**fussel (fuss)** - A Negative Regulator of BMP Signaling in *Drosophila melanogaster*

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Abstract

The TGF-ß/BMP signaling cascades control a wide range of developmental and physiological functions in vertebrates and invertebrates. In *Drosophila melanogaster*, members of this pathway can be divided into a Bone Morphogenic Protein (BMP) and an Activin-ß (Act-ß) branch, where Decapentaplegic (Dpp), a member of the BMP family has been most intensively studied. They differ in ligands, receptors and transmitting proteins, but also share some components, such as the Co-Smad Medea (Med). The essential role of Med is to form a complex with one of the two activating Smads, mothers against decapentaplegic (Mad) or dSmad, and to translocate together to the nucleus where they can function as transcriptional regulators of downstream target genes. This signaling cascade underlies different mechanisms of negative regulation, which can be exerted by inhibitory Smads, such as daughters against decapentaplegic (dad), but also by the Ski-Sno family. In this work we identified and functionally analyzed a new member of the Ski/Sno-family, fussel (fuss), the Drosophila homolog of the human functional suppressing element 15 (fussel-15). fuss codes for two differentially spliced transcripts with a neuronal expression pattern. The proteins are characterized by a Ski-Sno and a SAND homology domain. Overexpression studies and genetic interaction experiments clearly reveal an interaction of fuss with members of the BMP pathway, leading to a strong repression of BMP-signaling. The protein interacts directly with Medea and seems to reprogram the Smad pathway through its influence upon the formation of functional Mad/Medea complexes. This leads amongst others to a repression of downstream target genes of the Dpp pathway, such as *optomotor blind* (omb). Taken together we could show that fuss exerts a pivotal role as an antagonist of BMP signaling in *Drosophila melanogaster*.

Introduction

The TGF-ß/BMP cascades control a wide range of developmental and physiological functions in vertebrates and invertebrates. The enormous array of cellular processes spans events from proliferation, differentiation, cell migration, angiogenesis to mental and physiological functions in vertebrates and invertebrates. These authors contributed equally to this work.

Due to the discovery of a growing number of ligands, the BMP signaling cascades control a wide range of developmental and physiological functions in vertebrates and invertebrates. In *Drosophila melanogaster*, members of this pathway can be divided into a Bone Morphogenic Protein (BMP) and an Activin-ß (Act-ß) branch, where Decapentaplegic (Dpp), a member of the BMP family has been most intensively studied. They differ in ligands, receptors and transmitting proteins, but also share some components, such as the Co-Smad Medea (Med). The essential role of Med is to form a complex with one of the two activating Smads, mothers against decapentaplegic (Mad) or dSmad, and to translocate together to the nucleus where they can function as transcriptional regulators of downstream target genes. This signaling cascade underlies different mechanisms of negative regulation, which can be exerted by inhibitory Smads, such as daughters against decapentaplegic (dad), but also by the Ski-Sno family. In this work we identified and functionally analyzed a new member of the Ski/Sno-family, fussel (fuss), the Drosophila homolog of the human functional suppressing element 15 (fussel-15). fuss codes for two differentially spliced transcripts with a neuronal expression pattern. The proteins are characterized by a Ski-Sno and a SAND homology domain. Overexpression studies and genetic interaction experiments clearly reveal an interaction of fuss with members of the BMP pathway, leading to a strong repression of BMP-signaling. The protein interacts directly with Medea and seems to reprogram the Smad pathway through its influence upon the formation of functional Mad/Medea complexes. This leads amongst others to a repression of downstream target genes of the Dpp pathway, such as *optomotor blind* (omb). Taken together we could show that fuss exerts a pivotal role as an antagonist of BMP signaling in *Drosophila melanogaster*.
Smad2 has been described [21,22]. For mediating downstream activation, R-Smads need to form a complex with the unique Drosophila Co-Smad Medea (Med), in order to exert their task as transcriptional regulator of corresponding target genes [23].

Like in other pathway networks, there is an enormous amount of extracellular and intracellular cross talk. It has been shown, for example, that the activation of Mad can be mediated by interplay of Dpp and Act-ß signaling, resulting in a trimeric complex of Mad/Smox/Medea as an alternative to the signaling through the dimeric complexes Mad/Med and Smox/Med [15].

Besides the above described members of the BMP/Activin-ß cascade, another family of genes, the ski and closely related sno [ski-related novel gene] genes, are also involved in these signaling pathways. They were originally defined as oncogenes by their ability to transform chicken embryo fibroblasts upon overexpression and are widely described as potent negative regulators of the TGF-ß cascade interacting for example with Smad2/3/4 in order to repress a multitude of target genes [24]. Yet their role in the mammalian system is complex. Pro- and/or anti-oncogenic activity of the Ski-Sno family depends on cancer tissues, stages of tumorgenesis, cell lines and the availability of complexes forming co-activators and co-repressors [25,26]. In addition, the Ski complex seems to play an essential role in embryonic development repressing TGF-ß-responsive promoters to a basal level [27,28].

