# Characterization of dSki and its relationship to Decapentaplegic signalling in *Drosophila*

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**Abstract**

Vertebrate members of the *ski/snoN* family of proto-oncogenes antagonize TGFβ and BMP signalling in a variety of experimental situations. This activity of Ski/SnoN proteins is related to their ability to interact with Smads, the proteins acting as key mediators of the transcriptional response to the TGFβ superfamily members. However, despite extensive efforts to identify the physiological roles of the Ski/SnoN proteins, it is not yet clear whether they participate in regulating Activin and/or BMP signalling during normal development. It is therefore crucial to examine their roles *in vivo*, mostly because of the large number of known Ski/SnoN-interacting proteins and the association between the up-regulation of these genes and cancer progression. Here we characterise the *Drosophila* homolog to vertebrate *ski* and *snoN* genes. The *Drosophila* dSki protein retains the ability of its vertebrate counterparts to antagonize BMP signalling, it is localized to both the nucleus and the cytoplasm and it does not interfere with Mad phosphorylation. The elimination of *dski* reduces viability and causes female sterility, but the surviving homozygous flies have wings of normal size and pattern. We suggest that the presence of other TGFβ antagonists might make redundant the negative effects of dSki on signalling mediated by these pathways. The Ski/SnoN repression of the pathway might constitute a "security system" for the organism in cases where the TGFβ signal is unbalanced.

## Introduction

Decapentaplegic (Dpp), a member of the TGFβ/BMP family, plays essential roles during *Drosophila* embryonic and imaginal development, influencing cell fate choices, cell proliferation and differentiation (Gelbart, 1989). The core elements of the pathway include the ligand Dpp, the type I and II transmembrane receptors Thick veins (Tkv) and Punt (Pnt), and the intracellular transducers Mothers against Dpp (Mad, related to Smad1-5) and Medea (Med, related to Smad4) (for a reviews see (Affolter et al., 2001; Feng and Derynck, 2005; Massague and Wotton, 2000). Additional key components of the pathway are the transcription factors Brinker (Brk) and Schnurri (Shn) and the antagonist Daughters against Dpp (Dad), related to vertebrate Smad7 (Marty et al., 2000; Tsuneizumi et al., 1997). *Drosophila* also has a TGFβ/Activin pathway, involved in the control of cell growth during imaginal development, in which the ligand Activin binds the type I receptor Baboon (Babo) that phosphorylates dSmad2 (Brummel et al., 1999). The structure of these pathways is conserved and the mechanism of signal transduction is similar between vertebrates and invertebrates (Massague and Chen, 2000). In both pathways, binding of the ligand to receptor complexes triggers receptor activation, which leads to the phosphorylation of Mad (Tkv/Dpp) or dSmad2 (Babo/Activin). Once phosphorylated, Mad or dSmad2 interact with Med, forming complexes that translocate to the nucleus where they can interact with DNA and, in combination with a number of co-factors, regulate the transcription of their target genes (Wisotzkey et al., 1998). Transcriptional regulation mediated by Mad/Med complexes depends on the cell-type specific cofactors recruited to the DNA. For instance, in combination with Shn, Mad/Med negatively regulate the expression of the target gene *brk* (Marty et al., 2000; Torres-Vazquez et al., 2000). Brk, in turn, can recruit several

co-repressor complexes to the DNA, regulating negatively the transcription of target genes bearing Brk-binding sequences. In essence, the response of a target gene to Dpp signalling is determined both by the combination of Mad/Med and Brk with specific co-regulators and by the structure of the regulatory DNA regions bound by them (Barrio and de Celis, 2004; Kirkpatrick et al., 2001; Saller and Bienz, 2001; Winter and Campbell, 2004).

A variety of mechanisms regulate the activity of the Dpp pathway, including negative feedbacks that contribute to signal termination mediated by the E3-Ubiquitin ligase dSmurf and the antagonist effect of Dad. dSmurf interacts with activated Mad, triggering its degradation by the proteasome (Liang et al., 2003; Podos et al., 2001). In contrast, Dad, itself a Dpp target gene, interferes with the phosphorylation of Mad through interaction with the Dpp receptor complex (Inoue et al., 1998; Tsuneizumi et al., 1997). The TGFβ pathway has been widely studied in vertebrates, where it regulates a variety of physiological responses including growth inhibition, differentiation and induction of apoptosis (Massague et al., 2000). Major negative regulators of the TGFβ response are the proteins of the Ski/SnoN family, identified by their ability to induce morphological transformation in chicken and quail embryo fibroblasts and terminal muscle differentiation in quail embryo cells (Li et al., 1986; Stavnezer et al., 1986).

The Ski/SnoN proteins appear to antagonize TGFβ signalling using different cell-type specific mechanisms. First, Ski/SnoN, acting in the nucleus, displace the Smad co-activators p300/CBP and recruit the co-repressors N-CoR/SMRT and mSin3A, forming macromolecular complexes with the histone deacetylase complex (HDAC)

(Nomura et al., 1999). In this manner, in the presence of high levels of Ski/SnoN, gene expression is not activated in response to TGFβ signalling. Second, and to account for Ski/SnoN augmented DNA binding of Smad complexes in the absence of receptor activation, it was proposed that Ski/SnoN stabilize inactive Smad complexes bound to the DNA, explaining the ability of these proteins to antagonize TGFβ signalling by promoting gene expression, as it happens with some promoters (Luo et al., 1999; Sun et al., 1999). Finally, Ski/SnoN, acting in the cytoplasm, can interfere and reduce the ligand-dependent phosphorylation of Smad2/3, preventing their translocation to the nucleus (Prunier et al., 2003). The relationships between Ski/SnoN and TGFβ signalling has been primarily studied in experimental conditions of increased expression of these proteins, a situation that relates mainly to the progression of a large number of human tumours, where high levels of Ski/SnoN proteins are usually associated with poor clinical prognosis (Buess et al., 2004).

In addition to the Smads, several other Ski-interacting proteins have being identified in biochemical assays. Thus, Ski interacts with GATA1 and blocks the binding of GATA1 to DNA in a TGFβ signalling-independent manner (Ueki et al., 2004). Ski also interacts with c-Myb to negatively regulate its activity by recruiting HDAC complexes to the DNA (Nomura et al., 2004). In a similar manner, c-Ski is required for the transcriptional repression mediated by Retinoblastoma (Rb), which binds directly to c-Ski and HDAC (Prathapam et al., 2002; Tokitou et al., 1999). Ski also associates with the Retinoic Acid Receptor complex, and can repress transcription from a Retinoic Acid Response Element (Ritter et al., 2006). Finally, Ski has also been found in association with other cellular proteins not related to transcriptional control, such as α-tubulin in the mitotic spindle and centrosomal proteins (Marcelain and

Hayman, 2005).

The physiological roles of Ski/SnoN have also been analysed *in vivo*, by studying knockout mice deficient for the *ski* or *snoN* genes and analysing the *daf-5* mutant in *C. elegans* (da Graca et al., 2004; Tewari et al., 2004). DAF-5 is an orthologue of SnoN and Ski, being the first example of a Ski/SnoN protein with a genetically defined function in a TGFβ pathway, where in combination with Daf-3, it acts in a neurosecretory process to control *C. elegans* dauer developmental arrest. In the case of vertebrate SnoN, contradictory results have been reported because one *snoN*-null line shows embryonic lethality before E3.5 (Shinagawa et al., 2000) and two other *snoN* deletions are viable and only show defects in T cell activation (Pearson-White and McDuffie, 2003). In any case, it is not clear whether the observed phenotypes are related to a lack of repression of TGFβ signalling. Similarly, it is also not clear whether the typical abnormalities of *ski*-deficient mice, such as neural tube defects, postaxial polydactyly and eye defects, are related to TGFβ activity (Berk et al., 1997; Colmenares et al., 2002). Finally, HKC cells after transfection with *snoN* specific siRNA displayed a greater responsiveness to TGFβ-1 stimulation, suggesting that down-regulation of *snoN* lowers the threshold of TGFβ-1 responsiveness by amplifying TGFβ-1 signalling (Yang et al., 2003).

In this work, we describe the genetic and functional characterisation of the *Drosophila* gene *dski*, an orthologue of both vertebrate *ski* and *snoN*. To thoroughly analyse the relation between dSki and the TGFβ pathway, we have chosen the wing imaginal disc as experimental system. The wing disc is the epithelium that gives raise to the thorax and wing of the fly, and the role of both Dpp and Activin signalling in the control of growth and patterning of the disc have been carefully studied (Brummel et al.,

1999; Zecca et al., 1995). We identified *dski* in an over-expression screen aimed to identify genes affecting the differentiation of the veins, and found that increased expression of *dski* causes phenotypes reminiscent of loss of Dpp activity in the adult wing, legs and thorax. These phenotypes are associated to the failure of Dpp to regulate the expression of its target genes in the presence of high levels of dSki, a situation that mirrors the relations between vertebrate Ski/SnoN and the TGFβ signalling pathway. The augmented expression of dSki does not interfere with Mad phosphorylation, and its effect is completely abolished by mutations in the putative Smad 2/3 or Smad4-binding regions of dSki. Surprisingly, we did not find a significant phenotype in the wing upon removal of *dski*, and therefore we suggest that during normal wing development the contribution of several overlapping mechanisms makes redundant a role for *dski* to regulate Dpp signalling. In dSki over-expression conditions, the interaction between dSki and Mad/Med cannot be relieved by pathway activation, and the transcriptional regulation of Dpp-target genes is compromised.

## Results

### *Ectopic expression of dSki causes loss of Dpp signalling*

Genetic combinations between the P-UAS insertion *EP-M67* and a variety of Gal4 lines expressed in the imaginal discs result in striking adult phenotypes (Fig. 1). The modifications observed in the wing vary, depending on the particular Gal4 line, from moderate wing size reduction and vein loss (*Gal4-sal/EP-M67*; Fig. 1B) to strong wing size decrease accompanied by the absence of all veins (Fig. 1C-D; *nub-Gal4/EP-*

*M67* and *638-Gal4/+; EP-M67/+*). In the thorax, the combinations *ptc-Gal4/EP-M67* and *pnr-Gal4/EP-M67* display a complete failure in the closure between the two heminota (Fig. 1I and data not shown), whereas in the legs the combination *dll-Gal4/EP-M67* shows failures in anterior-posterior leg patterning and the absence of distal segments (Fig. 1K). Interestingly, all these phenotypes are similar to those caused by the reduction in *dpp* expression or signalling (Fig. 1E and see Campbell et al., 1993; de Celis, 1997). These facts suggest that the gene/s targeted by the *EP-M67* insertion interfere negatively with some aspect of the Dpp signalling pathway.

