Development 139, 3665-3674 (2012) doi:10.1242/dev.083550
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Dissecting the molecular bridges that mediate the function of Frizzled in planar cell polarity

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SUMMARY

Many epithelia have a common planar cell polarity (PCP), as exemplified by the consistent orientation of hairs on mammalian skin and insect cuticle. One conserved system of PCP depends on Starry night (Stan, also called Flamingo), an atypical cadherin that forms homodimeric bridges between adjacent cells. Stan acts together with other transmembrane proteins, most notably Frizzled (Fz) and Van Gogh (Vang, also called Strabismus). Here, using an in vivo assay for function, we show that the quintessential core of the Stan system is an asymmetric intercellular bridge between Stan in one cell and Stan acting together with Fz in its neighbour: such bridges are necessary and sufficient to polarise hairs in both cells, even in the absence of Vang. By contrast, Vang cannot polarise cells in the absence of Fz; instead, it appears to help Stan in each cell form effective bridges with Stan plus Fz in its neighbours. Finally, we show that cells containing Stan but lacking both Fz and Vang can be polarised to make hairs that point away from abutting cells that express Fz. We deduce that each cell has a mechanism to estimate and compare the numbers of asymmetric bridges, made between Stan and Stan plus Fz, that link it with its neighbouring cells. We propose that cells normally use this mechanism to read the local slope of tissue-wide gradients of Fz activity, so that all cells come to point in the same direction.

KEY WORDS: *Drosophila*, Planar cell polarity, Starry Night/Flamingo, Van Gogh/Strabismus, Abdomen, Gradient

INTRODUCTION

"This correspondence between Frizzled level and polarity suggests that cells can assess the Frizzled level or activity of neighbouring cells and use this to differentiate in a polarised way."

(Adler et al., 1997)

Planar cell polarity (PCP) is a property of multicellular organisms; for example, epithelial cells may singly or in groups make oriented structures (mammalian and insect hairs, bird feathers, fish scales) that are aligned with respect to organ or body axes. PCP depends on genes that have been conserved both in invertebrates and vertebrates (reviewed by Klein and Mlodzik, 2005; Wang and Nathans, 2007; Zallen, 2007; Strutt and Strutt, 2009; Goodrich and Strutt, 2011). In *Drosophila*, these genes are divided into two sets – the Starry night (Stan) and the Dachsous/Fat (Ds/Ft) systems – and we have shown previously that these two systems act independently to establish PCP (Casal et al., 2006; Lawrence et al., 2007).

Here, we are concerned with the Stan system, which depends on the receptor-like protein Frizzled (Fz) (Adler et al., 1990) that is thought to sense a long-range cue and to polarise epidermal cells in order to orient their outgrowths. There are two disparate models for this process, each of which has its supporters. The first model posits that graded activity of the Ds/Ft system provides the long-range cue that orients cells via the Stan system. This Ds/Ft signal would act directly on each cell by some yet unknown mechanism to cause the accumulation of Frizzled (Fz) protein on one side, generating an intracellular asymmetry that is then amplified by feedback interactions between neighbouring cells; there would be no long-range gradient of Fz activity (Yang et al., 2002; Ma et al., 2003; Axelrod, 2009). A second model, which we favour (Casal et al., 2006; Lawrence et al., 2007), finds the Stan system to be independent of the Ds/Ft system. It proposes that a morphogen, such as Hedgehog (Hh) in the *Drosophila* abdomen, drives a shallow long-range gradient of Fz activity; it is the orientation of this gradient that polarises the cell (Lawrence et al., 2004; Casal et al., 2006; Lawrence et al., 2007). In order to read the gradient, each cell would compare its level of Fz activity with its neighbours and point its hairs towards the cell with the lowest level (Adler et al., 1997; Lawrence et al., 2004). The capacity of cells to be polarised by differences in their levels of Fz activity was first demonstrated in the *Drosophila* wing by Gubb and Garcia-Bellido, who found that marked clones of cells lacking the *fz* gene can polarise wild-type cells nearby so that their hairs point in towards the clone, i.e. down the Fz gradient (Gubb and García-Bellido, 1982). Subsequently, Adler and colleagues ingeniously made a reversed Fz gradient in the wing and this reversed the hair polarity (Adler et al., 1997).

How might cells compare differences in Fz activity? Using genetic mosaics, we have already shown that the Stan protein (also called Flamingo), a receptor-like cadherin that forms homodimeric bridges between abutting cells (Chae et al., 1999; Usui et al., 1999), is essential for cells both to send and to receive information about their levels of Fz activity (Lawrence et al., 2004; Casal et al., 2006). Likewise, we have also demonstrated that such Stan bridges function asymmetrically to polarise cells, a process that depends on a third transmembrane protein, Van Gogh (Vang, also called Strabismus) (Taylor et al., 1998; Bastock et al., 2003; Lawrence et al., 2004). These findings were later corroborated by others (Strutt and Strutt, 2007; Chen et al., 2008; Strutt and Strutt, 2008; Wu and Mlodzik, 2008; reviewed by Lawrence et al., 2008).

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Accepted 5 July 2012

DEVELOPMENT

Several research groups have investigated how Fz and Vang polarise cells via their contribution to Stan bridges. A key finding is that Stan, Fz and Vang accumulate on the abutting proximal and distal surfaces of adjacent wing cells shortly before they form polarised hairs: Fz and Stan on the distal face of the cell with higher Fz activity, and Vang and Stan on the proximal face of the cell with lower activity (Usui et al., 1999; Strutt, 2001; Bastock et al., 2003). Three other conserved proteins of the Stan system, Dishevelled (Dsh), Diego (Dgo) and Prickle (Pk), also accumulate asymmetrically: Dsh and Dgo together with Fz, and Pk together with Vang, and they may help the actions of Fz and Vang (Axelrod, 2001; Tree et al., 2002; Das et al., 2004; reviewed by Strutt and Strutt, 2009). In addition, it has been proposed for the wing that the accumulation of Fz along the distal edge of each cell seeds the outgrowth of hairs that project distally, whereas that of Vang along the proximal edge suppresses their formation (Strutt and Warrington, 2008). Consequently, a favoured model has distinct Stan-Fz and Stan-Vang complexes that meet across cell-cell interfaces to polarise the behaviour of cells on both sides (Chen et al., 2008; Wu and Mlodzik, 2008; Strutt and Strutt, 2009).

This view is challenged by evidence that Stan, on its own, can form bridges with Stan-Fz complexes on abutting cells. An early indication for this was found in flies that lack *Vang*. In such flies, when *fz*$^{-}$ clones are made (making a patch of *Vang*$^{-}$ *fz*$^{-}$ cells), Stan accumulates on the interface between *Vang*$^{-}$ *fz*$^{-}$ cells and the *Vang*$^{-}$ surround, suggesting that Stan is stabilised at the cell surface when it can bridge with Stan-Fz in neighbouring cells (Strutt and Strutt, 2008). Such bridges can transmit polarising information in at least one direction, from the Stan to the Stan-Fz side of the bridge, as Stan on the surface of *Vang*$^{-}$ *fz*$^{-}$ cells can induce, in some instances, neighbouring wild-type cells to make hairs that point towards the mutant cells (Chen et al., 2008; Wu and Mlodzik, 2008). However, no polarising effects were reported in the other direction (from the Stan-Fz to the Stan side of the bridge), leaving unresolved the issue of whether the signal is transmitted in one or both directions.

Here, we use genetic mosaics to dissect the contributions of Fz and Vang to Stan bridges. We find that the heart of the Stan system is an asymmetric bridge formed between Stan on one cell and Stan plus Fz (Stan^{Fz}) on its neighbour. We report that such Stan-to-Stan^{Fz} bridges can polarise both cells so that they point in the same direction, even in the absence of Vang. By contrast, Vang has no detectable function in the absence of Fz.

It is surprising and illuminating that cells containing only Stan (i.e. cells that lack both Fz and Vang) can be polarised so that their hairs point away from neighbours that have Fz. It follows that there must be a mechanism for each cell to assess the number and types of Stan-based bridges it forms with its neighbouring cells. We propose that cells normally depend on this mechanism to read and be polarised by long-range gradients of Fz activity.