The Drosophila homolog of Sno, dSno, has been described independently by four different groups [4,29,30,31]. Overexpression studies show, that dSno is involved in pathway switching from Dpp to Activin signaling and produces phenotypes reminiscent of loss of Dpp activity. The protein seems to be required during optic lobe development to maintain a proper balance between differentiation and cell proliferation [29]. Interestingly no homolog of the vertebrate Ski has yet been described in Drosophila.

In this study we report the discovery and functional analysis of a novel gene, the Drosophila ortholog of the human functional suppressing element 15 (fussel-15), to which we refer as fussel (fuss). The gene has been identified in an in silico screen as the Drosophila homolog of human fussel-15 [32], a member of the Ski-Sno family in vertebrates. To study the function of Fuss in BMP/Act-ß signaling, we made use of GAL4 induced mis-expression of dSno in embryonic development repressing TGF-ß-responsive promoters to a basal level [27,28].

The Drosophila homolog of Sno, dsno, has been described independently by four different groups [4,29,30,31]. Overexpression studies show that dsno is involved in pathway switching from Dpp to Activin signaling and produces phenotypes reminiscent of loss of Dpp activity. The protein seems to be required during optic lobe development to maintain a proper balance between differentiation and cell proliferation [29]. Interestingly no homolog of the vertebrate Ski has yet been described in Drosophila.

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The Fuss protein itself is characterized by a Ski-Sno homology domain at the N-terminal part, which is present in all members of this proto-oncogene group (Fig. 2A). The domain shares a characteristic feature with members of the Dachshund family which is known as Dac-box, DS or DHD. It is present in worms, flies and mammals, resulting in an integration of Fuss within the Dachshund family (Fig. 2B). The folding pattern of this domain contains a helix turn helix and a beta-alpha-beta turn motif, suggesting a putative DNA binding activity.

The second domain with significant amino acid identity is the SMAD4 binding domain, which shares structural homology with the SAND domain, named after Sp100, AIRE-1, NucP41/75, DEAF-1 (Fig. 2A). It represents an evolutionarily conserved sequence motif found in nuclear proteins, which are involved in chromatin-dependent transcriptional regulation [35]. Interestingly only the N-terminal part of this domain shows high homology among the Ski-Sno members. Within this domain four residues Cys173, Cys176, His188 and His190 coordinate a bound zinc atom, contributing to the structural stability (arrows in Fig. 2C). These residues are a characteristic feature for the zinc binding ability of the Ski-family [36]. Finally, a coiled-coil region with a leucin zipper-like motif can be identified at the C-terminus, indicating a functional oligomerization or protein-protein interaction of Fussel (Fig. 2A; [36]).

Phylogenetic analysis of the protein on the basis of the neighbour joining method elucidates the relationship between the two Drosophila and human Fussel proteins, Drosophila dsnoN, the human and mouse Ski-Sno members and the Dachshund family (Fig. 2B). The vertebrate Ski-Sno family has a significant homology to the human and Drosophila fussel proteins. Their biological function is diverse, ranging from involvement during embryonic development of muscle, central and peripheral nervous systems or respiratory tissue to regulation of growth and differentiation of adult tissues like neural, muscle or hematopoetic cells. This group of proteins is known for its pro-oncogenic function due to their ability to antagonize the growth-inhibitory activity of the TGF-ß/Smad pathway [37,37]. From the phylogenetic tree it becomes obvious, that FussB and FussC form a homophilic group with h-FUSSEL-15 and 18, also known as Ladybird homeobox corepressor 1 (LBXCOR1) and Ladybird homeobox corepressor 1-like protein CORL2, and their homologues in mice, mCORL1 and mCORL2 [32,38–41]. This group has also been described as the CG11693 or Iceskate family [32,42] with fussel as its unique member in Drosophila.

fussel Acts as an Antagonist within the BMP Pathway

As already introduced, the dpp pathway represents a homolog to the vertebrate BMP2/4 cascade and its signaling is accomplished by proteins, which are widely conserved. One member of the Ski-Sno family in Drosophila is dsno, described to exert an inhibitory function within the BMP and Activin-ß signal transduction pathways [29–31]. Hence fussel, due to its structural similarity (see...
Fig. 2A), might also act as a transcriptional regulator within the dpp pathway. Due to the lack of fuss mutants (fourth chromosome localization) we used a reverse genetic approach to characterize the function of fuss. The expression of available fuss RNAi constructs from the National Institute of Genetics in Japan and from the Vienna Drosophila RNAi Center with actin-Gal4 (act-Gal4) or daughterless-Gal4 (da-Gal4) revealed conflicting results with either no phenotype at all or pupal lethality and possible off-target effects. In order to initiate the analysis of the function of Fussel on this pathway, two overexpression lines were established containing either fussC or fussB full length cDNAs. We first overexpressed fuss via A9-Gal4 in the wing, a favored system to study novel components of the dpp cascade, as morphological changes in wing shape, size and vein formation of the adult fly can easily be

Figure 1. Genomic organisation of the fussel locus CG11093 and its transcription pattern. (A) 20 kb of the reverse complemented 102F4 cytological region of chromosome IV and the transcripts fussB (RefSeq NM_001169358.1) and fussC (RefSeq NM_001169359.1) are shown. The position of the Tc1-2 family transposon and the size of the two alternate exons are indicated. In-situ hybridisation of fuss shows expression in (B) two segmental clusters of cells in stage 14 embryos and (C) cells in the mediolateral (arrow) and SE- (arrowhead) and Tv-neuron-region (open arrowheads) in L3 brains.

