The *Drosophila* functional Smad suppressing element *fuss*, a homologue of the human *Skor* genes, retains pro-oncogenic properties of the Ski/Sno family

Mathias Rass*, Laura Gizler, Florian Bayersdorfer, Christoph Irlbeck, Matthias Schramm, Stephan Schneuwly

Department of Developmental Biology, Institute of Zoology, University of Regensburg, Regensburg, Germany

*These authors contributed equally to this work.

**mathias.rass@ur.de**

Abstract

Over the years Ski and Sno have been found to be involved in cancer progression e.g. in oesophageal squamous cell carcinoma, melanoma, oestrogen receptor-positive breast carcinoma, colorectal carcinoma, and leukaemia. Often, their prooncogenic features have been linked to their ability of inhibiting the anti-proliferative action of TGF-ß signalling. Recently, not only pro-oncogenic but also anti-oncogenic functions of Ski/Sno proteins have been revealed. Besides Ski and Sno, which are ubiquitously expressed other members of Ski/Sno proteins exist which show highly specific neuronal expression, the SKI Family Transcriptional Corepressors (Skor). Among others Skor1 and Skor2 are involved in the development of Purkinje neurons and a mutation of Skor1 has been found to be associated with restless legs syndrome. But neither Skor1 nor Skor2 have been reported to be involved in cancer progression. Using overexpression studies in the *Drosophila* eye imaginal disc, we analysed if the *Drosophila* Skor homologue Fuss has retained the potential to inhibit differentiation and induce increased proliferation. Fuss expressed in cells posterior to the morphogenetic furrow, impairs photoreceptor axon pathfinding and inhibits differentiation of accessory cells. However, if its expression is induced prior to eye differentiation, Fuss might inhibit the differentiating function of Dpp signalling and might maintain proliferative action of Wg signalling, which is reminiscent of the Ski/Sno protein function in cancer.

Introduction

Transforming growth factor beta (TGF-ß) signalling is involved in a wide range of processes during development e.g. cell adhesion, bone morphogenesis and cell motility [1]. Upon binding of a ligand of the TGF-ß superfamily to a Type II receptor, the Type II receptor recruits a Type I receptor and activates the Type I receptor by phosphorylation. Then, the Type I receptor phosphorylates receptor regulated smads (R-Smads), which then can bind to the common mediator Smad SMAD4 and translocate as a R-Smad/Smad4 complex into the nucleus, where
these complexes interact with co-activators to activate gene expression [1]. Negative regulators of the TGF-β signalling pathway are inhibitory Smads (I-Smads), Smurfs and the Ski/Sno protein family [2–4]. Proteins of the latter group possess two structural domains: the Ski/Sno homology domain and the SMAD4-binding domain [5, 6]. With the help of these domains, Ski/Sno proteins can interact, among others, with R-Smads, N-CoR, Sin3a, SMAD4 and the histone deacetylase HDAC1 and this complex leads to transcriptional repression of target genes [7–11]. By their expression domains, Ski/Sno proteins can be further subdivided into ubiquitously expressed genes (human Ski and Sno), and mainly neurally expressed genes, the SKI Family Transcriptional Corepressors (Skor1 and Skor2 [8–11]). The Ski/Sno proteins fulfil a wide range of different physiological functions such as axonal morphogenesis [12], Purkinje cell development [13], myogenesis [14] and mammary gland alveogenesis [15].