The *EP-M67* insertion is localised between the 5' regions of two annotated genes, CG7233 and CG7231 (Fig. 2). Because the P-GS element carries UAS sequences at both ends, both genes can be simultaneously expressed in *EP-M67/Gal4* combinations (Molnar et al., 2006; Toba et al., 1999), and this is indeed what we observed (Fig. 2D and data not shown for CG7231). To identify unambiguously the gene responsible for the ectopic expression phenotypes, we generated UAS and UAS-RNAi transgenic lines for the coding regions of CG7233 and CG7231. We found that only the over-expression of CG7233 reproduces the phenotype of the *EP-M67* insertion (Fig. 1G). Likewise, only the *UAS-CG7233i* line is able to suppress the phenotype of *EP-M67/Gal4* combinations (Fig. 1F). These data indicate that the miss-expression of CG7233 accounts for all the phenotypes observed in combinations involving *EP-M67*. Furthermore, some suppressors of the *EP-M67* phenotype are characterized by amino acid changes in the CG7233 coding region (see below), further confirming its association with the *EP-M67* miss-expression phenotypes.

*Genomic structure and RNA expression of dski*

The gene CG7233, as annotated in Flybase, is formed by a unique exon with an open reading frame of 1017 bp with no EST clone associated (Fig. 2A). Therefore, we searched for cDNA clones containing the CG7233 sequence by PCR in cDNA libraries constructed from embryonic and imaginal polyA$^+$ RNA (Brown and Kafatos, 1988). We identified two polyA$^+$ cDNAs expressed throughout development, named *cDNA3.2* and *cDNA7.1*. *cDNA7.1* includes CG7233, whereas cDNA3.2 includes in addition part of CG7093, a gene annotated proximal to CG7233 (Fig. 2A). These data indicate that *dski* generate two alternative transcripts, which we named *dski-Short* (CG7233; *dskiS*) and *dski-Long* (CG7233 plus CG7093; *dskiL*). Thus, the intergenic region between CG7233 and CG7093 corresponds to a long intron of 80Kb. To verify the existence of this intron, we performed RT-PCR reactions using mRNA from embryos, larvae and wing imaginal discs and sets of primers specific for CG7233 (a), CG7093 (c) and the joint between the two of them (b). The results corroborated the existence of alternative splicing (Fig. 2B). Interestingly, the same genomic organization was found in *D. pseudoobscura* (not shown) and *A. gambiae* (Fig. 2C). However, sequences homologous to CG7093 were not found in other species.

By means of *in situ* hybridization using probes for the common region of *dskiS* and *dskiL* and for the unique exon of *dskiL*, we found that both transcripts are expressed under UAS control in combinations between *EP-M67* and Gal4 lines (Fig. 2D). However, the phenotype of *EP-M67* appears to be causally related only to the misexpression of *dskiS*, because the *UAS-dskiL* in combination with several Gal4 lines did not affect adult patterning or growth (data not shown). In wild type imaginal discs

the expression of both *dskiS* and *dskiL* is generalised (Fig. 2D for the wing disc and data not shown).

*dskiS* contains a Ski domain, present in the *ski*/*snoN* family of proto-oncogenes, and it has been identified as the *Drosophila* SnoN orthologue (Flybase). However, our phylogenetic analysis suggests that dSki is equidistant from the Ski and SnoN vertebrate paralogues (called previously Snowski (da Graca et al., 2004), indicating that the duplication *ski*/*sno* occurred after the separation of the arthropods (Fig. 3A). The fact that *T. castaneum* presents a unique *ski*/*sno* homologue equally distant from *ski* and *snoN* reinforces this idea (not shown). Other proteins containing Ski domains are grouped in two distinct families (Fig. 3A). Dachshund (Dac) proteins are negative regulators of the TGFβ signally through their interaction with Smad4, as reported in vertebrates (Wu et al., 2003). In *Drosophila*, Dac does not seem to have a function in the wing blade but is able to produce ectopic eyes when co-expressed with *eyes absent* (Chen et al., 1997; Hammond et al., 1998). The CG11093 family (previously called Iceskate (da Graca et al., 2004), which is poorly characterised, represents the third branch. Two subfamilies can be differentiated in vertebrates. In humans, the closer homologue to CG11093 is Fussel-18, which is expressed exclusively in the nervous system and interacts with Smad2 and 3 (Arndt et al., 2005). Closely related is the Ladybird homeobox corepressor 1 (LBXCO1) sub-family, which homologue in mice, Corl1, is expressed exclusively in brain and testis and interacts with the Ladybird homeobox protein (Arndt et al., 2005; Mizuhara et al., 2005). These data indicate that *Drosophila* has representative orthologues for the three branches of the Ski superfamily, but a unique member belonging to the Ski/SnoN family (Fig. 3A).

*Interactions between dSki and the Dpp pathway*

Vertebrate Ski/SnoN proteins have been extensively characterised by biochemical and cell culture approaches, behaving as strong antagonists of the Activin and BMP signalling pathways (see Introduction). The phenotype caused by increased expression of *dskiS* suggests that such antagonism is conserved in *Drosophila*. To understand the molecular bases of this interaction, we monitored the effects of *dski* over-expression on the expression of several Dpp targets in the wing imaginal disc. We used target genes that are activated by Dpp signalling such as *dad*, *spalt* (*sal)*, *optomotor blind* (*omb)* and *blistered* (*bs*) as well as *brk*, which expression is repressed by Dpp activity (Fig. 4). We compared the effects of dSki with those of the Dpp antagonist Dad and of the Brk repressor. We found that increased expression of *dski* reduces the expression of Sal and *dad* with high efficiency and interferes weakly with the expression of *omb* and Bs (Fig. 4G-J). In addition, the expression of *brk*, normally restricted to the most anterior and posterior part of the wing blade by Dpp repression, is now observed throughout the wing blade (Fig. 4K). These effects are very similar to those of ectopic Dad or Brk, the main differences being the degree with which each Dpp target gene is affected (Fig. 4M-Q,S-W). Thus, Brk is more efficient than Dad or dSki in repressing *omb* and *bs*, and dSki is more efficient that Dad or Brk in repressing *dad* (Fig. 4). Ectopic expression of dSki, Dad or Brk interfered the repression of *brk* in the centre of the wing blade with similar efficiency (Fig. 4K,Q,W). These data indicate that dSki antagonises Dpp targets gene expression, acting both on genes that are normally activated (*sal*, *dad*) or repressed (*brk*) by Dpp signalling. Because Mad/Med contribute to both activation and repression of Dpp targets (Muller et al., 2003), it is likely that the effect of dSki is exerted on the formation or activity of Mad/Med complexes.

*dSkiS does not modify Mad phosphorylation, but interferes with Mad/Med complexes*

To further characterise the relationship between dSki and the Dpp pathway, we analysed genetic combinations between dSki and several members of the pathway in misexpression experiments. Ectopic expression of activated-Tkv (Tkv*) or activated-Babo (Babo*) in the centre of the wing, the domain where the Dpp pathway is normally active, modifies wing size, the pattern of veins and the integrity of the wing (Fig. $5A^{I,II}$). In contrast, ectopic expression of Mad, Med or dSmad2 in the same central domain does not modify wing growth or patterning (Fig. $5A^{III-V}$). The co-expression of dSki rescues the phenotypes caused by ectopic Tkv* or Babo* (Fig. $5B^{I,II}$) resulting in wings very similar to those over-expressing only dSki (see Fig. 1). These results indicate that dSki functions downstream of the activated receptors. In contrast, the expression of Mad and Med suppresses the phenotype of dSki, giving rise to wings of normal size and pattern (Fig. $5B^{III,IV}$), suggesting that dSki interferes with the formation or activity of Mad/Med complexes. Similar results were obtained when the ectopic expression was driven in the entire wing, including the territories where the Dpp pathway is not normally active. In these cases, the over-expression of Mad, Med or dSmad2 caused a mutant phenotype consisting of alterations in the vein pattern and wing size (Fig. $5C^{III-V}$). The phenotypes of these combinations with over-expressed dSki confirms that only Mad or Med are able to suppress dSki, whereas dSki efficiently suppresses the phenotype of ectopic Tkv* or Babo* (Fig. $5D^{I-IV}$). Interestingly, we found a strong synergistic interaction between dSki and dSmad2, consisting in the generation of very small wings without veins, similar to those of strong *dpp*-disc alleles (Fig. $5D^{V}$). Possibly, the formation of

Med/dSmad2 dimmers could reduce the formation of Mad/Med functional dimmers, already severely compromised by the presence of dSkiS (see Discussion).

The genetic interactions between dSkiS and the elements of the Dpp and Activin pathways can be interpreted as a consequence of sequestering Mad or Med by dSkiS or, alternatively, by the failure to phosphorylate Mad in the presence of dSkiS. We studied *in vivo* the distribution of phosphorylated-Mad (PMad) in the presence of dSkiS or Dad, which is known to interfere with Mad phosphorylation (Fig. 5E-J). When dSkiS expression is increased, the phosphorylation of PMad is not modified (Fig. 5F), even in the presence of Tkv* (Fig. 5I). These data indicate that, at least in the wing disc, dSkiS does not interfere with Mad phosphorylation, suggesting that interactions between dSkiS and Mad or Med underlies the negative effects of dSkiS on Dpp signalling.