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discovered. The overexpression of one UAS-fussC copy with A9-Gal4 leads to vein truncations of L2, L5 and p-cv at 100% penetrance (Fig. 3B) and results in a smaller wing size (75% of the control flies; compare Fig. 3A to 3B). Expressing two copies of fussC causes wing size reduction up to 46% and a dramatic loss of vein and intervein tissue (Fig. 3C). Stronger phenotypes become manifested in unfolded wings (data not shown). Likewise the driver of nubbin (nub-Gal4), a gene expressed in the wing pouch cells of the wing disk [43], provokes defects in L2 and L5 which, similar to the overexpression with A9-Gal4, do not reach the distal margin of the adult wing (Fig. 3E). An even more conspicuous phenotype can be monitored, when the fussB form is driven with A9-Gal4. The male flies are lethal in the late pupal stage and the very few female escapers show a strong disruption in wing development. The wings are smaller (27% compared to the driver line) lacking veins and are poorly unfolded (Fig. 3D). This indicates, that the two genes differ in the way they exert their effect as antagonists of the dpp cascade. Furthermore we observe in A9-Gal4;UAS-fussC wings a loss of mechanosensory neurons, the campaniform sensilla located on L3 (Fig. 3G'/G'').

The modulation of vein development described above focused our interest on possible downstream regulating effects of fuss upon the BMP/Activin-ß cascade. The most prominent readouts of dpp signaling are the expression of optomotor blind (omb) and spalt major (salm) along the anterior/posterior (A/P) boundary of the imaginal wing disk [44,45]. Imaginal wing disks of third instar female omb-lacZ; UAS-fussB larvae show a wild type omb expression pattern along the anterior-posterior compartment boundary and in the mediolateral regions of the disk (Fig. 3H'). Yet, when fussB is mis-
expressed using the A9-Gal4 driver in the om8-lacZ background, we can monitor a clear reduction of omb expression within the entire disk of female larvae (n = 15). The center and part of the dorsal wing pouch compartment where A9-Gal4 is expressed shows the strongest decrease of the staining, strengthening the role of fuss as a negative regulator. In addition, the dorsal and ventral wing hinge regions are also affected (Fig. 3H’). Quantitative RT-PCR with third instar larvae confirms our histological observations. The level of omb mRNA is significantly reduced to 25% (Fig. 3J). Furthermore, salm, whose expression in the wing blade is strictly dependent on dpp signaling and which covers a broad central domain from the L2 provein to the anterior limit of the L5 [46], is reduced to 60% (Fig. 3J). This shows that fuss is able to negatively interact with BMP signaling in vivo and is consistent with its proposed role as an inhibitor. We also tested a known readout of the Activin pathway, namely the expression of the Ecdyson Receptor 1b (Er1b; [47]). In contrast to the clear downregulation of dpp signaling transcription targets omb and salm, we did not observe any significant changes of Er1b relative expression (Fig. 3J).

To further assay the modulating effects of fussel on BMP signaling and vein patterning we examined the expression of the Drosophila Serum Response Factor (DSRF) in third instar larval imaginal disks. The expression of DSRF protein is restricted to the wing pouch and the hinge region, representing the future intervein tissue and is repressed by dpp signaling [48]. Controls show that the DSRF protein is absent from the future wing margin and the area of the prospective wing veins (Fig. 3I). A9-Gal4;UAS-fussB larval disks reveal irregular DSRF expression with almost complete absence of the protein in the notably reduced dorsal part and a highly irregular pattern in the ventral part lacking clear vein primordia regions (Fig. 3I’).

As intracellular signaling of BMP in Drosophila is accomplished through the vertebrate R-Smad homolog Mad, we investigated if Fuss affects the phosphorylation or localization of Mad in the third instar wing disk. PMad concentration is a direct measure of the Dpp signaling activity [49,50] and can be assayed by use of an anti-phospho-SMAD1/5 antibody, which detects endogenous PMad in two prominent stripes (Fig. 3K”) [51,52]. When overexpressing fussel with A9-Gal4 the PMad staining persists, but clearly deviates from the sharp borders of activated Mad observed in control imaginal disks (Fig. 3K”). This indicates that Fuss does not repress Mad activation, but rather leads to a reallocation of Mad within the wing disk. Moreover, having a closer look at the morphology of A9-Gal4;UAS-fussB larval wing disks, a notable reduction of the dorsal compartment becomes obvious (Fig. 3H’, I’, K”). In consequence, despite the almost normal larval wing disk size (Fig. 3H”), the abnormal adult wing observed in fig. 3C and D can now be explained, by taking into account that the vein-to-intervein ratio is also affected during metamorphosis.