However, the Ski/Sno proteins were not discovered by their physiological functions but via the transforming capability of the viral ski (v-ski) homologue found in the Sloan-Kettering virus [16]. The first evidence that Ski/Sno proteins possess oncogenic capabilities came from overexpression experiments, where it was shown that not the truncation of v-ski is responsible for the transformation of chicken embryo fibroblasts, but that overexpression of v-ski, Ski or Sno is sufficient for this transformation [17]. Despite this background, their role in carcinogenesis is still not fully understood, if not even contradictory at times. Ski and Sno have been found to be upregulated in different types of cancer e.g. oesophagus squamous cell carcinoma [18], melanoma [19], and colorectal cancer [20]. Further evidence for a pro-oncogenic role was found in downregulation analyses of Sno or Ski. This downregulation resulted in decreased tumour growth in breast cancer cells [21] and pancreatic cancer cells [22]. But as stated before, there is some objection that Ski and Sno function purely as oncogenes. Mice, which were heterozygous mutant for Ski or Sno, showed an increased level of tumour induction after carcinogen treatment [23, 24]. In metastatic non-small cell lung cancer, Ski expression is significantly reduced, whereas increased expression of Ski in these cells reduced the invasiveness inhibiting epithelial–mesenchymal transition [25]. Therefore, this could reflect that the outcome of Ski or Sno expression in cancer cells is dependent on the cell type or the actual status of the cancer cells and cancer cells often exploit Ski or Sno to inhibit the anti-proliferative effects of TGF-β signalling. Whereas Ski or Sno have been found to be involved in a lot of different cancer types, there is sparse evidence for deregulation of Skor proteins in cancer cells. Endogenously, Skor proteins have been linked to neurodevelopmental processes. After Skor1 overexpression, genes involved in axonal guidance or post-synapse assembly were differentially expressed [26]. Skor2 is important for cerebellar Purkinje cell differentiation as in Skor2 knockout mice dendrite formation of Purkinje cells was impaired [9, 13]. Pathophysiologically, Skor1 has mainly been linked to restless leg syndrome [26] and localized scleroderma [27].

In Drosophila melanogaster, only one homologue of Ski and Sno, which is designated Snoo [28], and one homologue of Skor1 and Skor2, which is designated Fuss, exist [4, 29]. We have recently shown that Fuss is interacting with SMAD4 [4] and HDAC1 [30]. In overexpression assays, Fuss can inhibit Dpp signalling [4, 29] and endogenously, the Fuss/HDAC1 complex is required for bitter gustatory neuron differentiation [30] and fuss mutant flies pause more often during walking [31]. However, we were interested if the Skor/Fuss proteins retained their ability to inhibit differentiation and induce increased proliferation. For this purpose, we overexpressed Fuss in differentiating cells of the eye imaginal disc, an excellent model tissue to study regulatory gene function in the context of carcinogenesis [32–34]. This overexpression impaired photoreceptor axon guidance and inhibited the differentiation of accessory cells such as cone cells and primary pigment cells, which are all transformed into a basal pigment cell type. In a second approach we generated fuss overexpressing clones early during development
in the eye imaginal discs, when cells are still proliferating. This resulted in vast outgrowths of undifferentiated tissue of the eye imaginal disc because fuss overexpression most likely inhibited Dpp-signalling, a member of the TGF-ß superfamily. Our work shows that Fuss retained the ability of Ski/Sno proteins to inhibit the antiproliferative effects of TGF-ß signalling by analogous inhibition of Dpp-signalling, allowing proliferation to be sustained.

Results

fuss overexpression leads to a smooth eye surface and impairs axonal pathfinding

As Ski/Sno proteins have been found to be involved in cancer development and progression, we were interested if fuss overexpression can also inhibit differentiation and induce increased proliferation and hyperplasia, respectively. To answer this question, we chose the eye imaginal disc as a model tissue, because fuss is not endogenously expressed in the eye imaginal disc [30]. Furthermore, Fuss and its homologues are negative regulators of BMP/Dpp signalling in overexpression assays and consequently the eye imaginal disc enables us to investigate fuss overexpression in a Dpp independent and dependent context. First, we overexpressed fuss via the GMR-GAL4 driver line. GMR-GAL4 is active posterior to the morphogenetic furrow [35], which is the source of the Dpp morphogen during eye development [36] and thus Fuss cannot directly interfere with Dpp signalling.