*Generation and analysis of dski loss of function alleles*

We have shown that over-expression of dSki interferes with Dpp signalling probably through interactions with Mad and/or Med. In order to analyse the requirement of the gene during normal development, we generated a deficiency that removes all dSkiS coding sequences, as well as several point mutations that prevent the negative regulation of Dpp signalling. The *dski* deficiency is a 65Kb deletion induced by FRT-based recombination between the insertions *f00844* and *d02797*, and includes the genes *dskiS*, most of *dskiL*, CG7231 and most of CG7228. Homozygous flies *Df(2L)ski* are homozygous viable, albeit the viability is only of 30%. The surviving flies have wings of normal size and pattern, and the females are sterile. Furthermore, the expression of Sal and P-Mad is not affected in *Df(2L)ski* wing imaginal discs (data

not shown). Point mutation alleles in the *dski* coding region were induced by chemical mutagenesis as revertants of the *EP-M67* insertion, taking advantage of the strong *dpp*-like phenotype of the combination *Gal4-638/EP-M67* (Fig. 6B). Out of the eight mutants isolated and mapped to the coding region of *dSki*, three, $M67^{2C3}$, $M67^{3K1}$ and $M67^{3W1}$ behave as complete revertants (Fig. 6). $M67^{2C3}$ is caused by an F234I substitution in the dSkiS region corresponding to the Smad4 binding domain of vertebrate Ski (Fig. 6A). Interestingly, this phenylalanine is important to establish hydrogen bounds with several amino acids in Smad4 (Wu et al., 2002), suggesting that the 2C3 mutant protein has reduced ability to interact with Med/Smad4. The $dSki^{2C3}$ protein is still able to cancel some of the effects of ectopic Mad expression (Fig. 6J-M). $M67^{3W1}$ also maps in the Smad4 binding domain, and $M67^{3K1}$ is a P71S substitution in a Smad2/3 interacting domain identified by (Ueki and Hayman, 2003). We also identify several partial revertants, three of them causing amino acid substitutions in the Med/Smad4 binding domain (R102: S247F; S2: V269M and V2: F285L; Fig. 6A), in the Ski domain (F3: I182T; Fig. 6A) and in the C-terminal region of dSkiS (K2: E329L; Fig. 6A), suggesting that these regions are also critical for dSki activity. The C-terminal sequence of dSki (ELEHKKRKVRWV) includes a putative consensus nuclear localization signal (Fig. 7). However, no major differences were found between the sub-cellular localization of dSkiS-GFP or dSkiSΔC-GFP, a construction that lacks the C-terminal part of the protein (see below). We did not find any revertant in the non-conserved amino terminal part of dSkiS or in the dSkiL unique C-terminal region, even through these regions account for most of the dSki protein sequences. These data suggest that the effect of dSkiS on Dpp signalling requires an intact Smad4 and Smad2/3 interacting domain, as well as the Ski and the C-terminal domains.

All *EP-M67* revertant chromosomes were lethal in homozygosis, although the lethality is not related to mutations in *dski* but to second site mutations. Thus, (1) all pair-wise combinations between *EP-M67* revertants are viable and lack a visible phenotype, (2) heterozygous between these revertants and the *Df(2L)dski* are fully viable. Trans-heterozygous females between any *dski* revertant and *Df(2L)dski* are fertile and complete oogenesis normally, indicating that the defects observed in *Df(2L)dski* homozygous can not be directly attributed to dSki interacting with Mad/Med complexes.

*Subcellular localisation of dSki in imaginal discs*

We generated dSki-GFP fusion proteins and studied their subcellular localizations in the wing imaginal disc (Fig. 7). The level of GFP expression in the combination *Gal4-nub/UAS-GFP* was very similar in all wing cells during the third larval instar (data not shown). In contrast, we found that the levels and subcellular localisation of the GPF signal was extremely heterogeneous within each wing disc in *Gal4-nub/UAS-dSkiS-GFP* (Fig. 7E, G). In general, the protein is localised in both the nucleus and the cytoplasm, but the levels of GFP in these cellular compartments vary in different cells. In addition, GFP cannot be detected in cells distributed homogeneously in clusters throughout the wing blade. We did not find any correlation between the pattern of dSkiS-GFP expression and the domain of Dpp activity. Furthermore, the distribution of dSkiS-GFP is not modified when the activity of the pathway is altered, either by expressing Tkv* or Med (Fig. 7 F,H). All together, these data indicate that, in our experimental settings, Dpp signalling does not interfere with the stability and subcellular localization of dSkiS.

*Subcellular localization and activity of dSki in cultured cells*

To better understand the regulation of dSki subcellular localization, we introduced the Ski-GFP fusion proteins into mammalian cells with or without other components of the Dpp pathway (Fig. 8). When dSkiS-GFP is introduced alone, the protein appears both in the nucleus and the cytoplasm and this distribution does not change in the presence of Mad (Fig. 8A,E). However, when transfected together with Med, dSkiS-GFP is retained in the cytoplasm, as it has been reported for Med alone (Wisotzkey et al., 1998 and Fig.8C). The normal localization of dSkiS-GFP is recovered only when the Dpp pathway is active in the presence of Mad (Fig. 8H, compare to 8D and 8G). These results suggest that dSkiS interacts with Med in the cytoplasm and translocates to the nucleus, together with Med and Mad, when the pathway is active. Our results do not show any interaction between dSkiS and Mad. Whether this interaction exists or not, its nature seems to be weaker than the interaction of dSkiS with Med, as in the presence of Mad and Med, dSkiS is still retained in the cytoplasm (Fig. 8G). Interestingly, no major differences were seen in the localization of dSkiSΔC-GFP (data not shown), confirming that the C-terminal region is not necessary for the nuclear localization of the protein, neither for its interaction with Med. dSkiL-GFP behaves differently than dSkiS-GFP and is always retained in the nucleus despite the presence of Med or the activation of the Dpp pathway (Fig. 8I-J).

In order to understand the functionality of the dSki isoforms, we performed transcriptional assays using the mammalian TLX2 responsive element next to the *luciferase* reporter gene. This construction has been shown to respond to the *Drosophila* homologues of the TGFβ pathway (Brummel et al., 1999; Tang et al., 1998). The

reporter is moderately activated in presence of Tkv*, Mad and Med, but this activation diminishes significantly when either dSki isoform is introduced in the culture (Fig. 8K). These results indicate that the dSki proteins are able to inhibit the activation of a TGFβ responsive element in cell culture. The TGFβ pathway inhibits the cellular growth, probably through the inhibition of cell proliferation (Huang and Huang, 2005). When added to P19 cells, Tkv*, Mad and Med decrease cell viability by more than a 40% (Fig. 8L). However, this inhibition is counteracted significantly by the addition of either the dSki isoforms. Interestingly, the presence together of dSkiS and dSkiL reduces viability, suggesting that the two isoforms can counteract each other when expressed at high levels in cultured cells.

**Discussion**

We report here the characterization of the *Drosophila* homologue to vertebrates Ski and SnoN, and its relationships to Dpp signalling. *dski* is transcribed into two isoforms, *dskiS* and *dskiL*, with dSkiS being able to interfere with Dpp signalling when expressed at levels higher than normal. These effects of dSkiS are suppressed by increasing the expression of either Mad or Med, suggesting that, when over-expressed, dSkiS titrates out Mad/Med complexes, and renders them non-functional. In this manner, the antagonism of dSki on Dpp signalling affects indistinctively the Dpp-targets that are activated (*dad*, *sal* and *omb*) or repressed (*brk*) by the pathway. The elimination of dSkiS and dSkiL by a genetic deficiency did not affect Dpp signalling in the imaginal discs. This result is surprising because Ski/SnoN are among the best-known negative regulators of BMP and TGFβ signalling in vertebrate cells and several molecular mechanisms linking Ski/SnoN and the Smad proteins have been identified

(see introduction). Interestingly, the overwhelming majority of studies relating Ski/SnoN to BMP and TGFβ signalling pathways are based in experiments where Ski and/or SnoN are over-expressed (Luo, 2004). Our work shows that the ability of Ski/Sno to antagonise BMP signalling is conserved in Drosophila and vertebrate cell lines, but brings the physiological significance of these effects during normal development into question.

*dSkiS effects on Dpp signalling depends on its Mad and Med binding domains*

All the defects we observed in imaginal discs and adult patterns after increased expression of dSkiS can be constructed as the result of reduced Dpp signalling. Thus, the phenotypes of *dskiS* over-expression are extremely similar to those characteristics of *dpp* loss-of-function mutations, and the expression of Dpp target genes is severely impaired upon *dskiS* ectopic-expression. Finally, the interference with Dpp-target expression and the mutant phenotypes characteristic of increased dSki depend on the integrity of its Mad- and Med-binding domains. We could not observe effects of dSkiS over-expression on Mad phosphorylation, indicating that the pathway is activated in its normal spatial domain. Furthermore, ectopic expression of dSki cancels the effects of activated Tkv, while it is cancelled by augmented levels of either Mad or Med. These results indicate that the effect of dSki is related to its binding to the P-Mad and Med proteins, preventing them to form functional complexes. The subcellular location of dSkiS-GFP in cultured cells is compatible with direct interaction of dSkiS with Med, because these proteins are retained together in the cytoplasm when P-Mad is not present. In the wing disc, however, other mechanisms must contribute to dSkiS subcellular localization, because the protein is detected in both the nucleus and cytoplasm in cells where P-Mad is not present and Med is preferentially located at the

cytoplasm. In addition, other Dpp-independent mechanisms contribute to the stability of the fusion protein, because cells grouped in clusters in the wing disc fail to accumulate dSkiS-GFP. The importance of dSkiS interactions with Med is highlighted by the genetic interactions we observed between dSkiS and the Activin pathway members Babo* and dSmad2. Therefore, we suggest that dSkiS interferes with both the Dpp and Activin pathways, which share Med as a partner for Mad and dSmad2, respectively (Brummel et al., 1999).

In vertebrates, it appears that Ski/SnoN binds R-Smads (dSmad2/Smad2-3 and Mad/Smad1-5) and Smad4 independently, using different domains in the Ski protein (He et al., 2003; Ueki and Hayman, 2003; Wang et al., 2000; Wu et al., 2002). It is not clear, however, whether mutations in both the R-Smad and Smad4 ski-interacting domains are needed to suppress Ski/SnoN activities. Thus, in some assays, removing Smad4-Ski interactions is enough to cancel the effects of Ski on TGFβ (Suzuki et al., 2004) or BMP signalling (Takeda et al., 2004), whereas other reports suggests that Ski proteins defective in binding to only Smad2/3 or Smad4 retain their activity (He et al., 2003; Ueki and Hayman, 2003; Wu et al., 2002). We observed that single *dski* mutants in the dSmad2/Smad2-3 binding domain recently identified by (Ueki and Hayman, 2003) or in the Med/Smad4 binding domain severely compromise the ability of the dSki to interfere with Dpp signalling. In other words, *dski* mutants reducing or abolishing the phenotype of dSki ectopic expression map in either its Smad4 binding region (2C3, R102, S2, 2W1 and V2 see Fig. 6) or in its Smad2/3 binding domain (3K2). In this manner, although our in vivo data support the importance of both the R-Smad and Smad4 binding sites on dSkiS, they also indicate that the antagonism of dSki on Dpp signalling requires its interaction simultaneous with both P-Mad and Med.