Genetic Interaction of fussel with Members of the BMP Pathway

Due to the specific effects of fuss on dpp dependent target genes we performed genetic interaction experiments in the wing with wild type, constitutively active or dominant negative forms of various members of the BMP/Activin-B pathways. Saxophone, a type I serine-threonine receptor of the pathway [12] mediates signaling from Dpp and Gbb for patterning the wing imaginal disk (reviewed by O’Connor et al. 2006 [5]). Constitutively active Saxophone (SaxA) functions independently from endogenous signals [53] and its overexpression with A9-Gal4 leads to extra vein material between the region of L3 and L5 with slight wing blistering, also affecting the p-cv pattern (Fig. 4A). Interestingly, introducing one copy of UAS-fussC in these flies, the phenotype is almost completely rescued and the vein formation is close to wild type (Fig. 4B), suggesting a suppressive effect of fuss on the BMP pathway. In the case of Mad, an overexpression with A9-Gal4 leads to a dramatic reduction in wing size, ectopic vein tissue and blistering (Fig. 4C). Coexpression of Fuss restores not only the size of the wing, but leads also to a notable amelioration of the vein-, respectively intervein patterning (Fig. 4D). Similar to Mad, Fuss is able to restore wing shape and vein patterning of Mad overexpression to an almost wild type mode, with only slight failure of complete outgrowth of L5 and p-cv, reminiscent of the fussel phenotype (Fig. 4E, F). These data reveal that in the wing, fuss is able to interfere negatively with the BMP activity gradient. Mad is known to form a complex together with Med in order to actively translocate to the nucleus and transduce dpp signaling [54]. To this point we can not conclude if the interaction is based on a complex formation of Fuss with either Med or Mad, or if the effects we observe in the wing are due to titration effects, where Fuss for example sequesters Med, inhibiting the Mad/Med complex to perform its transcriptional functions.

For the analysis of interactions with the Activin-B cascade, we also investigated the coexpression of fussel with baboon, the type I receptor [14] and its associated mediator Sma2d. Neither the three different baboon isoforms nor two copies of Sma2d show any vein defects by themselves when overexpressed with A9-Gal4 [15]. Therefore it is not surprising that additional coexpression of fussel leads merely to a modest fuss phenotype, resulting in a slight truncation of L5 and a minor reduction in wing size but no signs of interaction (data not shown). Expression of a constitutively active form of baboon (UAS-babo) leads to tissue overgrowth in the wing and patterning defects in the veins [22]. However, in contrast to SaxA we could not observe any modification of this phenotype after co-overexpressing fussel in the wing (data not shown), which leads us to the conclusion, that fussel does not directly interact with the
Activin-β pathway. This confirms our data from the quantitative RT-PCR experiments, where we could not monitor any changes of \textit{EcR1b} expression (Fig. 3J), a readout of the Activin-β pathway. Taken together, our results indicate that \textit{fuss} exhibits its function as a negative regulator of the BMP cascade, most likely through interaction with the activating Co-Smad Medea.

Nuclear Translocation of Fussel through Medea

The presence of a SMAD binding domain in the Fuss Protein and the results we obtained from the genetic interactions provoked us to further study the interaction between Fuss and Med, respectively Mad, on a molecular level by a direct yeast two hybrid experiment. The \textit{fuss} cDNA was cloned in frame with the GAL4DB of the bait vector pDBLeu, \textit{mad} and \textit{med} were inserted downstream of the pPC86 GAL4 activation domain. The direct interaction of \textit{fussel} with \textit{med} could be clearly monitored through growth tests on the accordant selective media in four independent sets of experiments (Fig. 5A) and does not result from self activation of \textit{fuss} with the bait vector (5A: pdbLeu-\textit{fussC}+pPC86). However, we could not verify an interaction of Fuss with Mad on an in vitro level (data not shown). In addition we performed co-immunoprecipitations (CoIPs), for which HA and FLAG tagged \textit{fuss}, \textit{mad} and \textit{med} cDNAs were co-expressed in \textit{Drosophila} S2 cells. Analysis of the cell lysates showed that Fuss and Med do bind to each other whereas Fuss and Mad do not. To rule out effects that could result from the protein fusions with the HA/FLAG epitopes, we repeated the experiment with exchanged tags and obtained the same result (Fig. 5B). Taken together, these observations indicate that Fuss specifically interacts with Med to exert its inhibitory functions.