MATERIALS AND METHODS

Mutations and transgenes

FlyBase (McQuilton et al., 2012) entries of the mutations, transgenes and deletions referred to in the text are as follows. *Vang*$^{-}$: *Vang*$^{stbm\text{-}6}$. *fz*$^{-}$: *fz*^{21}. *fz2*$^{-}$: *fz2*^{C2}. *stan*$^{-}$: *stan*^{E59}. *ds*$^{-}$: *ds*^{UAO71}. *pk*$^{-}$: *pk*$^{pk\text{-}sple13}$. *pwn*: *pwn*^{1}. *sha*: *sha*^{1}. *UAS.Vang*: *Vang*$^{Scer\backslash UAS.cWa}$. *UAS.fz*: *fz*$^{Scer\backslash UAS.cZa}$ and *fz*$^{Scer\backslash UAS.cSa}$. *UAS.stan*: *stan*$^{Scer\backslash UAS.cUa}$. *UAS.w*: *w*$^{Scer\backslash UAS}$. *UAS.GFP*: *Avic\GFP*$^{Scer\backslash UAS.T:Hsap\backslash MYC,T:SV40\backslash nls2}$. *hs.FLP*: *Scer\FLP*$^{1hs.PS}$. *FRT42*: *P{FRT(w*^{hs}*)}42D*. *FRT80*: *P{neoFRT}80B*. *FRT2A*: *P{FRT(w*^{hs}*)}2A*. *tub.Gal4*: *Scer\GAL4*$^{alphaTub84B.PL}$. *tub.Gal80*: *Scer\GAL80*$^{alphaTub84B.PL}$. *tub.fz*: *fz*$^{tub.T:Hsap\backslash MYC}$. *CD2y*$^{+}$: *Rnor\CD2*$^{hs.PJ}$. *Df(2R)Exel6072*.

Experimental genotypes

Clones of *pwn sha* cells with altered Stan system activity were generated using Flp-mediated mitotic recombination (Golic, 1991) and the MARCM technique (Lee and Luo, 1999) as previously described (Lawrence et al., 2004; Casal et al., 2006). Both mutations appear gratuitous for PCP, as any hairs or bristles made by abdominal cells in entirely *pwn* or *sha* mutant flies have normal polarity. When such clones are made in a genetic background that is wild type for the Stan system but lacks the function of the Ds/Ft system, non-autonomous effects of the clones can be increased (Adler et al., 1998; Casal et al., 2006). Therefore, we performed many of our experiments in *ds*$^{-}$/*ds*$^{-}$ and/or in *ds*$^{-}$/+ flies (asterisks below indicate genotypes in which both backgrounds were tested). However, in *fz*$^{-}$, *Vang*$^{-}$ or *Vang*$^{-}$ *fz*$^{-}$ backgrounds, the range of repolarisation is essentially limited to one cell and no significant differences were observed in the strength of polarisations regardless of whether these mutant flies were homozygous or heterozygous for *ds*. The genotype for each experiment is designated below with the corresponding number (column) and letter (row) from Fig. 1. Clones are marked only with *pwn* for genotypes 2A-H and only with *sha* for genotypes 6A-C.

1A,B: *y hs.FLP; FRT42 pwn «x» sha/FRT42 CD2y*$^{+}$, where *«x»* is *Vang*$^{-}$ (1A) or *stan*$^{-}$ (1B).

1C,D: *y w hs.FLP; FRT42 pwn «x» sha/FRT42 tub.fz; fz*$^{-}$ *CD2y*$^{+}$ *ri FRT80/fz*$^{-}$ *CD2y*$^{+}$ *ri FRT2A*, where *«x»* is nothing (1C) or *Vang*$^{-}$ (1D).

1G: *y w hs.FLP; FRT42 pwn Vang*$^{-}$ *stan*$^{-}$ *sha/FRT42 tub.fz Df(2R)Exel6072; fz*$^{-}$ *CD2y*$^{+}$ *ri FRT80/fz*$^{-}$ *CD2y*$^{+}$ *ri FRT80.*

2A-C: *w/y w hs.FLP; FRT42 pwn cn bw/FRT42 tub.Gal80 CD2y*$^{+}$*; «x»/tub.Gal4*, where *«x»* is *UAS.Vang* (2A), *UAS.stan* (2B) or *UAS.fz* (2C).

2F: *y w hs.FLP; FRT42D pwn Vang*$^{-}$*/FRT42 tub.Gal80 tub.fz; UAS.stan fz*$^{-}$ *CD2y*$^{+}$ *ri FRT2A/fz*$^{-}$ *UAS.w tub.Gal4.*

2G,H: *w/y w hs.FLP; FRT42 pwn stan*$^{-}$*/FRT42 tub.Gal80 CD2y*$^{+}$*; «x»/tub.Gal4*, where *«x»* is *UAS.Vang* (2G) or *UAS.fz* (2H).

3A-C*: *y w hs.FLP; ds*$^{-}$ *CD2y*$^{+}$ *FRT42 pwn Vang*$^{-}$ *sha/ds*$^{-}$ *FRT42 Vang*$^{-}$ *tub.Gal80; «x»/tub.gal4*, where *«x»* is *UAS.Vang* (3A), *UAS.stan* (3B) or *UAS.fz* (3C).

3D: *y w hs.FLP; ds*$^{-}$ *CD2y*$^{+}$ *FRT42 pwn Vang*$^{-}$ *sha/FRT42 Vang*$^{-}$ *tub.Gal80 tub.fz Df(2R)Exel6072; fz*$^{-}$ *ri FRT2A/fz*$^{-}$ *CD2y*$^{+}$ *ri FRT2A.*

3E,F: *y w hs.FLP; FRT42 Vang*$^{-}$ *tub.Gal80 tub.fz Df(2R)Exel6072/ds*$^{-}$ *FRT42 pwn Vang*$^{-}$ *sha; fz*$^{-}$ *UAS.w tub.Gal4/fz*$^{-}$ *CD2y*$^{+}$ *«x»*, where *«x»* is *UAS.Vang* (3E) or *UAS.stan* (3F).

3G,H: *y w hs.FLP; ds*$^{-}$ *CD2y*$^{+}$ *FRT42 pwn Vang*$^{-}$ *stan*$^{-}$ *sha/FRT42 Vang*$^{-}$ *tub.Gal80; «x»/tub.Gal4*, where *«x»* is *UAS.Vang* (3G) or *UAS.fz* (3H).

4A,B*: *y w hs.FLP tub.Gal4 UAS.GFP/y w hs.FLP; ds*$^{-}$ *CD2y*$^{+}$ *FRT42 pwn sha/ds*$^{-}$ *FRT42 tub.Gal80 CD2y*$^{+}$*; fz*$^{-}$ *CD2y*$^{+}$ *«x»/fz*$^{-}$ *ri FRT2A*, where *«x»* is *UAS.Vang* (4A) or *UAS.stan* (4B).

4C*: *y w hs.FLP; ds*$^{-}$ *CD2y*$^{+}$ *FRT42 pwn sha/ds*$^{-}$ *FRT42 tub.Gal80 ; fz*$^{-}$ *ri FRT2A UAS.fz/fz*$^{-}$ *UAS.w tub.Gal4*.

4D*: *y w hs.FLP/y w hs.FLP tub.Gal4 UAS.GFP; ds*$^{-}$ *CD2y*$^{+}$ *FRT42 pwn Vang*$^{-}$ *sha/ds*$^{-}$ *FRT42 tub.Gal80 CD2y*$^{+}$*; fz*$^{-}$ *CD2y*$^{+}$ *ri FRT80/fz*$^{-}$ *ri FRT2A*.

4E,F: *y w hs.FLP; FRT42 pwn Vang*$^{-}$ *sha/ds*$^{-}$ *FRT42 tub.Gal80; fz*$^{-}$ *CD2y*$^{+}$ *«x»/fz*$^{-}$ *UAS.w tub.Gal4,* where «x» is *UAS.fz* (4E) or *UAS.stan* (4F).

4H: *y w hs.FLP; ds*$^{-}$ *FRT42 pwn stan*$^{-}$ *sha/FRT42 tub.Gal80 CD2y*$^{+}$*; fz*$^{-}$ *CD2y*$^{+}$ *UAS.fz/fz*$^{-}$ *UAS.w tub.Gal4*.

5A-C*: *y w hs.FLP; ds*$^{-}$ *CD2y*$^{+}$ *FRT42 pwn Vang*$^{-}$ *sha/ds*$^{-}$ *FRT42 Vang*$^{-}$ *tub.Gal80; fz*$^{-}$ *CD2y*$^{+}$ *ri FRT2A «x»/fz*$^{-}$ *UAS.w tub.Gal4*, where *«x»* is *UAS.Vang* (5A), *UAS.stan* (5B) or *UAS.fz* (5C).