Interestingly, in adult Drosophila flies, where fuss was overexpressed with GMR-GAL4 during eye development, this overexpression leads to massive differentiation defects exhibiting a smooth, red coloured eye surface devoid of any typical eye structures such as ommatidia or bristles and we observed little to no phenotypic variability in these flies (Fig 1A and 1B). To see if photoreceptor induction is also affected by fuss overexpression, eye imaginal discs of late third instar larvae were stained with antibodies against Elav, a marker for neurons [37] and Chaoptin, a marker for photoreceptors [38]. In eye imaginal discs overexpressing fuss, neither loss of Elav nor Chaoptin was detected (Fig 1C and 1D, arrow). This shows that cells overexpressing fuss still acquired a neuronal- as well as a photoreceptor fate. In the central nervous system (CNS), fuss is expressed in postmitotic interneurons during development, and its expression is maintained in adulthood [30]. Therefore, we focused in more detail on photoreceptor development. After acquiring photoreceptor fate, these cells start to protrude their axons into the larval optic lobe. Photoreceptors R1-R6 target the lamina neuropil (Fig 1C, arrow), whereas R7 and R8 axons migrate deeper into the medulla neuropil (Fig 1C, arrowhead). This leads to a very specific pattern, which can be observed in larval optic lobes of control larvae (Fig 1C), but upon fuss overexpression in developing photoreceptors, this pattern is strongly disturbed (Fig 1D, arrowhead).

RNAseq reveals downregulation of the PAX2 homolog Shaven after fuss overexpression

To achieve a better overview of genes and processes which become dysregulated by fuss overexpression in the larval eye imaginal disc, we extracted RNA from eye discs of late third instar larvae of controls (GMR-Gal4 > w1118) and experimental flies overexpressing fuss (GMR-Gal4 > UAS-fuss), reverse transcribed it and prepared NGS libraries, which then were sequenced. Comparing gene expression profiles of both genotypes, we found that Fuss was highly enriched in GMR-GAL4; UAS-fuss eye discs, which was a proof of principle that the experiment was successful (Fig 2A). Among 360 genes which showed an altered expression in contrast to controls with an adjusted p-value < 0.01, one gene, shaven (sv), was especially
Fig 1. *fuss* overexpression leads to a smooth eye surface and impairs axonal pathfinding. (A) Eye from control flies (GMR-GAL4 > UAS-nGFP). (B) Eyes from *fuss* overexpressing flies (GMR-GAL4 > UAS-*fuss*). (C) Developing photoreceptors from control flies express Elav and Chaoptin. R1-R6 photoreceptor axons are terminating in the lamina plexus (arrows), whereas R7 and R8 photoreceptor axons are terminating in the developing medulla (arrowhead). (D) Photoreceptors overexpressing *fuss* are expressing Elav and Chaoptin, but photoreceptor axons are impaired in targeting accurately the lamina plexus and the medulla (arrows). (C, D) Confocal images are Z-stacks. Scale bars: 100 μm.
Fig 2. *fuss* overexpression leads to Ct and Sv downregulation and inhibits eye differentiation. (A) Heatmap depicts the top 21 dysregulated genes in *fuss* overexpressing eye imaginal discs in contrast to control eye imaginal discs. (B) Shaven (Sv) expression in control eye discs. (C) Shaven (Sv) expression in *fuss* overexpressing eye discs. (D) Cut (Ct) expression in control eye discs. (E) Cut expression in *fuss* overexpressing eye discs. (F) Adult eyes expressing UAS-*Stinger* with GMR-Gal4. (G) Adult eyes expressing UAS-*Med-IR* with GMR-Gal4. (H) Adult eyes expressing UAS-*Tkv-IR* with GMR-Gal4. (I) Adult eyes expressing UAS-*sv-IR* with GMR-Gal4. (J) Adult eyes expressing UAS-*fuss* with GMR-Gal4.