However, what is the cellular consequence of a Fuss/Med interaction and what are the possible reasons for a downregulation of Dpp target genes? On the one hand there is the possibility of Fuss/Med heterodimer degradation in the cytoplasm, on the other hand, the heterodimer could translocate to the nucleus and act as a transcription-complex altering gene expression. To gain insight into the molecular processes initiated through the binding of Fuss to Med, we decided to study the subcellular localization of Fuss. First we established a \textit{fuss}-eGFP line. The 105 kDa protein carries the GFP tag at the C-Terminus and is fully functional: overexpression with actin-Gal4 is lethal in pupae and the wing phenotype with A9-Gal4 can even be considered as an enhancement to the one we observe with the \textit{fuss} cDNA, namely a stronger reduction in wing size to 62% of wild type and a more severe vein loss of L2, L5 and p-cv (Fig. 5C). This might be a result of the GFP fusion which could lead to an increase of the Fuss protein stability. Confocal analysis of GFP staining in third instar wing discs shows an almost exclusive localization of the protein in the cytoplasm, becoming most apparent dorsal to the wing blade margin (Fig. 5D–D’). However, when \textit{fuss}-GFP and Med are co-

Figure 4. Genetic interaction of fussel with members of the BMP pathway. (A) A9-Gal4/y; UAS-saxA. Overexpression of constitutively active \textit{sax} causes growth of additional vein material between L3–L5 (B) A9-Gal4/y; UAS-saxA; UAS-fussC. Coexpression of \textit{fussC} ameliorates vein overgrowth. (C) A9-Gal4/y; UAS-mad. Overexpression of \textit{mad} transforms most of the intervein tissue into vein tissue and eventually results in a blistered wing. (D) A9-Gal4/y; UAS-mad; UAS-fussC. Coexpression of \textit{fussC} ameliorates blistering and considerably improves vein patterning. (E) A9-Gal4/y; UAS-med. Overexpression of med causes distinct overgrowth and duplication of wing veins. (F) A9-Gal4/y; UAS-med; UAS-fussC. Coexpression of \textit{fussC} rescues the vein overgrowth and restores the \textit{fussC}-phenotype.

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Figure 5. Formation of a Fuss/Med protein complex and its translocation into the nucleus. (A) Yeast Two Hybrid experiment showing physical interaction of Fuss and Med: pPC97-Fos + pPC86-Jun: interaction control; pCL1 + pPC86: Gal4- growth control and empty prey vector; pdbLeu-Fuss + pPC86: negative control; pdbLeu-Fuss + pPC86-Med: positive interaction of Fuss and Med. (B) Coimmunoprecipitations: in lysates from S2-cells

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fussel as a Negative Regulator of BMB Signaling

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expressed with A9-Gal4, we detect a noticeable displacement of the GFP signal to the nucleus (Fig. 5E–E’’). As already mentioned, the R-Smad Mad usually forms a complex with Med in order to fulfill its role as a transcriptional regulator in the nucleus [23]. Knowing that Med influences the localization of Fuss, we wanted to study possible effects of Fuss on Mad localization, too. We analyzed the subcellular localization of pMad in the dorsal compartment of L3 wing disks. In control disks pMad accumulates in a sharp stripe of cell nuclei (Fig. 5F–F’’). Although the overall distribution of pMad in A9-Gal4; UAS-fussB disks is changed, there is no noticeable difference of the subcellular pMad localization (Fig. 5G–G’’).

All together, these results indicate that Fuss is able to bind to Med in the cytosol and to translocate into the nucleus, either as a Fuss/Med complex, or as part of a Mad/Medea complex.

Discussion

In this report we have characterized a new gene in Drosophila melanogaster, fussel (fuss), an ortholog of the human functional Smad suppressing element 15 (fusel-15), also known as SKOR1, Coril or LBXcor1 (UniProtKB ID: P84550). Fuss is characterized by a Ski-Sno proteins and a SAND homology domain and can be classified as a proto-oncogene. The two transcripts, fussB and fussC, diverge in the N-terminus and represent two phylogenetically different versions of the gene: fussB is the original form of the CG11093 locus, whereas the fussC transcript is spliced differently due to the subsequent integration of a Tcl-2 transposon (Fig. 1A). The ubiquitous mis-expression of both forms is lethal in pupal stages. Its endogenous expression pattern during embryogenesis and larval development is neuronal, which is similar to the vertebrate genes fussel-15 and fussel-18 which also show a restricted pattern of expression mainly limited to neuronal tissue such as the developing murine cerebellum and the spinal cord [32,38,41].

As Ski-Sno proteins are described to repress TGF-β signaling through their interaction with Smad proteins [55,56] we investigated if fuss is able to inhibit the TGF-β/BMP cascade, which is represented in Drosophila by BMP/Activin-β signaling. We made use of the wing blade and the adult wing, an amenable and widely used tissue to analyse function and crosstalk of members within this signaling pathway. The ectopic expression of fuss in the wing affects the overall vein structure (Fig. 3B/C). While fussC produces defects in L2, L5 and p-cv and results in a loss of the campaniform sensilla on L3, the fussB isoform overexpression leads to a complete wing disruption or poorly unfolded extremities in the few female escapers which hatch. Together these phenotypes are highly reminiscent of loss of function phenotypes within the dpp pathway and provoked us to investigate if and how fuss is able to interact negatively within this cascade. While the contribution of BMP signaling to Drosophila development is enormous, including cell-fate specification, imaginal disk patterning or growth organization, the Activin-β branch has only been elucidated recently. It could be shown, that its components regulate neuronal wiring and proliferation, mushroom body remodeling and the morphogenesis of neurons in the adult [47,57]. We were wondering if we can decipher the pathway affected by fuss overexpression and examined the expression pattern of prominent read-outs, namely omβ and sal for BMP signaling and Erib for the Activin-β cascade. Our results show a clear reduction of omβ and sal on a histological and also molecular level, which leads us to the conclusion, that fuss is indeed an inhibitor of the BMP pathway. Moreover, the coexpression of dpp-cascade activators like the typel receptor saxophone or the Smads mad and medea with fuss results in a clear rescue of vein patterning and wing size. In the case of medea we observed an almost complete rescue of the A9-Gal4; UAS-mad wing phenotype and postulated a direct interaction of fuss with medea via its SMAD4 binding domain (Fig. 2A), like it has been described for dSno [29] or c-ski [55]. In contrast to the BMP pathway, no effects could be observed on the expression of one of the main target genes of the Activin-β pathway, erb1. This result was further supported by a failure to detect genetic interactions of fuss with members of the Activin-β branch, which further supports a specific inhibitory function of fuss on the BMP pathway.