5D*: *y w hs.FLP; ds*$^{-}$ *CD2y*$^{+}$ *FRT42 pwn Vang*$^{-}$ *sha/ds*$^{-}$ *FRT42 Vang*$^{-}$ *tub.Gal80; fz*$^{-}$ *ri FRT2A/fz*$^{-}$ *UAS.w tub.Gal4*.

5G: *y w hs.FLP; UAS.Vang FRT42 pwn Vang*$^{-}$ *sha/ds*$^{-}$ *FRT42 Vang*$^{-}$ *tub.Gal80; fz*$^{-}$ *CD2y*$^{+}$ *ri FRT2A UAS.stan/fz*$^{-}$ *UAS.w tub.Gal4*.

5H: *y w hs.FLP; ds*$^{-}$ *CD2y*$^{+}$ *FRT42 pwn Vang*$^{-}$ *stan*$^{-}$ *sha/ds*$^{-}$ *FRT42 Vang*$^{-}$ *tub.Gal80; fz*$^{-}$ *CD2y*$^{+}$ *ri FRT2A UAS.fz/fz*$^{-}$ *UAS.w tub.Gal4*.

6A-C: *y w hs.FLP; ds*$^{-}$ *CD2y*$^{+}$ *FRT42 pk*$^{-}$ *Vang*$^{-}$ *sha/FRT42 pk*$^{-}$ *Vang*$^{-}$ *tub.Gal80;«x»/tub.Gal4*, where *«x»* is *UAS.fz* (6A), *UAS.Vang* (6B) or *UAS.stan* (6C).

DEVELOPMENT

Generation of clones

Clones were induced by heat shocking third instar larvae for 1 hour at 34°C. Adult abdominal cuticles were mounted in Hoyer's and examined by Nomarski optics as previously described (e.g. Lawrence et al., 2004; Casal et al., 2006). Images were obtained using a Nikon D300 camera and Camera Control Pro (Nikon UK Ltd., Kingston upon Thames, UK), assembled with Helicon Focus (Helicon Soft, Kharkov, Ukraine) and processed with Adobe Photoshop (Adobe Systems, San José, CA, USA).

Analysis of clones

The primary data are the results of 21 experiments (Fig. 1, columns 3-5); each tests the effect of 'sending' cells of one genotype on the polarity of hairs formed by adjacent 'receiving' cells of a second genotype. Each experiment was assessed qualitatively, and for nine key experiments also quantitatively (Fig. 3; Table 1), to determine whether the receiving cells were biased to make hairs that point towards or away from the sending cells. For both the qualitative and quantitative assessments, experiments were scored double blind as follows. Slides with one or two mounted abdomens were coded by a third party, and multiple coded slides of each of several genotypes, including negative control genotypes, were mixed and scored as a group, with the observer ignorant of the number, or types, of genotypes represented within the group. For negative controls, we used clones of identical genotype to the surround (except for the *pwn sha* marker). Corroborating and extending previous findings, we find that the removal of the *stan* gene from any sending cells blocks polarisation effects, giving outcomes that are indistinguishable from negative controls (Fig. 1, rows G and H).

Qualitative assessment

All qualitative assessments were made independently on duplicate preparations of the same mosaic genotypes by at least two observers. Typically, each mounted abdomen carries 5-20 useable clones and at least 100 clones (each surrounded by ~50-100 hairs) were scored for each experiment. In all cases, both observers were able to classify each abdomen as having clones that generally cause receiving cells to point towards or away from the clone, or as having no effect on the surround, relative to negative controls (as summarised in Fig. 1).

Quantitative assessment

Every hair surrounding each of 15-30 clones of each experimental genotype (~1000-3000 hairs) was scored as pointing inwards or outwards from the clone, except for a small fraction of hairs (8% of all hairs), which was more or less parallel to the clone border (see Fig. 2). The number of such parallel hairs was divided and added equally to the inwards and outwards sets. The percentage of the outwards hairs was calculated for each of the 15-30 clones and the arcsine transformation applied to normalise the values and to allow valid *t*-tests to be carried out (Sokal and Rohlf, 1995; Zar, 2010). This quantitative data is represented in the form of boxplots (Fig. 3), which provide information about the distribution of percentages for all clones of the same experimental genotype. Statistical comparisons of the results obtained for each experimental genotype were performed by pair-wise *t*-tests as shown in Table 1. Transformations and statistical analysis were performed using the *R* programming language and software environment (R Development Core Team, 2011). The results of this quantitative analysis were unequivocal and in agreement with the results of the qualitative analysis.

RESULTS

Experimental design and terminology

We removed or overexpressed various combinations of Fz, Vang and Stan in clones of 'sending' cells and assayed the effects on the polarity of hairs made by adjacent 'receiving' cells that are wild type, *Vang*$^-$, *fz*$^-$ or *Vang*$^-$ *fz*$^-$. The clones are marked by the double mutant combination *pwn sha*, which blocks the formation of hairs, allowing assessment of the effects of sending cells on receiving cells with single cell resolution (Casal et al., 2006). We used the ventral abdominal epidermis (sternites and pleura), which makes a continuous lawn of hairs, all of which point posteriorly in wild-type flies. We used wild-type, *ds*$^-$/+ and *ds*$^-$/*ds*$^-$ flies; the last condition removes any Ds/Ft signaling. The loss of Ds/Ft signaling can increase the range of the polarising effects of Stan-system clones on the surrounding cells (Adler et al., 1998; Casal et al., 2006). However, we detected no differences in outcome in our main experiments (flies lacking endogenous Vang, Fz or both), in which the polarising effects of clones are always limited to about one cell, regardless of the *ds* genotype (Fig. 1, columns 3-5; Fig. 3; Table 1; see Materials and methods).

Previous authors (Lawrence et al., 2004; Klein and Mlodzik, 2005; Le Garrec et al., 2006; Chen et al., 2008; Strutt and Strutt, 2008; Wu and Mlodzik, 2008), have posited three distinct forms of Stan: these are Stan on its own (Stan), Stan with Vang (Stan^{V}) and Stan with Fz (Stan^{Fz}). Possible intercellular bridges between Stan, Stan^{V} and Stan^{Fz} are shown in this way: Stan<<Stan^{Fz}, with the form of Stan in the sending cell placed first, that in the receiving cell placed second, with the direction of the chevrons indicating the polarising effect of the bridge, causing the hairs made by receiving cells to point towards (<<) or away (>>) from the sending cell.

In Fig. 1, each experiment is coded by column and row: experiment 1A refers to column 1, row A. In all cases tested, removal of Stan from the sending cells blocks any polarisation of the receiving cells (genotypes 1G; 2G,H; 3G,H; 4H and 5H in Fig. 1), indicating that signals pass across Stan bridges, as previously shown (Lawrence et al., 2004; Casal et al., 2006).

The main results can be stated simply: *Vang*$^-$ receiving cells can be polarised by clones of cells overexpressing Stan, Fz or Vang, or lacking Fz (column 3 in Fig. 1), but *fz*$^-$ and *Vang*$^-$ *fz*$^-$ cells can be polarised only by clones overexpressing Fz (columns 4 and 5 in Fig. 1). Representative examples are shown in Fig. 2, and quantitative results of key genotypes are shown in Fig. 3 and Table 1. We detail these results below.

Vang$^-$ receiving cells can be polarised by Stan, Stan^{V} and Stan^{Fz} signals

Vang$^-$ receiving cells, like wild-type receiving cells, are polarised by sending cells that overexpress Vang, Stan or Fz, or that lack Fz (*UAS.Vang*, *UAS.stan*, *UAS.fz* or *fz*$^-$ clones in *Vang*$^-$ flies; genotypes 3A-D in Fig. 1). Moreover, the direction of polarisation is as in wild-type flies (away from *UAS.fz* clones, and towards *UAS.stan*, *UAS.Vang* and *fz*$^-$ clones; genotypes 1 and 2 in Fig. 1). In wild-type flies, the polarising effects of all four kinds of clones on the surround are strong and can extend up to five rows of cells away from the clone. In *Vang*$^-$ receiving cells, the polarisation can be weaker (compare genotypes 3A-D,F with corresponding clones in columns 1 and 2 in Fig. 1; Table 1) and is limited largely or entirely to cells adjacent to the clone (Fig. 2). We draw two main conclusions from these findings.