*dSki subcellular location*

The subcellular localization of Ski proteins varies in different cell types and physiological situations. In most cases, Ski has been reported as a nuclear protein, although it can be kept in the cytoplasm, restricting TGFβ signalling by Smad2 sequestration (Kokura et al., 2003). In mouse liver hepatocytes, C184M, a small leucine-rich protein that represses growth cell inhibition by TGFβ, could retain Ski in the cytoplasm (Kokura et al., 2003). In pre-invasive melanomas Ski is located only in the nucleus while it appears in the cytoplasm in primary invasive and metastatic melanomas (Reed et al., 2001). SnoN is predominantly cytoplasmic in normal tissues and in non-tumorogenic or primary epithelial cells. After cell-cycle arrest, SnoN translocates into the nucleus (Krakowski et al., 2005). In the *Drosophila* wing disc the localization of the protein when over-expressed occurs in both the nucleus and the cytoplasm, and this pattern appears independent of Dpp signalling, because it does not correlate with the spatial domain of Dpp activation (P-Mad expression) and it is not modify upon manipulation of pathway activity. In mammalian cells, however, the location of dSkiS can be modulated by the activation of the Dpp pathway, mainly through the interaction of dSkiS with Med. Thus, the phosphorylation of Mad by Tkv* promotes its translocation to the nucleus accompanied by Med, which in turn can transport dSkiS. Taking dSkiS to the nucleus along with the activated Smads could ensure the down-regulation of the pathway to avoid an excess of signal.

*Developmental role of dSki in Drosophila*

Despite the fact that the wing is an excellent system to demonstrate the capacity of dSkiS to antagonize the Dpp pathway, our genetic analysis does not reveal a role of this protein during normal wing patterning. To explain the lack of a prominent function

for dSkiS in the wing, we could argue that dSkiS function is carried out by another homologue of the Ski super-family in *Drosophila* (see Fig. 3). However, we think this is unlikely. Dac is not expressed in the wing blade, except in some cells at the wing margin, and it does not seem to have a requirement there (Mardon et al., 1994). However, similarly to dSkiS, Dac is also able to interfere with wing development in over-expression experiments (Chen et al., 1997). Also, CG11093 is not a likely candidate; it is not expressed in the wing imaginal disc and, as it happens for its mammalian counterparts, its expression seems to be restricted to the nervous system (F. Casares, personal communication). Alternatively, a requirement for dSki during normal wing development might be hidden due to the existence of complementary mechanisms that contribute to Dpp signal down-regulation. For example, the lack of dSkiS could augment Dpp signalling and, consequently, the amount of negative regulators of the pathway, such as Dad, whose expression is regulated by the pathway (Briones-Orta et al., 2006; Tsuneizumi et al., 1997). Increased expression of Dad might counteract the effect of reduced dSki. Interestingly, *dad* homozygous flies only have minor phenotypes in the wing, which is compatible with a cooperative effect between different repressors (J. F. de C., unpublished). In this context, it is remarkable the paucity of data that relate Ski/Sno activity to BMP/TGFβ signalling in loss-of-function experiments. The analysis of *ski* or *sno* mutant mice show a variety of developmental defects consistent with their role in various signalling pathways but it does not immediately relate them to BMP/TGFβ signalling (Berk et al., 1997; Colmenares et al., 2002). Only in *C. elegans* mutants for the *ski*/*snoN* homologue *daf-5* suggests its involvement in one aspect of TGFβ signalling (da Graca et al., 2004; Tewari et al., 2004).

Although the capacity of Ski proteins to interact directly with a wide variety of factors (see introduction) makes feasible a involvement for these proteins in multiple developmental pathways, the promotion of oncogenic transformation by Ski or SnoN proteins is related to their capacity to inhibit the TGFβ pathway (Buess et al., 2004; Poser et al., 2005; Reed et al., 2001; Zhang et al., 2003; Zhu et al., 2005). Thus, the function of dSkiS, which is not apparent during normal wing development, might be manifested when Dpp/TGFβ signalling is des-regulated. For example, mice deficient for *ski* are more susceptible to generate tumours when exposed to a chemical carcinogen (Shinagawa et al., 2001). In the same way, dSkiS could be important for cell behaviour when challenged with factors or mutations that alters the levels of Dpp signalling. In this manner, dSki repression of the pathway might constitute a "security system" for the organism in cases where the TGFβ signal is unbalanced. Interestingly, interfering with the ability of dSki to interact with Smad proteins does not seem to compromise any critical physiological function of these proteins, making Ski/Sno a suitable target for cancer therapies.

## Materials and Methods

*Drosophila melanogaster strains and phenotypic análisis*

We isolated one P-GS insertion in the 5' UTR of CG7233 (*EP-M67*) in a mutagenesis screen designed to identify genes affecting vein differentiation (Molnar et al., 2006). We also used the following *Drosophila* UAS lines: *UAS-tkv*$^{QD}$ (Nellen et al., 1996), *UAS-dpp* (Staehling-Hampton and Hoffmann, 1994), *UAS-brk*, *UAS-Mad*, *UAS-Med*, *UAS-babo*$^{act}$, *UAS-dSmad2* and *UAS-GFP* (Ito et al., 1997). We also used the Gal4 lines *Gal4-shv*$^{3kpn}$ (Sotillos and de Celis, 2006), *Gal4-dll*, *Gal4-nub* and *Gal4-salEPv* (Barrio and de Celis, 2004), *Gal4-638* and *Gal4-253* (de Celis et al., 1999) and the P-

*lacZ* reporter lines *dad-lacZ*, *brk-lacZ*, *tkv-lacZ* and *omb-lacZ*. All stocks not described in the text can be found in Flybase (http://www.flybase.net/). All phenotypes were analysed at 25ºC and flies were mounted for microscopic examination in lactic acid-ethanol (1:1). Pictures were taken in an Axiophot microscope with a Spot digital camera and processed using Adobe Photoshop.

*Generation of dski alleles by chemical mutagenesis*

Loss-of-function alleles were induced by chemical mutagenesis, treating isogenic males of *w; EP-M67*/*EP-M67* genotype with 20mM EMS or ENU, according to (Lewis and Bacher, 1968). Treated males were mass-crossed with *Gal4-638* females and viable progeny of genotype *Gal4-638;EP-M67*/+ were isolated to make the stocks *w; EP-M67*$^{rev}$/CyO. The *Gal4-638; EP-M67*/+ males do not hatch from the pupal case, and the sibling females (*Gal4-638*/+*; EP-M67*/+) display a strong reduction in wing size and elimination of wing pattern elements. Among 15000 progeny (viable females with wing size reduction phenotype) we isolated three complete revertant, named *EP-M67*$^{2c3}$, *EP-M67*$^{3K1}$ and *EP-M67*$^{3W1}$and six partial revertants: *EP-M67*$^{1D1}$, *EP-M67*$^{F3}$, *EP-M67*$^{S2}$, *EP-M67*$^{V2}$ *EP-M67*$^{K2}$ and *EP-M67*$^{R102}$. The presence of mutations in all revertants was confirmed by sequencing the coding region of *dskiS* (Supplementary Materials and Methods).

*Generation of dski deficiencies by FRT-mediated recombination*

We used the Exelixis flanking insertions *f00844* and *d02797* (Parks and al., 2004) separated by 65 Kb including *dskiS*, CG7231 and most of *dskiL* and CG7228 coding regions. FRT recombination was induced by a daily one hour heat shock at 37ºC to the progeny of *hsFLP1.22*/+*; f00844* / *d02797* females and *w;CyO*/*If* males. 40 putative

*f00844-d02797/CyO* offspring males were individually crossed to *w,CyO/If* females, and after three days were used to extract genomic DNA to confirm by PCR the existence of FRT recombination.

*cDNA isolation and expression analysis*

*cDNAs 3.2* and *202* were isolated from a 12-24 hr embryonic library and *cDNA 7.1* from an imaginal disc library by PCR (Brown and Kafatos, 1988). Accession numbers are: *cDNA7.1*, AM282580; *cDNA3.2*, AM282581; *cDNA202*, AM282582. For RT-PCR experiments mRNA was prepared from *D. melanogaster* embryos 12h to 24h old, larva 3 L3, and wing imaginal discs. The mosquito amplifications were obtained from an adult abdomen cDNA library. Detailed protocols and sequences for all the primers will be provided upon request.

*DNA constructions, sequence analysis and generation of transgenic flies*

*dskiS* was generated by a combination of *cDNA202* with *cDNA7.1*. *dskiL* is a combination of *cDNA202* with *cDNA3.2* and EST GH28569 (Flybase). Accession numbers are: *dskiS*, AM282583; *dskiL*, AM282584. Cloning of *dskiS* and *dskiL* GFP-fusion proteins and UAS constructs, as well as generation of *UAS-CG7231*, of templates for in situ probes, of CG7233 RNAi construct and of CG7093 RNAi construct, together with the sequences of all the primers used and the accession numbers of the sequences included in the analysis will be provided upon request. All the clones were verified by sequencing and all the UAS constructs were introduced in the germ line by *P*-element transformation as described (Spradling and Rubin, 1982). For sequence comparison and analysis we used ClustalW package.

*Tissue culture, transfection, immunocytochemistry and reporter assays*

Mouse embryonic carcinoma P19 cells (ATCC) and Cos1 cells were transformed in 24 well plates with the appropriated plasmids. For immunohistochemistry, Cos1 cells were grown on coverslips and stained with mouse monoclonal anti-Ha 12CA5 (dilution 1:200; Sigma) and rabbit anti-Flag antibodies (dilution 1:2000; Sigma) using standard procedures. Samples were analyzed in a Leica confocal microscope. Luciferase assays and viability assays were done in P19 cells using the Dual-Luciferase Reporter Assay System and CellTiter-Glo, respectively (Promega). Average, standard deviation and P-test values for each condition originate from at least 20 independent experiments and were calculated using Microsoft Excel.