To further strengthen our hypothesis of a specific interaction of Fuss with Medea, the direct interaction of these two proteins was identified by a yeast two hybrid experiment and confirmed by CoIP in Drosophila cell culture. Interestingly we could not detect an interaction between Fuss and Mad in vitro, although the genetic interaction of Fuss with Mad revealed a partial rescue of the wing phenotype. The fact that pMad concentration in wing disks is not reduced in the presence of Fuss clearly indicates, that Fuss function is downstream of R-Smad activation. One possibility could be that Fuss is able to titrate out pMad/Med or possibly forms a trinary complex with pMad/Med affecting BMP target gene expression like omβ.

Is there a functional difference between dSno and fuss, both belonging to the ski family? Although dSno exerts its effects also through med, it is supposed to act as a BMP-to-Activin-β pathway switch, at least in brain development. By forming a complex with dsMAD2 it directs differentiation of neuroblasts towards proliferation [29,31], a role we can not ascertain for fuss. However, we can detect a clear difference in vein patterning defects comparing dSno and fuss overexpression using an identical driver line (A9-Gal4) further supporting an individual and different inhibitory effect of the closely related ski/sno/fuss proteins.

The interaction of fuss with med and the subsequent inhibitory effects on BMP signaling, led us to the assumption, that the subcellular localization of Fuss might undergo changes during dpp activation transmitted by med overexpression. In general, the subcellular localization of homologous proteins such as Ski and SnoN is variable and depends on several conditions, such as morphological differentiation of cells or activity of proteins in normal versus tumor tissues; for example SnoN localization in nontumorigenic cells is preferentially cytoplasmic, while in tumor cells it is constitutively nuclear [58]. Our GFP tagged Fuss protein is predominantly localized in the cytoplasm, when it is overexpressed by itself. Here it might sequester Med and prevent its nuclear translocation in response to dpp signaling or another yet unidentified factor. However, overexpression of med together with fuss leads to a clear relocation from the cytoplasm into the nucleus. Fuss thereby antagonizes the BMP cascade, which is
overstimulated by excessive med signaling leading to an almost complete rescue of the med overexpression wing phenotype. Interestingly, we observed that pMad still translocates into the nucleus upon *fuss* overexpression. Considering the genetic interaction results, it is very likely that pMad/Med/Fuss enter the nucleus already as a trimmer, which then might lead to a change in DNA binding or regulatory capabilities of the Smad complex.

Our results also need to be discussed in respect to recent data on the mammalian Fussel genes, in particular the isolation and characterization of a transposon induced mouse null allele of Fussel-18 (Skorl-2) [41]. Interestingly one prominent signaling phenotype in these mice is a strong repression of sonic hedgehog (Shh) signaling. In particular the authors could show that Fussel-18 is able to bind R-Smads and Co-Smads leading to a specific reduction of BMP- but not TGF-β-signaling. This nicely fits our genetic interaction results, which show that both Mad and Med interact with Fuss, although we could only show physical protein interaction for Mad and not for Med. The Shh repression effect in the mouse mutant can be explained through a repressing function of the BMP signaling on Shh, which in a wildtype background, is repressed by Fussel-18 itself, reestablishing Shh expression [41].

The exact mechanism through which *fuss* exerts its endogenous function remains to be elucidated. As mentioned before, the protein exhibits a DHD motif, known to be responsible for DNA binding. Although a direct interaction with DNA could neither be shown for the human Ski-Sno proteins, nor for *Drosophila* SNO [29,37,59] we can not rule out the possibility that *fuss* translocates to the nucleus (with or without med) and binds itself to DNA. Yet we rather propose an association of Fuss with other protein partners, such as the corepressors Smrter or dSin3A or that it forms a complex with the histone deacetylase Rpd3, such an association of Fussel-18 with HDAC1 has been described in the mouse [41]. It is also possible that *fuss* displaces coactivators, such as the *Drosophila* homolog of the p300/CBP complex called *nejire* [60–62] or stabilizes inactive Smad complexes [55].

Further investigations will elucidate the endogenous role of *fuss* during *Drosophila* development and the processes by which it antagonizes BMP signaling.