First, in the absence of Vang, the only asymmetric bridges that can form between any given sending and receiving cell are Stan<<Stan^{Fz} and Stan^{Fz}>>Stan, and the ratio between these two types of bridges directs the receiving cell to make hairs that point towards or away from the sending cell. For clones of *fz*$^-$ cells in Vang$^-$ flies, all such bridges must be Stan<<Stan^{Fz} and the receiving cells make hairs that point towards the clones (genotype 3D), corroborating previous findings (Chen et al., 2008; Wu and Mlodzik, 2008) that Stan, on its own, can function as a polarising signal (compare genotypes 1D,2F with genotype 1G in Fig. 1). Regarding *UAS.fz* clones in *Vang*$^-$ flies (genotype 3C), these clones overexpress Fz and confront surrounding cells that express endogenous Fz at a lower level; the majority of the asymmetric bridges would therefore be Stan^{Fz}>>Stan and the hairs of the receiving cells point away.

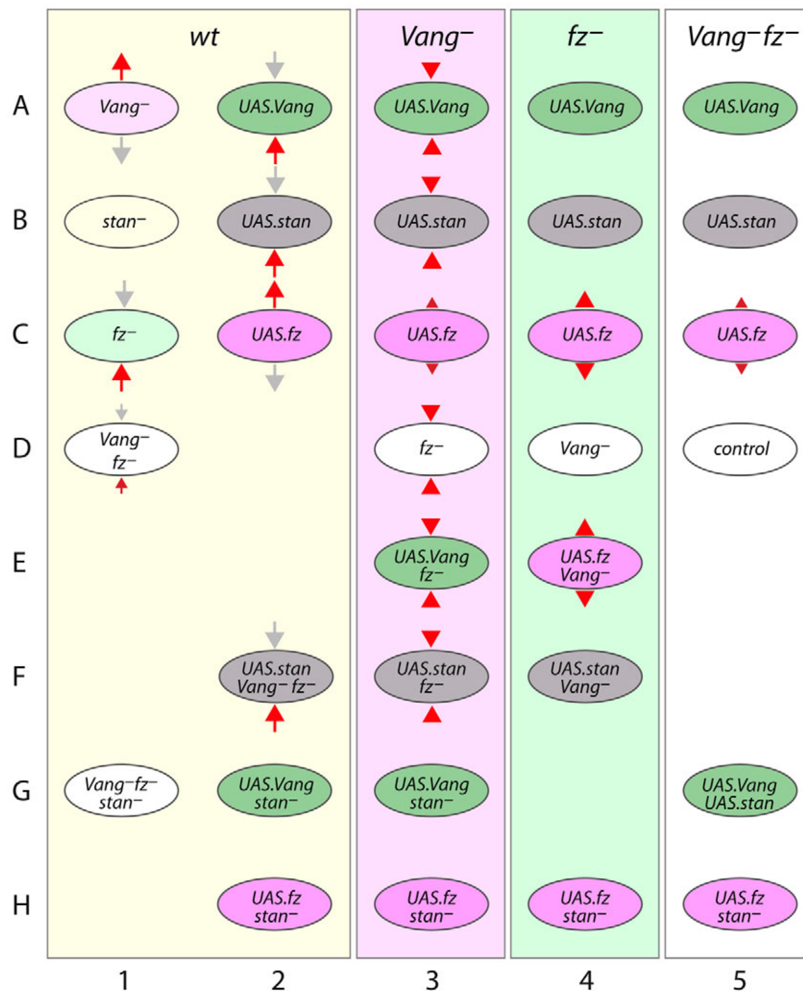

Fig. 1. A summary of the experiments. The experiments are shown as clones (ellipses) that affect (or not) the polarity of the wild-type (wt) or mutant surround. Anterior of the fly is shown towards the top of the figure; all hairs made by the ventral abdominal epidermis normally point posteriorly, towards the bottom. Large red arrows indicate a change in polarity spreading up to several cells from the clone, and large red arrowheads indicate a change that is limited largely or only to the abutting cells. Small red arrows and arrowheads indicate that the polarity effects are weak. Arrows in grey indicate an effect (surmised) that is co-oriented with the extant polarity and therefore cryptic. The pale green and pale pink colours represent endogenous Vang and Fz. Vang and Fz together (*wt*) give pale yellow; absence of both gives white. Dark colours indicate overexpression; Stan is shown as grey. Complete experimental genotypes are listed in the Materials and methods according to this scheme: e.g. 1A is a *Vang*⁻ clone in a wild-type background. Polarising effects depicted are based on assessments described in the Materials and methods.

Second, Vang functions to enhance the signalling activity of Stan in asymmetric Stan<<Stan^{Fz} bridges. In particular, both *UAS.Vang* and *UAS.stan* clones in *Vang*⁻ flies cause neighbouring cells to point towards the clone (genotypes 3A,B in Fig. 1), suggesting that adding back Vang activity to the sending cell is functionally equivalent to increasing the level of Stan activity. The same result is obtained when either *UAS.Vang* or *UAS.Stan* clones also lack *fz* (genotypes 3E,F in Fig. 1) confirming that, whether Fz is present or not in the sending cell, Vang enhances Stan activity.

These findings establish that Vang is dispensable for the central function of the Stan system; namely, to communicate differences in Fz activity between neighbouring cells and polarise them to point away from cells with higher activity and towards cells with lower activity. Furthermore, they argue that, in the absence of Vang, the ratio of Stan<<Stan^{Fz} and Stan^{Fz}>>Stan bridges that a given cell forms with each of its neighbours is sufficient to determine its polarity.

The function of Pk is not understood but it is distributed like Vang in the cell (Tree et al., 2002; Bastock et al., 2003; Das et al., 2004), raising the possibility that Pk has a partially redundant role to Vang that allows *Vang*⁻ receiving cells to respond to Stan, Stan^{V} and Stan^{Fz} signals. However, this does not appear to be the case, as we find that *UAS.stan*, *UAS.Vang* and *UAS.fz* clones in *pk*⁻ *Vang*⁻ flies polarise adjacent cells as they do in *Vang*⁻ flies (genotypes 6A-C; data not shown).

Our present results differ from earlier work (Lawrence et al., 2004) when we saw no polarisation by *UAS.fz*, *UAS.Vang* or *fz*⁻ clones in *Vang*⁻ flies; however, they agree with more recent reports of polarisation of *Vang*⁻ cells by wild-type or *fz*⁻ cells (Strutt and Strutt, 2007; Strutt and Warrington, 2008; Gomes et al., 2009). The discrepancy was due to the differing marker mutations. Our present positive results are obtained using *pwn sha*; previously, we depended on *multiple wing hairs* (*mwh*), a marker that gave negative results in 2004, and, as we have since confirmed, appears to interfere with polarisation of *Vang*⁻ cells. Our earlier findings misled us and others: e.g. they were cited as support for a model in which Fz acts unidirectionally on Vang (Wu and Mlodzik, 2008), a hypothesis that is not consistent with our present results. We regret this.

fz⁻ receiving cells can be polarised by Stan^{Fz} signal, but not by either Stan or Stan^{V} signals

UAS.fz clones generated in *fz*⁻ flies cause adjacent cells to make hairs that point away strongly: genotype 4C in Fig. 1; Fig. 3 (Lawrence et al., 2004; Casal et al., 2006). The effect is as with *UAS.fz* clones in wild-type flies (genotype 2C in Fig. 1), but is restricted to receiving cells immediately adjacent to the clone. Thus, *fz*⁻ receiving cells are strongly polarised by a Stan^{Fz} signal coming from sending cells. By contrast, *UAS.Vang*, *UAS.stan*, *Vang*⁻ or *Vang*⁻*UAS.stan* clones all fail to polarise cells in *fz*⁻ flies (genotypes 4A,B,D,F in Fig. 1) showing that *fz*⁻ receiving