*Drosophila inmunocitochemistry and in situ hybridization*

We used mouse monoclonal anti-DSRF (Hybridoma bank) and rabbit anti-Sal (dilution 1:200; Barrio et al., 1999) and anti-βGal (dilution 1:200; Cappel). Secondary antibodies were from Jackson Immunological Laboratories (used at 1/200 dilution). Third instar imaginal discs were dissected, fixed and stained as described in (de Celis, 1997). Confocal images were captured using a BioRad confocal microscope. In situ hybridization in imaginal discs was carried out as described in (de Celis, 1997). Sense and anti-sense digoxigenin-labeled RNA probes were prepared with T7, T3 or SP6 RNApolymerases using the appropriated plasmid clones as templates.

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## Figure legends

### Figure 1

**Wing phenotypes caused by increased expression of dSki**

(A) Wild-type wing showing the longitudinal veins (L2-L5). (B-D) Adult wings of genetic combinations between the P-UAS insertion *EP-M67* and the Gal4 lines *Gal4-sal* (B), *Gal4-nub* (C) and *Gal4-638* (D). (E) Homozygous $dpp^{d5}$ wings. (F) Adult wing of *Gal4-sal/UAS-idSkiS; EP-M67/+* genotype, showing a complete suppression of the *EP-M67* phenotype shown in B. (G) Adult wing of *Gal4-nub/UAS-dskiS* genotype, compare with C. (H) Wild-type notum. (I) *Gal4-ptc/EP-M67* notum, showing a failure to close the two heminota (arrow). (J) Wild-type legs. (K) *Gal4-dll/EP-M67* legs, showing distal truncations characteristic of $dpp^{disc}$ alleles. All wings, nota and legs correspond to female flies, and pictures were taken at the same magnification.

### Figure 2

**Molecular analysis and expression of *dski***

(A) Schematic representation of the genomic region 28D2-D3 according to Flybase. Blue boxes represent predicted ORFs and green boxes represent ESTs. The red triangle indicates the insertion site of *EP-M67*. The location of the two *dski* isoforms, dSkiS and dSkiL, is indicated. The *dskiS* and *dSkiL* cDNAs (cDNA7.1 and cDNA3.2) are represented by red rectangles and arrows. a, b and c indicate the pairs of primers used in the RT-PRC reactions shown in B. (B) RT-PCR products using the pairs of primers a, b and c from *D. melanogaster* embryonic (E), third instar larvae (L) and wing imaginal discs (wd) mRNA. The asterisk indicated the amplified band. Rp49 amplification was used as control. (C) PCR using *A. gambiae* cDNA library as template

and specific a, b and c pairs of primers. (D) in situ hybridisation using anti-sense RNA probes for *dskiS* (7233) or *dskiL* (7093) transcripts, either on wild type (WT) or *EP-M67/+;Gal4-sal/+* (M67) third instar wing imaginal discs.

**Figure 3**

**Phylogenetic analysis of the Ski superfamily of proteins**

(A) Unrooted dendogram showing three main branches: In blue, the Ski/Sno family that includes CG7233 from *Drosophila* (Ski domain consensus RLCLPQ); in orange, the Dachshund family that includes the only *C. elegans* homolog Dac1 (consensus LICLPQ); in purple the CG11093-like family that includes also the LBXCO1 members (consensus RLCLAQ). See also Supplementary Fig. 1. (B, C) Alignments of the Ski-domain (B) and the Smad4 binding domain (C) from the Ski/Sno family members. Red arrows indicate the amino acid changes in the *EP-M67* revertant mutants. Ag, *A. gambiae*; Ce, *C. elegans*; Dm, *D. melanogaster*; Dp, *D. pseudoobscura*, Dr, zebrafish; Gg, chicken; Hs, human; Mm, mouse; LBXCO1: Ladybird homeobox corepressor 1.

**Figure 4**

**Effects of dSki, Dad and Brk on the expression of Dpp targets in wing discs**

(A-E) Wild-type third instar wing discs showing the expression of Sal (A), *omb-lacZ* (B), Bs (C), *dad-lacZ* (D) and *brk-lacZ* (E). *Gal4-sal/EP-M67* (G-K), *Gal4-sal/UAS-brk* (M-Q) and *Gal4-sal/UAS-dad* (S-W) third instar wing showing the expression of Sal (G,M,S), *omb-lacZ* (H,N,T), Bs (I,O,U), *dad-lacZ* (J,P,V) and *brk-lacZ* (K,Q,W). (F, L, R, X) Adult wings of wild-type (F), *Gal4-sal/EP-M67* (L), *Gal4-sal/UAS-brk* (R) or *Gal4-sal/UAS-dad* (X) genotypes.

**Figure 5**

**Genetic interactions between dSki and different members of the Dpp pathway**

(A) Adult wings of combinations between the Gal4 line *Gal4-sal* and the UAS lines *UAS-tkv** ($A^{I}$), *UAS-babo** ($A^{II}$), *UAS-Med* ($A^{III}$), *UAS-Mad* ($A^{IV}$) and *UAS-dSmad2* ($A^{V}$). (B) Adult wings co-expressing dSki and different members of the Dpp pathway in the *sal* domain of expression, *Gal4-sal, EP-M67/UAS-tkv** ($B^{I}$), *Gal4-sal, EP-M67/UAS-babo** ($B^{II}$), *Gal4-sal EP-M67/UAS-Med* ($B^{III}$), *Gal4-sal EP-M67/UAS-Mad* ($B^{IV}$) and *Gal4-sal EP-M67/UAS-dSmad2* ($B^{V}$). (C) Adult wings over-expressing in the entire wing blade (*Gal4-nub*) different members of the Dpp pathway. *UAS-tkv** ($C^{I}$), *UAS-babo** ($C^{II}$), *UAS-Med* ($C^{III}$), *UAS-Mad* ($C^{IV}$) and *UAS-dSmad2* ($C^{V}$). (D) Adult wings co-expressing dSki and different members of the Dpp pathway in the *nub* pattern of expression. *Gal4-nub, EP-M67/UAS-tkv** ($D^{I}$), *Gal4-nub, EP-M67/UAS-babo** ($D^{II}$), *Gal4-nub, EP-M67/UAS-Med* ($D^{III}$), *Gal4-nub, EP-M67/UAS-Mad* ($D^{IV}$) and *Gal4-nub, EP-M67/UAS-dSmad2* ($D^{V}$). (E-J) P-Mad expression in third instar wing imaginal discs from wild type (E) or from the genetic combinations *Gal4-nub/EP-M67* (F), *Gal4-nub /UAS-tkv** (G), *Gal4-nub /UAS-dad* (H), *Gal4-nub, EP-M67/UAS-tkv** (I) or *Gal4-nub, EP-M67/UAS-dad* (J).

**Figure 6**

**Identification and characterization of *dSki* mutants**

(A) Representation of the dSkiS protein, showing the Ski domain (orange) and the Smad4-binding domain (red). The amino acid substitutions present in each mutant protein are shown below the black lines. Numbers correspond to the localization of the affected amino acids. (B-J) Adult wings of combinations between *Gal4-638* and

different *EP-M67* revertant chromosomes: (B) *Gal4-638/EP-M67$^{1D1}$*, (C) *Gal4-638/EP-M67$^{3K1}$* (D) *Gal4-638/EP-M67$^{F3}$*, (E) *Gal4-638/EP-M67$^{2C3}$*, (F) *Gal4-638/EP-M67$^{R102}$*, (G) *Gal4-638/EP-M67$^{S2}$*, (H) *Gal4-638/EP-M67$^{3W1}$* (I) *Gal4-638/EP-M67$^{V2}$*, (J) *Gal4-638/EP-M67$^{K2}$*. Below each wing is shown the position of the corresponding amino acid substitution. (K-M) Partial rescue of Mad by *EP-M67$^{2C3}$* over-expression. (K) *Gal4-nub/UAS-Mad*, (L) *Gal4-nub/EP-M67; UAS-Mad/+*, (M) *Gal4-nub/EP-M67$^{2C3}$; UAS-Mad/+*.