**Materials and Methods**

**Fly Strains and Drosophila Genetics**

Flies were kept under standard conditions. All Gal4-lines, except of *sub-Gal4* (J.F. deCelis, Madrid), were obtained from the Bloomington Stock Center, the *UAS-fuss-C-IR* lines (transformant IDs: 11093R-1 and 11093R-3) were obtained from the National Institute of Genetics, Japan and the Vienna Drosophila RNAi Center (transformant ID 15478). Other UAS-constructs used were: *UAS-SaxA* [52], *UAS-Med* [63], *UAS-Mad* [64], and *UAS-babo* [65]. To create the *fuss* overexpression constructs, the full-length *fuss*-cDNA was cloned from the *Drosophila* Genome Project (IP13014), excised from pOT2 and directly cloned into pUAST using the BglII and XbaI restriction sites. The 5′-region of *fuss*-cDNA was isolated from CantonS flies by RT-PCR using 5′-ATGGATTTAAATGAAAATTTTAAAA-3′ and 5′-CTTATTGGACTCCGCCAC-3′, *fuss*: 5′-AGTTGGAGTAACGGGAGGTAG-3′ and 5′-TTGGTTAAGGGCTGATATA-3′, *salm*: 5′-GCCGCGTTTGTGATCTTAC-3′, *mad*: 5′-GGGCGAATCTTGATGATAT-3′, *med*: 5′-GCCGCGTTTGTGATCTTAC-3′ and 5′-GGGCGAATCTTGATGATAT-3′, *ecr*: 5′-GCCGCGTTTGTGATCTTAC-3′ and 5′-GCCGCGTTTGTGATCTTAC-3′, *rp49*: 5′-GAGTTGGAGTAACGGGAGGTAG-3′ and 5′-CTTATTGGACTCCGCCAC-3′. All *fuss*- and *med* cDNAs were amplified and 5′-BamHI or 5′-BglII or 5′-BamHI and XbaI restriction sites. The 5′- region of *fuss*-cDNA was isolated from CantonS flies by RT-PCR using 5′-ATGGATTTAAATGAAAATTTTAAAA-3′/5′-ACCGGAAAGTCGTCAGGTAG-3′ primers and T/A- cloning in pGEM-T Easy, the coding sequences were transferred into the expression vectors pSR-2 (Roche, Germany) and transferred at 90% confluency in 6-well plates using FuGeneHD transfection reagent (Roche, Switzerland). Cells were harvested after 24 hours of incubation with 1 mM CuSO4, washed with PBS and lysed in ES2 cell lysis buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 2.5 mM EDTA, 5 mM DTT, 0.05% Triton X-100, 5% Glycerol) supplemented with 50 mM EDTA, 50 mM DTT, 0.05% Triton X-100, 5% Glycerol) supplemented with Complete Mini protease inhibitor cocktail (Roche, Switzerland). After removal of the cell debris and pre-clearing with Protein-G Sepharose (Sigma-Aldrich, MO) the lysates were incubated for two hours with anti-Flag M2 antibody at 4°C (Sigma-Aldrich, MO). To precipitate the complexes, Protein-G Sepharose was added and the mixture was incubated for another two hours at 4°C. After four washes, the precipitates were resuspended in ES2 supplemented with the

**Expression Analysis**

cDNA was produced by extraction of RNA from one hundred embryos, ten L3-larvae, ten prepupae, ten pupae or ten adult CantonS flies using *ployGOLD* TriFast (Peqlab, Germany) and reverse transcription with QuantiTect Reverse Transcription Kit (Qiagen, Germany). Primers used for the confirmation of the predicted 5′-ends of the transcripts by PCR were 5′-ATGGCACTGTTCCGCAGAT-3′/5′-AACGCAAAGTCGTCAGGTAG-3′ for *fuss*, and 5′-ATGGATTAAATTAAAGAAAAATTTAAA-3′/5′-AACGCAAAGTCGTCAGGTAG-3′ for *fuss*. For the confirmation of the 3′- splice sites, primers were 5′-ACAGGTTCCTTCCCTCA-3′/5′-CTACTTCCGTGTCGTCATCT-3′ spanning the intron between exon 5 and 4 in *fussB/fussC* and 5′-GACGGAAAGTATGAGACAT-3′/5′-CTATTGGAGACGTCCGAC-3′ spanning the intron between exon 4 and 5 in *fussB/fussC*.

**Real Time PCR**

cDNA samples from three different individual crosses were tested in the Lightcycler- System (Roche, Switzerland) using the *Quantitex* SYBR Green RT- PCR- Kit (Qiagen, Germany). For relative quantification, we applied the delta- delta CT algorithm. Primer pairs used for the experiments were: *rp49*: 5′-GCGGTTGGCGTTTGGTAC-3′ and 5′-CCAG- GACCTTCTCCGACCGG-3′, *fuss*: 5′-AGTTGGAGTAACGGGAGGTAG-3′ and 5′-TTGGTTAAGGGCTGATATA-3′, *salm*: 5′-ACTGGGACCGAATGTG-3′ and 5′-GCGGGAATCTTGATGATAT-3′, *med*: 5′-GCCGCGTTTGTGATCTTAC-3′ and 5′-GCCGCGTTTGTGATCTTAC-3′, *mad*: 5′-GCCGCGTTTGTGATCTTAC-3′ and 5′-GCCGCGTTTGTGATCTTAC-3′. For the confirmation of the 3′- splice sites, primers were 5′-ACAGGTTCCTTCCCTCA-3′/5′-CTACTTCCGTGTCGTCATCT-3′ spanning the intron between exon 5 and 4 in *fussB/fussC* and 5′-GACGGAAAGTATGAGACAT-3′/5′-CTATTGGAGACGTCCGAC-3′ spanning the intron between exon 4 and 5 in *fussB/fussC*.