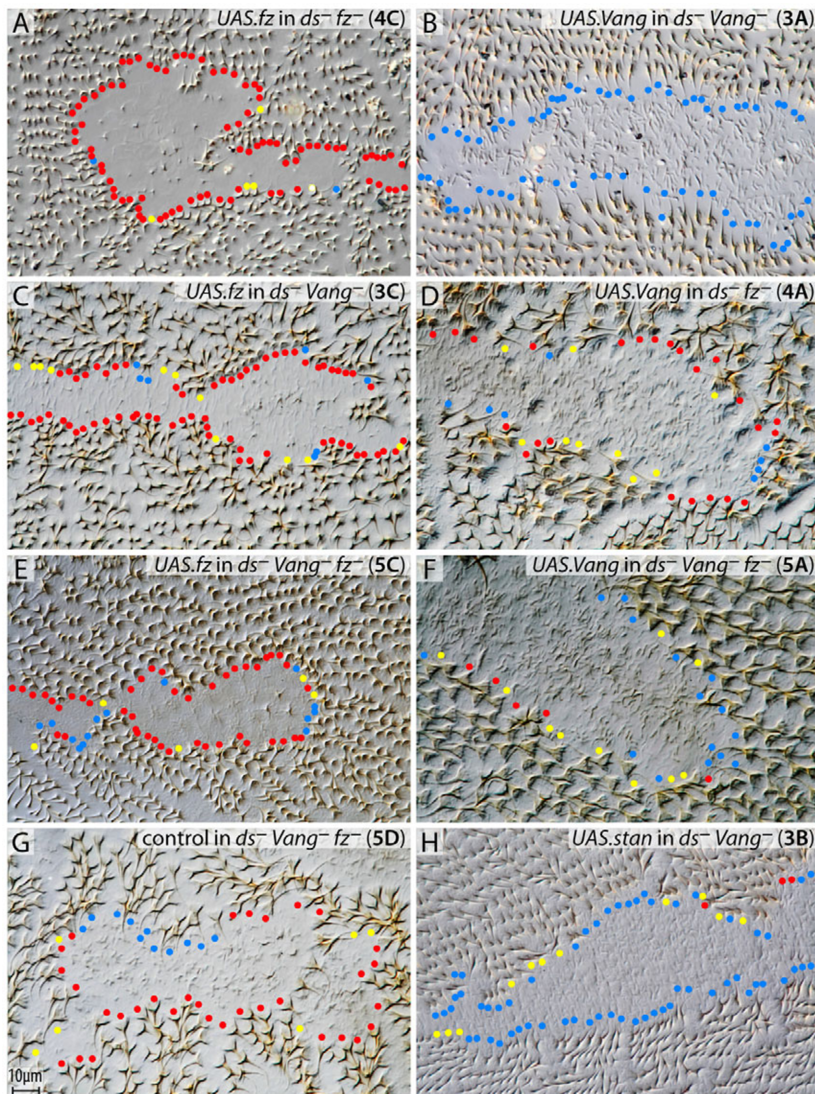

Fig. 2. Examples of clones and polarity effects in the pleura. (**A-H**) Genotypes of the clones and the backgrounds are indicated, coded by column and row as in Fig. 1 and in the Materials and methods. The clones are marked by *pawn* and *shavenoid*, mutations that together remove hairs cell-autonomously, producing a naked patch; the direction of each hair made by adjacent receiving cells is indicated by a coloured dot (red, outwards; blue, inwards; yellow, parallel). The clones either affect (A-C,E,H) or not (D,F,G) the orientation of hairs adjacent to the clone. The adjacent hairs tend to be oriented outwards (A,C,E), inwards (B,H) or more or less randomly (D,F,G). This figure is intended to provide representative examples of the different polarising effects observed, and not to provide quantitative data, which are presented in Fig. 3 and Table 1. All pictures are at the same magnification; hair sizes vary according to the segment as well as to the position within each segment. Anterior is towards the top.

cells do not respond to Stan or to Stan^{V} signals. We interpret these results as follows.

In the absence of Fz, the only intercellular Stan bridges that could form are symmetrical and asymmetrical combinations between Stan and Stan^{V}. It follows that the failure of all experimentally induced disparities in Vang or Stan to polarise cells in *fz*$^-$ flies (genotypes 4A,B,D,F in Fig. 1) indicates that such bridges either do not form or, if they do, lack polarising activity. By contrast, when Fz is added to either receiving cells (compare genotype 1D with 4D in Fig. 1) or sending cells (compare genotype 4E with 4D in Fig. 1), Stan^{Fz} is now available to bridge asymmetrically with both Stan and Stan^{V}, and the sending cells polarise the receiving cells accordingly, causing them to make hairs that point towards the clone (1D) or away (4E). Thus, of all the possible intercellular bridges that can form between Stan^{Fz}, Stan and Stan^{V}, only asymmetric bridges that link Stan^{Fz} to either Stan or Stan^{V} have a polarising effect.

Vang$^-$ *fz*$^-$ receiving cells can be polarised by a Stan^{Fz} signal, but not by either Stan or Stan^{V} signals

UAS.stan, *UAS.Vang* or even *UAS.Vang UAS.stan* clones all fail to polarise adjacent cells in *Vang*$^-$ *fz*$^-$flies (genotypes 5A,B,G in Fig. 1), as expected given that only asymmetric bridges involving Stan^{Fz} have polarising activity. By contrast, *UAS.fz* clones have polarising activity in *Vang*$^-$ *fz*$^-$ flies: they can induce abutting cells to make hairs that point away from the clone (genotype 5C in Fig. 1; Fig. 3), although the effect is significantly weaker than in *fz*$^-$ flies (genotype 4C in Fig. 1; Fig. 3). These results have two important implications.

First, the finding that *UAS.fz* clones have polarising activity in *Vang*$^-$ *fz*$^-$ flies establishes that Stan, on its own, can receive and transduce an incoming Stan^{Fz} signal via Stan^{Fz}>>Stan bridges – just as other experiments show that it can send an outgoing Stan signal via Stan<< Stan^{Fz} bridges (compare genotypes 1D, 2F and 3D,F in Fig. 1 with genotype 1G) (Chen et al., 2008; Wu and Mlodzik, 2008). We conclude that asymmetric bridges between Stan and Stan^{Fz} serve as bidirectional conduits for polarising information, with the two forms of Stan having reciprocal signalling and receiving functions.

Second, *UAS.fz* clones polarise less strongly in *Vang*$^-$ *fz*$^-$ flies (than in *Vang*$^+$ *fz*$^-$ flies), showing that the polarising activity of Stan^{Fz}>>Stan bridges is influenced by Vang, and allowing us to test whether Vang is required on the Stan or Stan^{Fz} side of such bridges by restoring it selectively to one or the other side. In the first case, we compare the polarising activity of *Vang*$^-$ *UAS.fz* clones in *fz*$^-$ flies with that of *UAS.fz* clones in *Vang*$^-$ *fz*$^-$ flies (genotypes 4E and 5C in Fig. 1). In both genotypes, the sending cells are identical

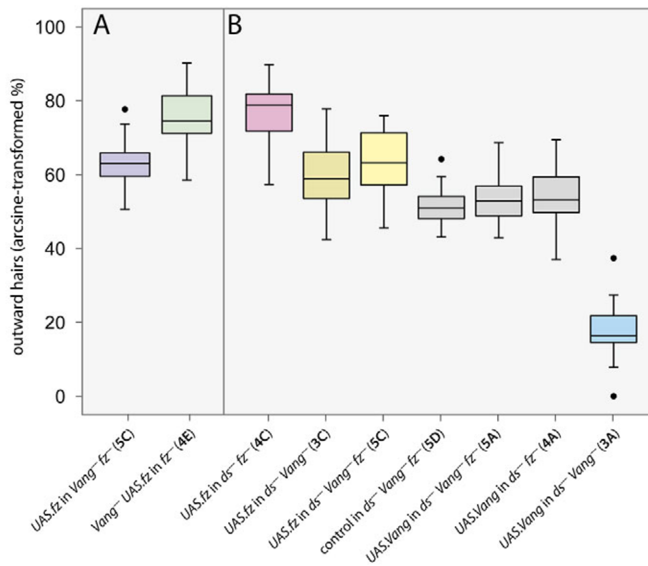

Fig. 3. Quantification of the polarising effects of Stan^{Fz} and Stan^V signals on receiving cells. Boxplots of key experimental genotypes, showing the percentage of hairs facing outwards from the clones (see Materials and methods; Table 1). (**A**) Experiments performed in *ds*$^-$/+ flies to test the requirement for Vang in the response of *fz*$^-$ receiving cells to Stan^{Fz} signal. *Vang*$^-$ *UAS.fz* clones repolarise surrounding cells to point away strongly in *fz*$^-$ flies (4E; box 2; green) but only weakly in *Vang*$^-$ *fz*$^-$ flies (5C; box 1; purple). The weak repolarising activity of *UAS.fz* clones in *Vang*$^-$ *fz*$^-$ flies is similar, irrespective of the *ds* genotype (compare the first box in A with the third box in B), but significantly different from the negative control (fourth box in B) and from the strong repolarising activity in *fz*$^-$ flies (second box in A, first box in B). (**B**) Experiments performed in *ds*$^-$ flies. *UAS.fz* clones polarise surrounding cells strongly to make hairs that point away in *fz*$^-$ flies (4C; first box; pink), but only weakly in *Vang*$^-$ and *Vang*$^-$ *fz*$^-$ flies (3C, 5C; second and third boxes; yellow). *UAS.Vang* clones have no detectable effect in *Vang*$^-$ *fz*$^-$ and *fz*$^-$ flies (5A, 4A; fifth and sixth boxes; grey; the fourth box is a negative control, grey, 5D), whereas they polarise strongly to point inwards in *Vang*$^-$ flies (3A; seventh box; blue).

