicates that a protein–protein interaction occurs.