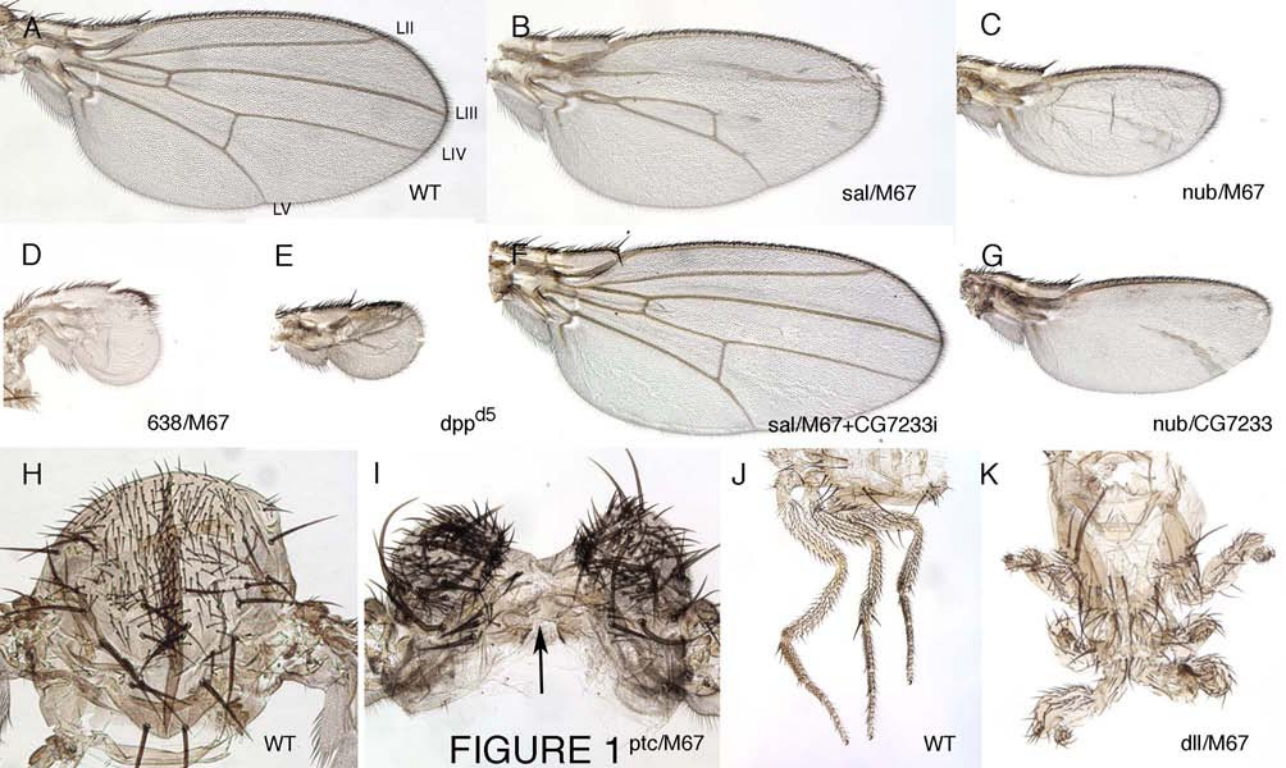
**Figure 7**

**Subcellular localization of dSkiS-GFP in wing discs**

(A) Representation of the different dSki-GFP fusion proteins. Coloured lines indicate the Ski, Smad4 and Smad2/3 binding domains. The C-terminal peptide is underlined in blue. Amino acids VRWV are spliced out in dSkiL. (B-D) Adult wings of the following genotypes: *Gal4-nub/EP-M67* (M67; B); *Gal4-nub/UAS-dSkiS-GFP* (dSkiS-GFP; C) and *Gal4-nub/UAS-dSkiSΔC-GFP* (dSkiSΔC-GFP; D). (E-E') Expression of GFP (green in E and E') and Sal (red in E) in *Gal4-nub/UAS-dSkiSΔC-GFP*. (F) Expression of GFP (green in F and F') and Het1 (red in F) in *Gal4-sal/UAS-dSkiSΔC-GFP* third instar discs wing imaginal discs. (G-G') Expression of GFP (green in G and G') and Sal (red in G) in *Gal4-nub/UAS-dSkiS-GFP* third instar wing discs. (H) Expression of GFP in *Gal4-nub/UAS-dSkiS-GFP; UAS-tkv*/+* third instar wing discs (I-I') Expression of GFP (green) and Sal (red) in *Gal4-nub/UAS-dSkiS-GFP; UAS-Med/+* third instar wing discs. I' is a tangential sections of the disc shown in I.

**Figure 8**

Differential subcellular localisation of dSki in cultured cells and its effect on transcriptional activation and cell viability. (A-J) Immunocytochemistry on Cos1 cells transfected with the indicated constructs. (A-H) Cells are transfected with dSkiS-GFP in the absence (A,C,E,G) or presence (B,D,F,H) of Tkv*. (I-J) Cells are transfected with dSkiL-GFP in the absence (I) or presence (J) of Tkv*. (K,L) Graphical representations where the Y-axes represent times of activation of TLX2-Lux (K) or percentage of viable cells (L) in P19 cultures under to the presence of the indicated constructs. T-test values are shown. Bars indicate standard deviation.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LV
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C
nub/M67
D
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F
sal/M67+CG7233i
G
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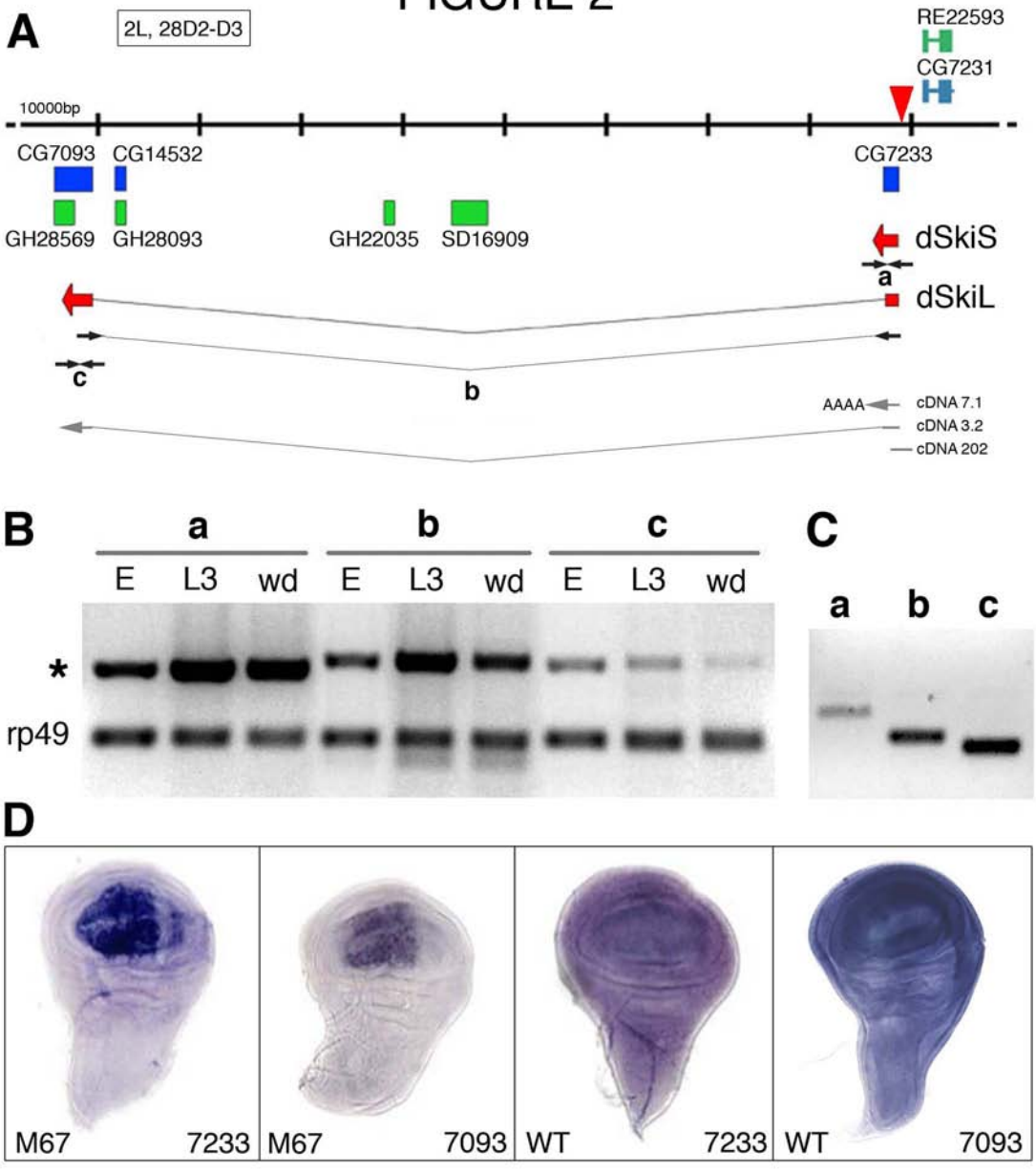
H
WT
I
ptc/M67
J
WT
K
dll/M67