**Yeast Two-Hybrid**

The Yeast two hybrid tests were performed as described in the instruction manual from PROQUEST (Life Technologies, CA) using the full-length *fussC* cDNA cloned into pDBLeu. For the direct two-hybrid tests with *medea* and *med*, the full length cDNAs LD22279 and RE72705 were cloned into pPC86.
protease inhibitor cocktail. The cell lysates and precipitates were analysed by standard SDS-PAGE followed by western blotting. The blots were blocked and incubated overnight with anti-FLAG M2 or anti-HA 12CA5 (Abcam, UK) primary antibodies and Alexa Fluor 680 goat anti-mouse A-21057 (Life Technologies, CA) secondary antibody. Signals were detected using an Odyssey infrared imaging system (Li-Cor, NE).

**Analysis of Wing-venation and Size**

Wings were mounted on microscope slides in DePeX and fixed under a glass cover slide. Digital pictures were taken on an Axioshot Microscope (Zeiss, Germany) at a resolution of 1560 x 1024 Pixels. Overall wing size was measured in ImageJ 1.44e using the ‘Huang’ thresholding algorithm to create binary pictures. The function ‘analyse particles’ with activated ‘exclude holes’-option was used to measure the size of the wing in pixels.

**RNA in situ Hybridisation, X-Gal-staining and Immunohistochemistry**

RNA in situ hybridization on embryos and third instar larval brains were carried out according to Tautz and Pfeifle (1989) \[66\]. The **fuss** sense and anti-sense digoxigenin-labeled RNA probes were prepared with T7 and SP6 RNA polymerases using the **fuss** cDNA as template with the following primers: 5' TATCAGCA-GAGGAAATTGCAAAGGATTAAGGC-3' (for) and 5' CATCG-TAATCATTTCCACTCAGAGAC-3' (rev). For signal detection, alkaline phosphatase-conjugated anti-digoxigenin antibody and NBT/BCIP stock solutions (Roche, Switzerland) were used. For the X-Gal stainings, larvae were dissected in ice cold PBS and fixed with 1% GuHtaraldehyde. The tissue was incubated for 2 hours at 37°C with 0.4% X-Gal in staining solution (10 mM Phosphate Buffer pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 3 mM tetrapotassium hexacyanoferrate, 3 mM tripotassium hexacyanoferrate, 0.3% Triton X-100), washed in PBS and incubated at 4°C in 70% glycerine/PBS overnight before mounting in glycerol. For immunohistochemistry, L3 wing disks were dissected in PBS and fixed for 1 hour in 4% paraformaldehyde in PBS (PBS containing 0.1% Triton X-100). Tissues were washed five times in PBS and blocked 2 h with 10% serum in PBS. Primary antibody incubation was carried out over night at 4°C in blocking solution, followed by five washes in PBS and 4 h incubation with the secondary antibody mounted at room temperature in blocking solution. After five washes in PBS, disks were mounted in VectaShield (Vector Labs, CA) or glycerol. Primary antibodies anti-dSRE 39093 (Active Motif, CA), anti-phospho-SMAD1/5 #9516 (Cell Signaling, MA) anti-GFP A-6435 (Invitrogen, CA) and anti-histone ga199 (A. Hofbauer, Regensburg, Germany) were used at 1:250 dilutions. Secondary goat anti-mouse-AF4368 A-11031, goat anti-rabbit-AF460 A-11034, goat-anti-mouse-AF647 A-21236 (Invitrogen, CA) and biotinylated goat anti-mouse BA-9200 (Vector Labs, CA) were used at 1:300 dilutions. The tissue was analysed on a LSM 510 META confocal microscope or an Axioshot Microscope (Zeiss, Germany).

**Bioinformatics**

For the identification of proteins similar to FussB and FussC the UniProtKB Database (http://www.uniprot.org) was analyzed using BLAST. Protein sequences with significant sequence similarity to FussB or FussC were aligned in Clustal-X 2.0 \[67\]. The unrooted phylogenetic tree and the estimate significance of branch points were calculated in MEGA4 \[68\] using the neighbour-joining method with bootstrap resampling. Coils 2.2 \[69\] was used for the identification of coiled coil motifs.

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**Author Contributions**

Conceived and designed the experiments: AKB SS. Performed the experiments: SF EH RR SA. Analyzed the data: SF AKB SS. Contributed reagents/materials/analysis tools: SA. Wrote the paper: SF BB SS.

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