(they overexpress Fz but lack Vang). However, the receiving cells differ: in *fz*$^-$ flies, they retain Vang and are strongly repolarised, whereas in *Vang*$^-$ *fz*$^-$ flies they lack Vang and are weakly repolarised (Table 1; Fig. 3). Thus, Vang appears to function in the receiving cells to help Stan respond to an incoming Stan^{Fz} signal. In the second case, we compare the polarising activities of *UAS.fz* and *UAS.fz Vang*$^-$ clones in *fz*$^-$ flies (genotypes 4C,E in Fig. 1). In this instance, the receiving cells are identical (they lack Fz but retain Vang), whereas the sending cells differ (they express Fz, but retain or lack Vang). Nevertheless, both types of clones repolarise strongly (genotypes 4C,E in Fig. 1; Table 1; Fig. 3), indicating that Vang is not required in sending cells to help Stan^{Fz} signal to receiving cells. We conclude that Vang acts specifically on Stan to help Stan form effective intercellular bridges with Stan^{Fz}. Of course, in the wild type, Stan^{Fz}>>Stan^V and Stan^V<<Stan^{Fz} bridges would form across all cell-cell interfaces. Hence, Vang would act in all cells to facilitate the exchange of polarising information. This could explain why entirely *Vang*$^-$ flies show a PCP phenotype similar to that of flies lacking Fz or Stan activity.

DISCUSSION

In *Drosophila* and other animals, including vertebrates, there appear to be at least two conserved genetic systems responsible for planar cell polarity (PCP) (Lawrence et al., 2007; Goodrich and Strutt, 2011); here we are concerned with the Stan system. The introduction presents evidence that, in *Drosophila*, epithelial cells become polarised by a multicellular gradient of Fz activity. To read this gradient, the Stan system builds intercellular bridges of Stan-Stan homodimers that allow neighbouring cells to compare their levels of Fz activity. Under this hypothesis, Fz and Stan are essential components, as without Fz there is nothing to compare and without Stan there is no means to make comparisons. The Stan system also depends on a third protein, Vang, which appears to act in a complementary way to Fz. Here, we dissect the function of these proteins by confronting adjacent cells of different *fz*, *Vang* and *stan* genotypes, and assaying the effects on PCP. Our main finding is that, even in the absence of Vang, Fz can function to polarise cells if it is present in at least one of the two abutting cells. By contrast, Vang has no detectable function when Fz is absent. Based on these and on other results, it follows that, at the core of the Stan system, intercellular bridges form between Stan on its own and Stan complexed with Fz (Stan^{Fz}), and these act to polarise cells on both sides. We conclude that Vang acts as an auxiliary component, helping Stan bridge with Stan^{Fz}. Furthermore, we posit the numbers and disposition of asymmetric Stan<<Stan^{Fz} bridges linking each cell with its neighbours are the consequence of the Fz activity gradient and serve to polarise the cell.

Cell polarisation by the Stan system: building a model

We now build a model for how bridges between Stan and Stan^{Fz} might determine the polarity of a cell (Fig. 4). In the absence of Vang, expression of Fz in a sending cell can bias the polarity of a receiving cell that lacks Fz (genotype 5C in Fig. 1; Fig. 4A**)**. Previous results (Strutt and Strutt, 2008) indicate that within the receiving cell, Stan should accumulate only on the surface that faces the sending cell – because it is the only interface where it can form bridges with Stan^{Fz} – and we now propose that it is this localised accumulation of Stan that biases the *Vang*$^-$ *fz*$^-$ receiving cell to make hairs on the other side, pointing away from the sending cell **(**Fig. 4A). A parsimonious hypothesis is that the apical membrane of each cell would have an unpolarised propensity to form hairs, and that an excess of Stan on one side locally inhibits this propensity, directing the production of hairs to the opposite side where there is least Stan. The response by a *Vang*$^-$ *fz*$^-$ cell eloquently suggests that the local accumulation of Stan bridged to Stan^{Fz} in neighbouring cells is the main, and possibly the only, intracellular transducer of Stan system PCP.

Next consider the finding that Vang functions in receiving cells to help Stan interact productively with Stan^{Fz} in sending cells. The key experiment is shown in Fig. 4B (genotype 4E in Fig. 1), and is identical to that shown in Fig. 4A, except that Vang is now added to just the *Vang*$^-$ *fz*$^-$ receiving cell: this cell is now more strongly polarised by Stan^{Fz} signal coming from the sending cell (comparing experiments Fig. 4A,B). Thus, Vang can act in the same cell as Stan to help it receive incoming Stan^{Fz} signal. The model also explains why the polarising effect of the Fz-expressing cell propagates only one cell into the *fz*$^-$ surround, even when Vang activity is restored to the receiving cells – as Stan-Stan bridges do not form, and/or do not function, between neighbouring cells that lack Fz.

Last, consider the finding that cells lacking Fz can polarise cells devoid of Vang (Fig. 4C; genotype 3E in Fig. 1). In this case, only Stan<<Stan^{Fz} and Stan^V<<Stan^{Fz} bridges can form between the two cells, and as a consequence, only the Stan^{Fz} form of Stan will

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Table 1. Statistical comparisons of quantified experiments

	Vang$^-$ *UAS.fz* in *fz*$^-$ (4E)
UAS.fz in *Vang*$^-$ *fz*$^-$ (5C)	8.2×10^{-4}

	UAS.fz in *ds*$^-$ *Vang*$^-$ (3C)	*UAS.fz* in *ds*$^-$ *Vang*$^-$ *fz*$^-$ (5C)	Control in *ds*$^-$ *Vang*$^-$ *fz*$^-$ (5D)	*UAS.Vang* in *ds*$^-$ *Vang*$^-$ *fz*$^-$ (5A)	*UAS.Vang* in *ds*$^-$ *fz*$^-$ (4A)	*UAS.Vang* in *ds*$^-$ *Vang*$^-$ (3A)
UAS.fz in *ds*$^-$ *fz*$^-$ (4C)	8.6×10^{-10}	2.2×10^{-6}	1.7×10^{-13}	2.3×10^{-14}	6.0×10^{-14}	$<2.0\times10^{-16}$
UAS.fz in *ds*$^-$ *Vang*$^-$ (3C)		0.044	0.011	0.018	0.030	$<2.0\times10^{-16}$
UAS.fz in *ds*$^-$ *Vang*$^-$ *fz*$^-$ (5C)			2.2×10^{-5}	1.7×10^{-5}	3.8×10^{-5}	$<2.0\times10^{-16}$
Control in *ds*$^-$ *Vang*$^-$ *fz*$^-$ (5D)				0.636‡	0.531‡	$<2.0\times10^{-16}$
UAS.Vang in *ds*$^-$ *Vang*$^-$ *fz*$^-$ (5A)					0.829‡	$<2.0\times10^{-16}$
UAS.Vang in *ds*$^-$ *fz*$^-$ (4A)						$<2.0\times10^{-16}$

The *P* values are the result of a pairwise *t*-test with a post-hoc Benjamini and Hochberg adjustment, with the exception of the Welch's *t*-test comparison between *UAS.fz* clones in *Vang*$^-$ *fz*$^-$ flies and *Vang*$^-$ *UAS.fz* clones in *fz*$^-$ flies.
‡$P>0.05$.

accumulate on the surface of the *Vang*$^-$ receiving cell where it abuts the *fz*$^-$ sending cell. We conjecture that Fz, when in a complex with Stan, acts to inhibit the normal action of Stan to block hair outgrowth. Therefore, the only place within the *Vang*$^-$ receiving cell where Stan can accumulate and block hair formation is on the far side, where it can form intercellular bridges with Stan^{Fz} in the next *Vang*$^-$ cell (Fig. 4C). Accordingly, the receiving cell would be directed to make a hair on the near side, where it abuts the *fz*$^-$ sending cell. This reasoning also explains why the polarising effect of *fz*$^-$ sending cells on *Vang*$^-$ receiving cells appears to be limited mostly to the adjacent *Vang*$^-$ cell; because Stan<<Stan^{Fz} and Stan^{Fz}>>Stan bridges should form and/or function poorly between this cell and the next *Vang*$^-$ cell. Nevertheless, some imbalance between these two kinds of bridges probably does spread further than one cell; indeed *fz*$^-$ sending cells can polarise receiving cells up to two rows away in *Vang*$^-$ pupal wings (Strutt and Warrington, 2008).