FIGURE 1

# FIGURE 2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dSkiL
a
b
c
AAAA
cDNA 7.1
cDNA 3.2
cDNA 202
B
a
b
c
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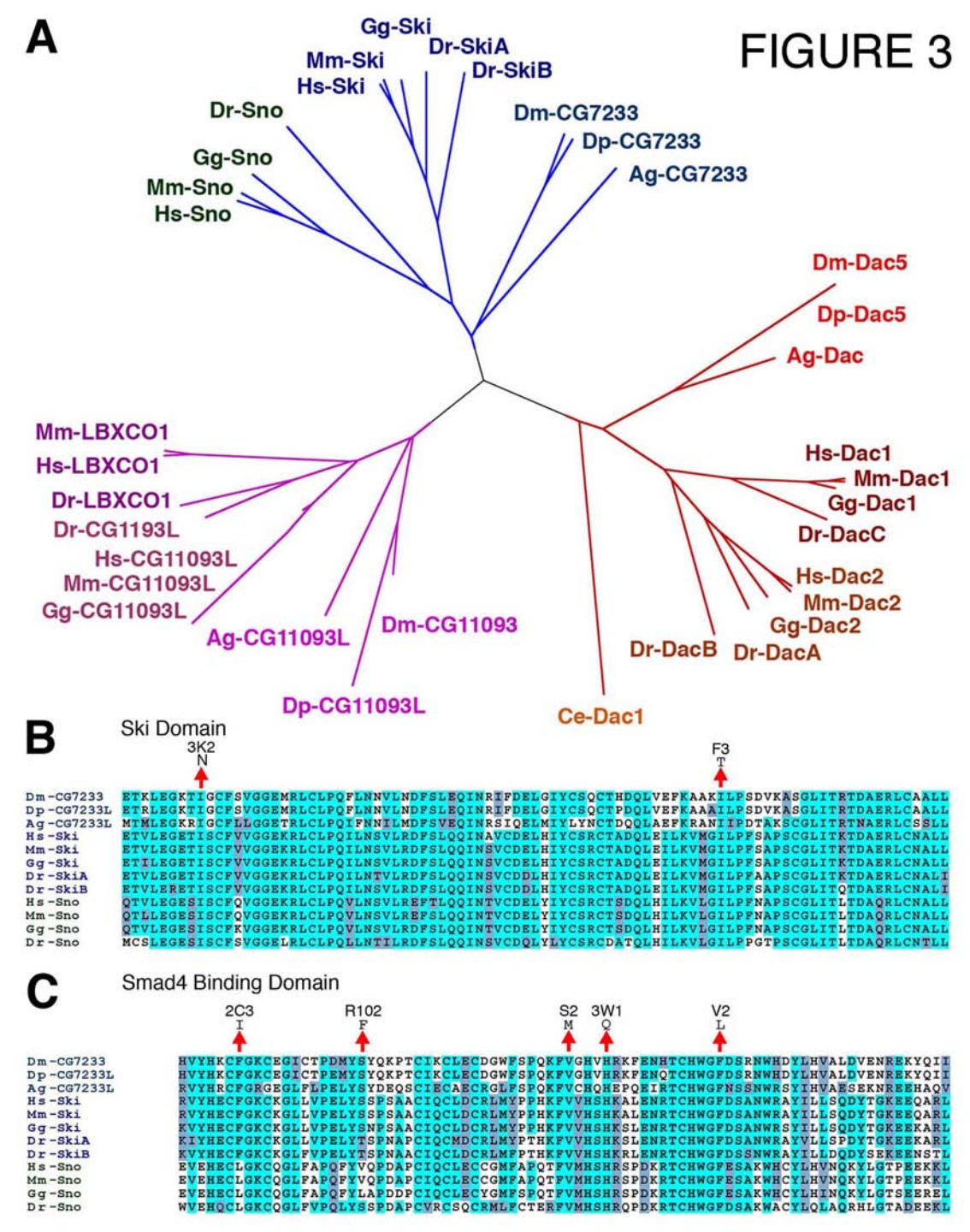
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FIGURE 3
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Mm-Ski
Dr-SkiB
Hs-Ski
Dr-Sno
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Gg-Sno
Ag-CG7233
Mm-Sno
Hs-Sno
Dm-Dac5
Dp-Dac5
Ag-Dac
Mm-LBXCO1
Hs-LBXCO1
Hs-Dac1
Mm-Dac1
Dr-LBXCO1
Gg-Dac1
Dr-CG1193L
Dr-DacC
Hs-CG11093L
Hs-Dac2
Mm-CG11093L
Mm-Dac2
Gg-CG11093L
Gg-Dac2
Ag-CG11093L
Dm-CG11093
Dr-DacB
Dr-DacA
Dp-CG11093L
Ce-Dac1
B
Ski Domain
3K2
N
F3
T
Dm-CG7233 ETKLEGKTIGCFSVGGEMRLCLPQFLNNVLNDFSLEQINRIFDELGIYCSQCTHDQLVEFKAAKILPSDVKASGLITRTDAERLCAALL
Dp-CG7233L ETRLEGKTIGCFSVGGEMRLCLPQFLNNVLNDFSLEQINRIFDELGIYCSQCTPDQLVEFKAAAILPSDVKASGLITRTDAERLCAALL
Ag-CG7233L MTMLEGKRIGCFLLGGETRLCLPQIFNNILMDFSVEQINRSIQELMIYLYNCTDQQLAEFKRANIIPDTAKSCGLITRTNAERLCSSLL
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Smad4 Binding Domain
2C3
I
R102
F
S2
M
3W1
Q
V2
L
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Mm-Ski RVYHECFGKCKGLLVPELYSSPSAACIQCLDCRLMYPPHKFVVHSHKALENRTCHWGFDSANWRAYILLSQDYTGKEEQARL
Gg-Ski KVYHECFGKCKGLLVPELYSNPSAACIQCLDCRLMYPPHKFVVHSHKSLENRTCHWGFDSANWRSYILLSQDYTGKEEKARL
Dr-SkiA KIYHECFGKCKGLLVPELYTSPNAPCIQCMDCRLMYPTHKFVVHSHKSLENRTCHWGFDSANWRAYVLLSPDYTGKEEKARL
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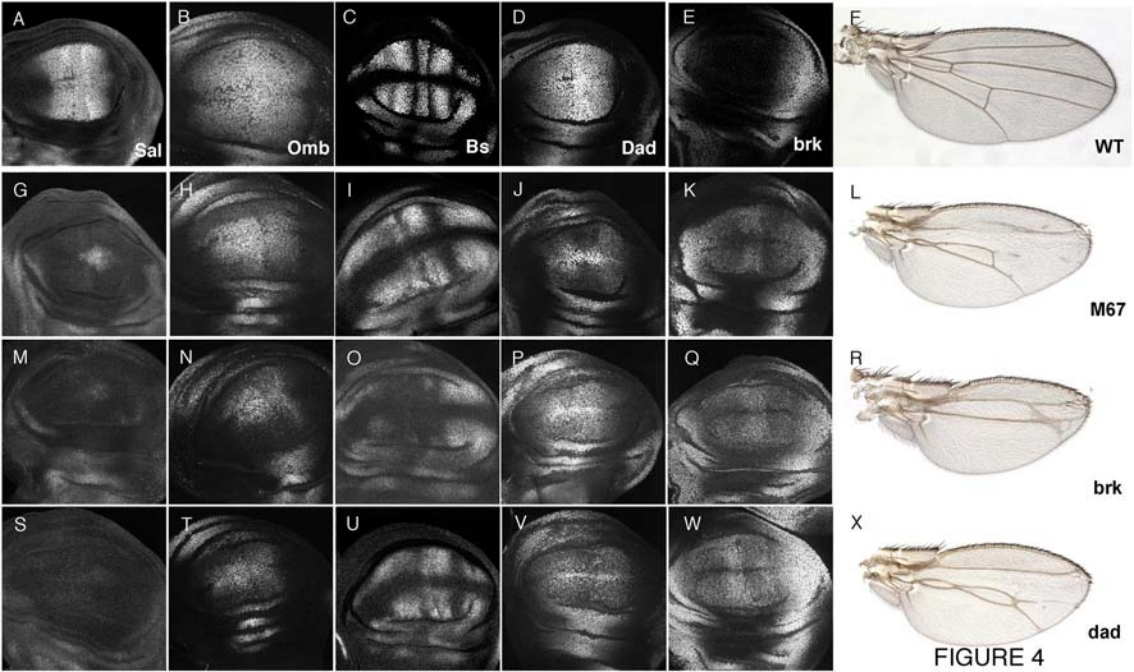
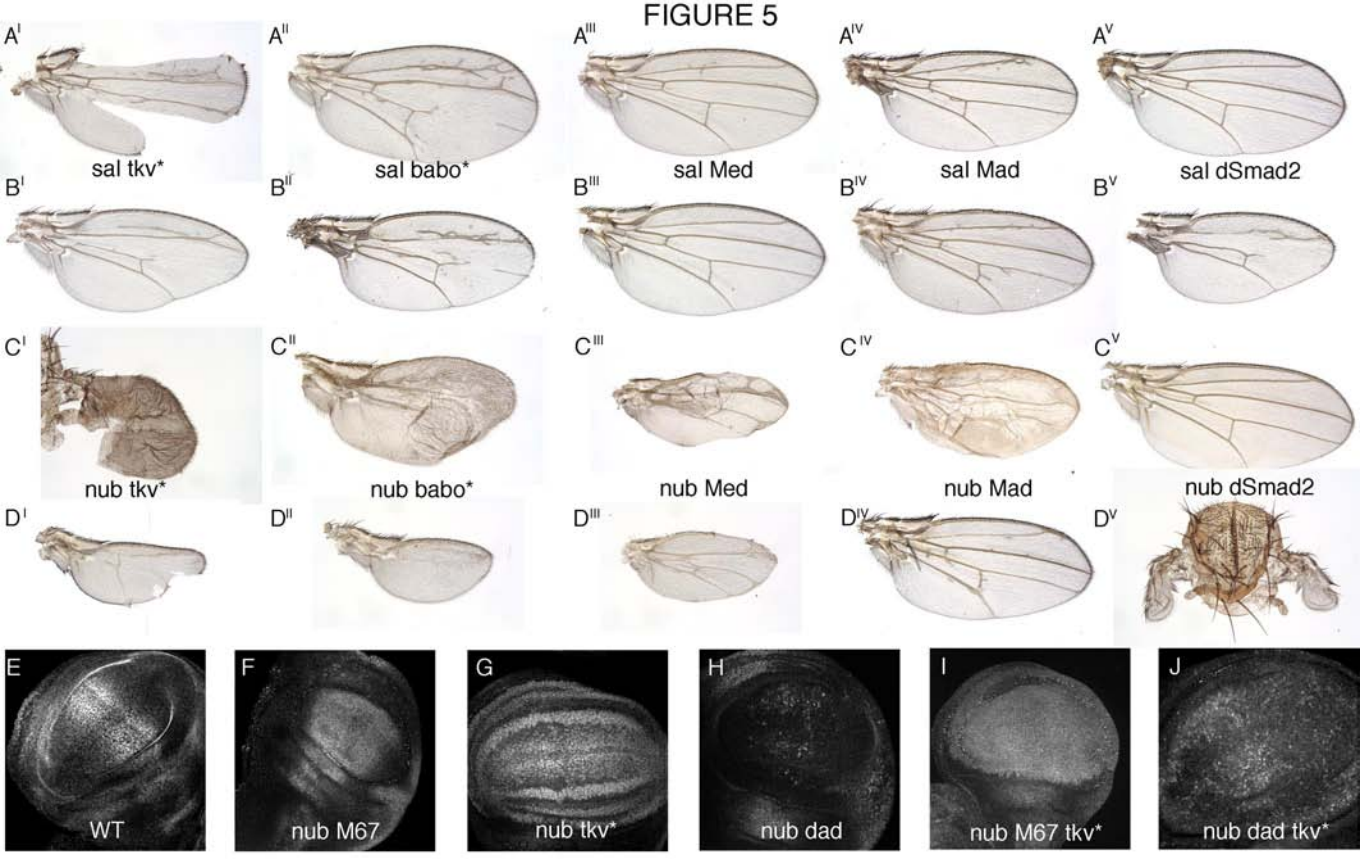



FIGURE 4

FIGURE 5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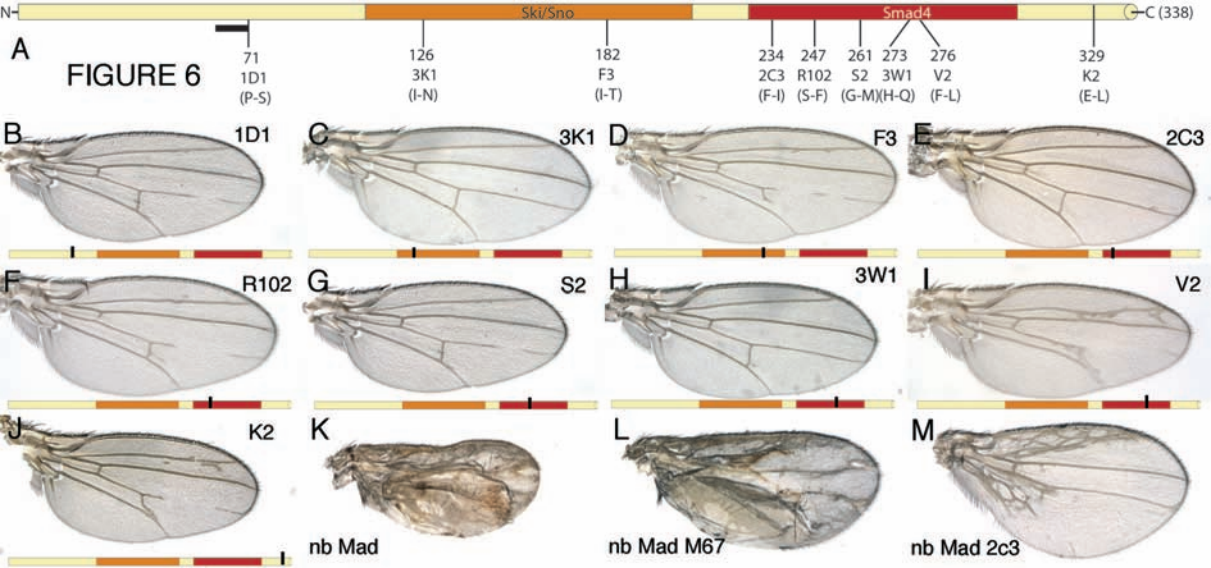
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Ski/Sno
Smad4
C (338)
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FIGURE 6
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182
F3
(I-T)
234
2C3
(F-I)
247
R102
(S-F)
261
S2
(G-M)
273
3W1
(H-Q)
276
V2
(F-L)
329
K2
(E-L)
B
1D1
C
3K1
D
F3
E
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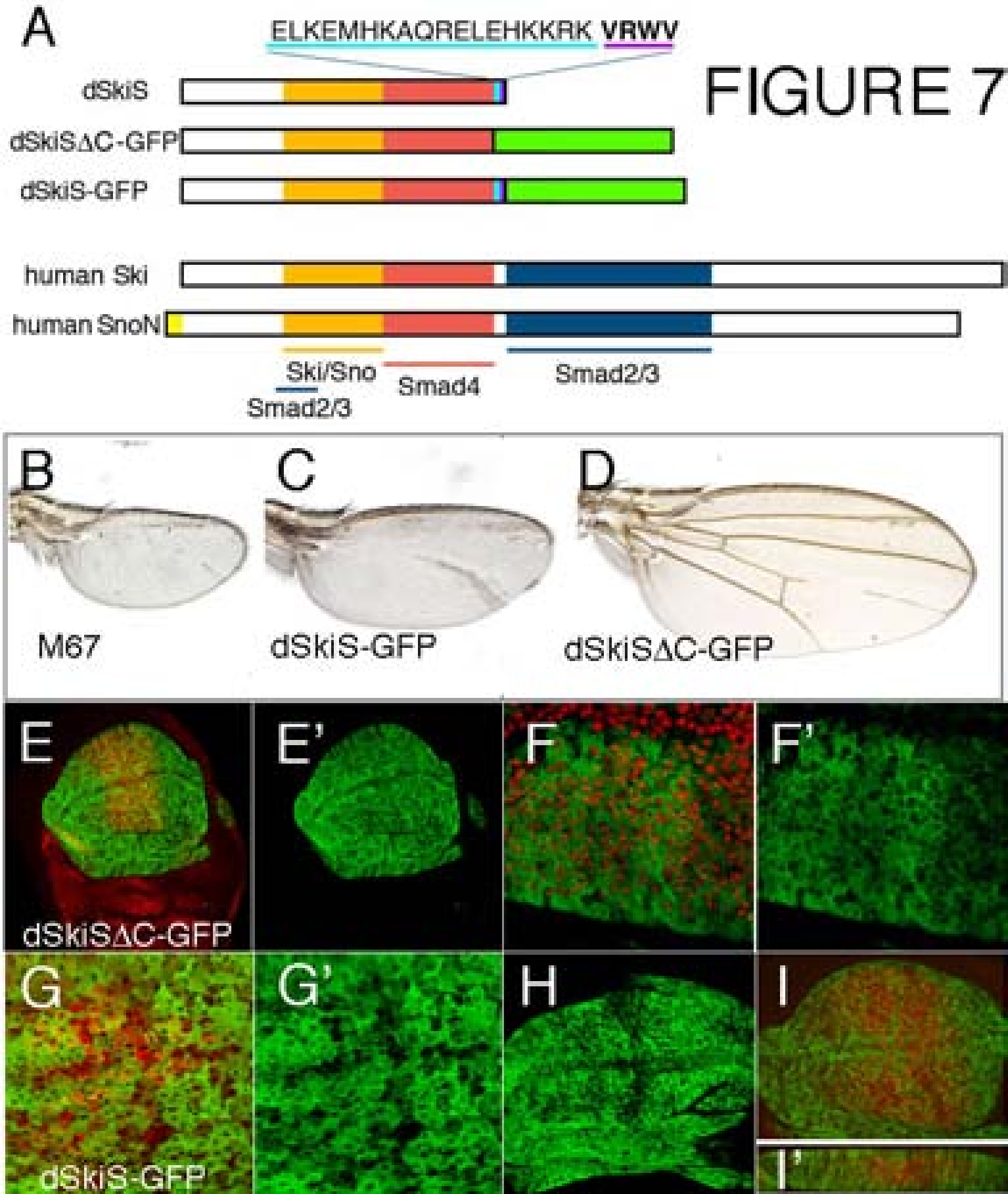
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Smad2/3
Smad4
Smad2/3
Smad2/3
B
C
D
M67
dSkiS-GFP
dSkiSΔC-GFP
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G
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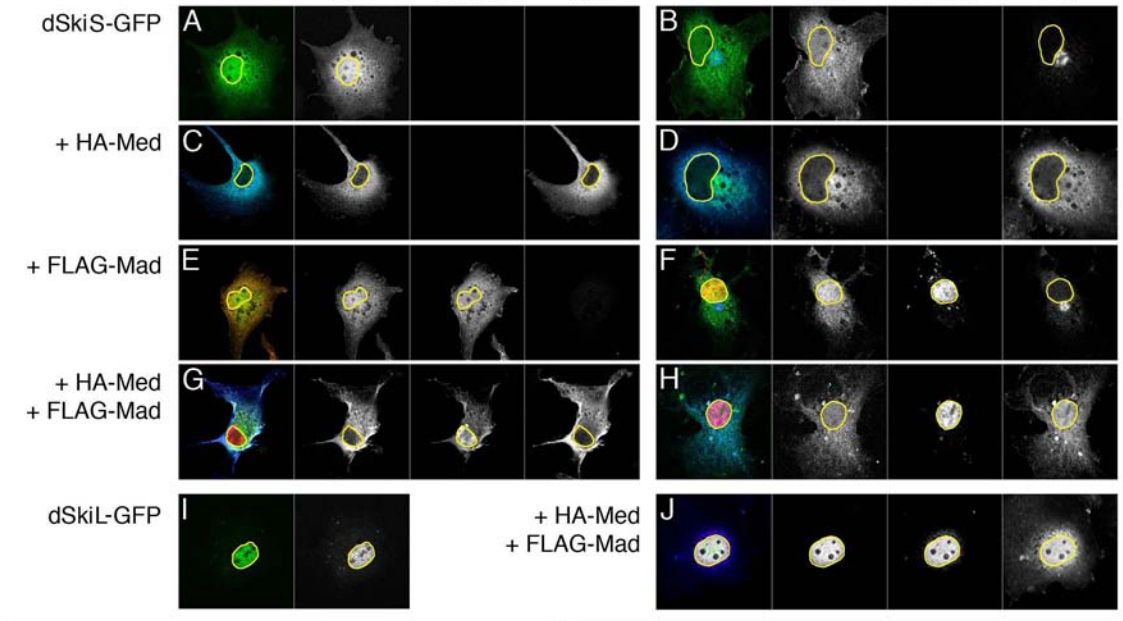
H
I
I'
dSkiS-GFP

FIGURE 7

# FIGURE 8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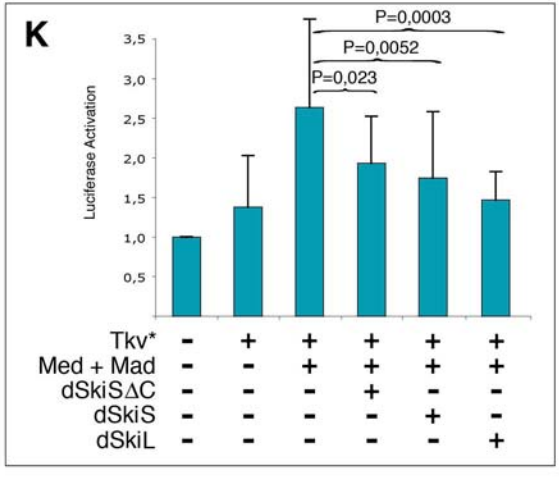
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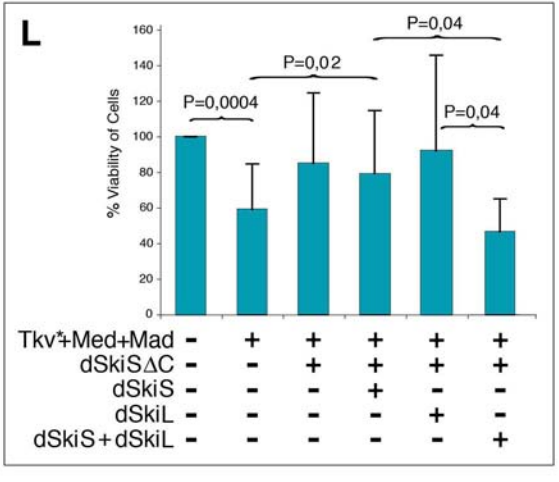
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P=0,02
P=0,04
P=0,04
Tkv*+Med+Mad - + + + + +
dSkiSΔC - - + + + +
dSkiS - - - + - -
dSkiL - - - - + -
dSkiS+dSkiL - - - - - +