All the many other experiments in the results section (Fig. 1) fit with the simple model shown in Fig. 4, in which Stan accumulates at the cell surface only where it can form intercellular bridges with Stan^{Fz}, and each cell is polarised by differences in the amounts of Stan that accumulate along each of its interfaces with adjacent cells. Vang is not essential for these bridges, but by acting on Stan it helps them form (Strutt and Strutt, 2009) and/or makes them more effective.

How do wild-type cells acquire different numbers and dispositions of asymmetric bridges on opposite sides of the cell? In the *Drosophila* abdomen, in the anterior compartment of each segment, we have argued that the Hh morphogen gradient drives a gradient of Fz activity (Lawrence et al., 2004; Lawrence et al., 2007). The slope of the vector of the Fz gradient would then be read by each cell via a comparison of the amount of Stan in its membranes, as shown in Fig. 4D. Within each cell, most Stan will accumulate on the cell surface that abuts the neighbour with most Fz activity, whereas most Stan^{Fz} will accumulate on the opposite surface, where it confronts the neighbour with least Fz activity. This differential would then be amplified by feedback interactions both between and within cells (Adler et al., 1997; Lawrence et al., 2004; Amonlirdviman et al., 2005; Klein and Mlodzik, 2005; Le Garrec et al., 2006). The result in each cell is a steep asymmetry in Stan activity that represses hair formation on one side, while allowing it at the other, directing all cells to make hairs that point 'down' the Fz gradient. Our model differs in various and simplifying ways from the several and overlapping hypotheses published before (Adler et al., 1997; Lawrence et al., 2004; Amonlirdviman et al., 2005; Klein and Mlodzik, 2005; Le Garrec et al., 2006; Strutt and Strutt, 2007; Chen et al., 2008; Lawrence et al., 2008; Wu and Mlodzik, 2008). It makes Stan, rather than Fz, the main mediator of PCP, with differences in Fz activity between cells serving to regulate the local accumulation and transducing activity of Stan within cells.

A central premise of our model is that morphogen gradients do not act directly on each cell to polarise Fz activity, but rather indirectly, by first specifying stepwise differences in Fz activity between adjacent cells. We favour such an indirect mechanism for two reasons. First, PCP in much of the abdominal epidermis is organised by Hh, which is transduced primarily by its effects on the transcription factor Cubitus interruptus (Ci) (Méthot and Basler, 2001; reviewed by Ingham et al., 2011). It is difficult to understand how graded extracellular Hh could act directly – without cell interactions and only through the regulation of transcription – to polarise Fz activity within each cell. In addition, Adler and colleagues used temperature to drive tissue-wide gradients of transcription of a *fz* transgene under the control of a heat shock promoter (Adler et al., 1997); they nicely establish that cell-by-cell differences in Fz activity generated by transcriptional regulation are sufficient to polarise cells (as in Fig. 4D). Second, we have previously shown that the polarising action of Hh depends on the Stan system. Specifically, cells in which the Hh transduction pathway is autonomously activated by the removal of the negative regulator Patched require Stan to polarise neighbouring cells (Casal et al., 2006). That result adds to evidence that graded Hh creates differences in Fz activity between cells – presumably via transcriptional regulation – that lead to asymmetries in Fz and Stan activities within cells, as in Fig. 4D. The target gene could be either *fz* itself or any other gene whose activity might bias the formation of Stan<<Stan^{Fz} versus Stan^{Fz}>>Stan bridges (Fig. 4D).

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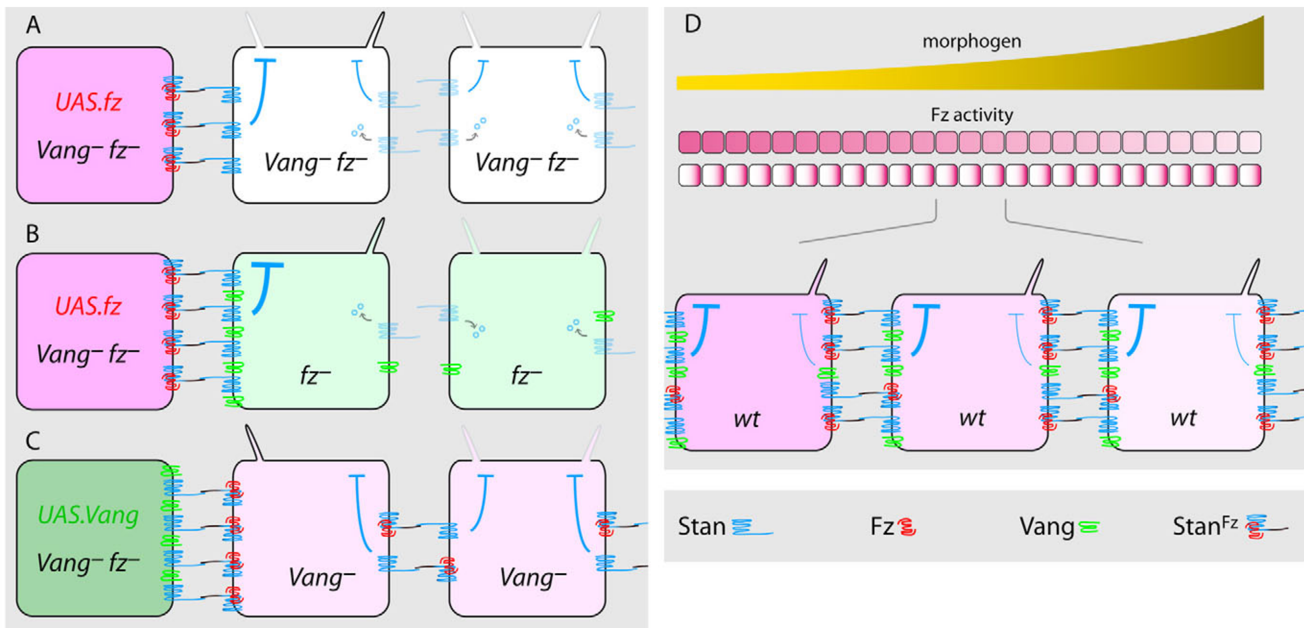

Fig. 4. The Stan system in PCP – a model. (A-C) The imagined disposition of Stan (blue), Fz (red) and Vang (green) proteins in sending (left) and receiving (middle and right) cells in three experiments. Stan, alone or in complex with Fz (Stan^{Fz}), accumulates on the apical cell membrane only when engaged in asymmetric Stan<<Stan^{Fz} or Stan^{Fz}>>Stan bridges; otherwise, it dwells transiently on the apical surface before being endocytosed, as suggested by Strutt et al. (Strutt et al., 2011). Fz complexes with Stan to make Stan^{Fz} that binds to Stan on the surface of abutting cells (the extracellular domain of the Stan molecule in the complex is shown in black to depict its altered state). Fz also blocks the hair-repressing activity of Stan (the latter is indicated by the blue inhibitory arrows, the thickness of the arrows reflects the number of Stan molecules not in complex with Fz, and hence able to repress hair outgrowth). Vang helps Stan form stable intercellular bridges with Stan^{Fz}. (**A**) For *UAS.fz* clones in *Vang*$^-$ *fz*$^-$ flies, the only bridges that can form are Stan^{Fz}>>Stan bridges between the *UAS.fz* sending cell and the abutting *Vang*$^-$ *fz*$^-$ receiving cell. Thus, higher amounts of Stan would accumulate on the left side of the *Vang*$^-$ *fz*$^-$ receiving cell than on the right side, repressing hair outgrowth on the left side, and biasing the cell to project its hair from the right side. No Stan<<Stan^{Fz} or Stan^{Fz}>>Stan bridges can form between this cell and the next *Vang*$^-$ *fz*$^-$ receiving cell to the right, limiting the polarising effect of the sending cell to only the abutting *Vang*$^-$ *fz*$^-$ receiving cell. (**B**) The situation for *UAS.fz* clones in *fz*$^-$ flies is similar to that in A, except that Vang is now present in the *fz*$^-$ receiving cell, helping to drive Stan in that cell to form stable bridges with Stan^{Fz} on the sending cell. Thus, higher levels of Stan accumulate on the left side of the receiving cell than in A, resulting in a stronger polarising effect. (**C**) *fz*$^-$ *UAS.Vang* clones in *Vang*$^-$ flies create a confrontation between *fz*$^-$ sending cells and *Vang*$^-$ receiving cells. Such sending cells can form only Stan<<Stan^{Fz} bridges with the abutting receiving cells; moreover, excess Vang in the sending cell will promote the formation of these bridges. Thus, large amounts of Stan^{Fz}, which lack the capacity to repress hair formation, will accumulate on the left side of *Vang*$^-$ receiving cell. This first *Vang*$^-$ cell engages with a second *Vang*$^-$ cell on its right and could form both Stan<<Stan^{Fz} and Stan^{Fz}>>Stan bridges. Any Stan<<Stan^{Fz} bridges that form between the first and second *Vang*$^-$ cells will locally repress hair outgrowth on the right side of first cell, biasing the hair on this cell to the left side and towards the *fz*$^-$ sending cell. (**D**) Control of Stan PCP by morphogen gradients. A morphogen gradient directs stepwise changes in the level of Fz activity from one cell to the next. The resulting differences in Fz activity between cells determine the number of Stan molecules in each cell that are engaged in asymmetric Stan<<Stan^{Fz} bridges with each neighbour. This number should be highest along the interface with the neighbour with the most Fz activity, and lowest along the interface with the neighbour with least. Formation of such bridges stabilises both Stan and Stan^{Fz}, on the apical cell surface, protecting them from endocytosis and recycling; these accumulations may be increased by intra- and intercellular feedbacks. Stan and Stan^{Fz} have opposite effects on cell polarity on each side of asymmetric Stan<<Stan^{Fz} bridges, repressing hair formation on the Stan side, while allowing it on the Stan^{Fz} side – thus directing all cells to point hairs in the same direction, down the tissue-wide gradient of Fz activity.

Molecular observations on bridges and their implications

Two staining experiments of Strutt and Strutt (Strutt and Strutt, 2008) provide further support for our model with respect to Stan<<Stan^{Fz} bridges. First, when *Vang*$^-$ clones are made in *fz*$^-$ flies (generating patches of *Vang*$^-$ *fz*$^-$ cells within *fz*$^-$ territory), a situation in which no Stan<<Stan^{Fz} bridges can form, there is no accumulation of Stan near or at the border between the clone and the surround – and indeed we now find no polarisation of the *fz*$^-$ cells across the clone border (genotype 4D in Fig. 1). Second, and by contrast, when *fz*$^-$ clones are made in *Vang*$^-$ flies (generating patches of *Vang*$^-$ *fz*$^-$ cells within *Vang*$^-$ territory) Stan accumulates strongly along cell interfaces at the clone borders (Strutt and Strutt, 2008). Moreover, it is depleted from the cytoplasm of those cells of a clone that abut that border, indicating that Stan in *Vang*$^-$ *fz*$^-$ cells is accumulating at the apicolateral cell membrane where it can form stable intercellular Stan<<Stan^{Fz} bridges. Previously, there was no evidence that this localisation of Stan within such *Vang*$^-$ *fz*$^-$ cells would polarise them (Strutt and Strutt, 2008). However, we now show that the *Vang*$^-$ *fz*$^-$ cells are polarised by their Fz-expressing neighbours (genotype 5C in Fig. 1), and, also that the effect is reciprocal, their Fz-expressing neighbours are polarised in the same direction (genotype 3D in Fig. 1).

The molecular mechanisms by which Fz and Vang control the formation and activity of Stan bridges remain unknown. Consistent with a direct action of Fz on Stan, both in vivo and in vitro studies suggest a physical interaction between the two proteins (Chen et al., 2008; Strutt and Strutt, 2008). Thus, Fz might act in a Stan^{Fz}

complex to regulate both the bridging and transducing activities of Stan, as we have discussed. There is no comparable evidence in *Drosophila* for direct interactions between Vang and Stan. However, their mammalian counterparts have been shown to interact with each other (Devenport and Fuchs, 2008). But *Drosophila* Vang does interact directly with Pk (Jenny et al., 2003), while a different Pk-related protein, Espinas, appears to interact directly with Stan during neuronal development (Matsubara et al., 2011). Hence, Vang and Pk might form a cis-complex with Stan in epidermal cells, allowing Vang to act directly on Stan and help it form intercellular bridges with $Stan^{Fz}$. Intriguingly, there is some evidence that Vang in one cell can interact directly with Fz in adjacent cells (Strutt and Strutt, 2008; Wu and Mlodzik, 2008). Such an interaction might enhance the capacity of Stan to bridge with $Stan^{Fz}$ by providing an additional binding surface between the two forms of Stan. Alternatively, Vang might affect the formation or stability of Stan<<$Stan^{Fz}$ bridges indirectly, consistent with evidence implicating it in the trafficking of proteins and lipids to the cell surface (Lee et al., 2003). For example, Strutt and Strutt have presented evidence that any Stan or $Stan^{Fz}$ on the cell surface that is not engaged in Stan<<$Stan^{Fz}$ bridges is rapidly endocytosed and recycled to other sites on the cell surface (Strutt and Strutt, 2008; Strutt et al., 2011). Vang activity could bias this process in favour of Stan, thereby enhancing its capacity to form bridges with $Stan^{Fz}$.

Parallels between the Stan and Ds/Ft PCP systems: a common logic?

Our results point to parallels between the Stan and Ds/Ft systems of PCP. First, both systems depend on the formation of asymmetric intercellular bridges between two distinct protocadherin-like molecules. For the Ds/Ft system, these are the Ds and Ft proteins themselves (Matakatsu and Blair, 2004; Casal et al., 2006; Matakatsu and Blair, 2006); for the Stan system, we argue that these are two forms of Stan, either alone or in complex with Fz ($Stan^{Fz}$). Second, morphogens may organise both systems by driving the graded transcription of target genes to create opposing gradients of bridging molecules. For the Ds/Ft system, at least two such target genes have been identified: *ds* itself and *four-jointed* (*fj*), a modulator of Ds/Ft interactions (Zeidler et al., 1999; Casal et al., 2002; Strutt et al., 2004; Ishikawa et al., 2008; Brittle et al., 2010; Simon et al., 2010). For the Stan system, we inferred the existence of at least one such target gene induced by Hh (Casal et al., 2006). Third, for both systems, the two kinds of asymmetric bridges become distributed unequally on opposite faces of each cell, providing the information necessary to point all cells in the same direction. Thus for the Ds/Ft system, we proposed that different amounts of Ds-Ft heterodimers would be distributed asymmetrically in the cell (Casal et al., 2006) and this has been recently observed (Bosveld et al., 2012; Brittle et al., 2012). Similarly, for the Stan system, there is plenty of evidence showing that Stan, Fz and Vang are unequally distributed within each cell (reviewed by Strutt and Strutt, 2009). Finally, both systems have self-propagating properties: sharp disparities in Stan, Vang or Fz activity repolarise neighbouring cells over several cell diameters, even in the absence of the Ds/Ft system (Casal et al., 2006), and the same is true of sharp disparities in Ds or Ft activity in the absence of the Stan system (Casal et al., 2006). Thus, the Stan and Ds/Ft systems may share a common logic that links morphogen gradients via the oriented assembly of asymmetric molecular bridges and feedback amplification, to cell polarisation.

Acknowledgements
We thank Xiao-Jing Qui for technical assistance, Bill Amos and Anthony Edwards for advice on statistics, Tülay Atamert for stock keeping, the Bloomington *Drosophila* Stock Center at Indiana University, and David Strutt for advice and discussion.

Funding
We thank the Wellcome Trust [WT086986MA] for welcome support. G.S. is an Investigator of the Howard Hughes Medical Institute. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

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