DAB staining visualized by light microscopy, images were taken from the posterior side of the eye imaginal disc and the focus was set to the plane, where cone cell nuclei are localised (B-E). Scale bars: 10 μm.

https://doi.org/10.1371/journal.pone.0262360.g002
Fuss overexpression results in loss of cell types and increased apoptosis

To understand the developmental defects behind the smooth eye phenotype, pupal development of the overexpression eyes was analysed. In control eye imaginal discs of pupae (GMR-Gal4; UAS-nGFP), which have pupariated for 40 h, a highly ordered pattern of different cell types can already be observed. In the middle of an ommatidium, four cone cells, which secrete the lens, are surround by two primary pigment cells. The single ommatidia are separated from each other by secondary and tertiary pigment cells as well as bristle cells (Fig 3A). In pupal eye imaginal discs, where fuss was overexpressed via the GMR-GAL4 driver line (Fig 3B) most distinct cell types are lost on the surface of the pupal retina in contrast to controls, except for some single bristle cells which are visible (Fig 3B, arrows) but most of the cells which are present are of indefinable cell fate. However, these cells might develop to pigment cells, because the adults develop flat, structureless, but red eyes. With a fluorescent apoptosis sensor called GC3Ai [42], the fuss overexpressing retinas show an increase in apoptotic events in a deeper layer of the developing retina (Fig 3D) which could also account for the reduced size of the adult eye field if compared to the sv knockdown. As observed in larvae, the photoreceptor axons show a perturbed arrangement compared to controls (Fig 3D), but are still expressing the neuronal marker Elav (Fig 3F), although the positioning of photoreceptor nuclei is deranged. Additionally, large gaps can be observed between individual photoreceptor cell groups, which could be the result of apoptosis. (Fig 3F).

The final adult eye differentiation pattern was analysed by paraffin sections of heads of GMR-Gal4; UAS-fuss flies (Fig 4A and 4B) as well as controls (GMR-Gal4; UAS-nGFP). In fuss overexpressing eyes (Fig 4C and 4D) the photoreceptors and their rhabdomeres, which can be observed by a bright fluorescent signal in the controls (Fig 4A) are completely lost. Although they were determined to become photoreceptors as observed in Fig 1D, they are probably removed by apoptosis (Fig 3D) during pupal development, which also affects the integrity of the adult lamina. Furthermore, vacuoles can be observed (Fig 4D, arrows) which are probably also the outcome of the cell death observed during pupal stage (Fig 3D). These sections show, that the indefinable cells observed in pupal retinas have a completely different shape than those of secondary or tertiary pigment cells in controls (Fig 4A), but still contain...
Fig 3. In *fuss* overexpressing pupal retinas cell organisation is completely lost and cell death is increased. (A) Organisation of cone cells, primary, secondary, tertiary and bristle cells in a pupal retina 40 hrs after puparium formation of control flies visualized with DE-CADHERIN (DE-CAD, gray) staining. No G3CAi (magenta) signal can be observed. (B) After *fuss* overexpression with GMR-Gal4 mainly one indefinable cell type differentiates and some single bristle cells are visible on the pupal retina surface (arrows) visualized with DE-CADHERIN (DE-CAD, gray) staining. G3CAi signal (magenta) is from cells directly below the retinal surface. (C) In a lower layer of the pupal retina photoreceptor axons are arranged circularly in controls and no G3CAi signal (magenta) can be observed. (D) After *fuss* overexpression circular arrangement of photoreceptor axons is strongly impaired (gray, arrows) and many apoptotic cells marked with G3CAi (magenta) signal can be observed. (E) In pupal retinas photoreceptor nuclei...
pigment granules (Fig 4C). Thus, these cells rather adopt a basal pigment cell fate but don’t acquire the correct shape.

Obviously, overexpressing fuss in differentiating cells posterior to the morphogenetic furrow highly impacts their specification. Photoreceptors are determined but are abolished during development and residual cells are prevented to adopt their natural fate and nearly all are marked with ELAV expression (yellow) are arranged in a hexagonal array in controls (green). (F) In pupal eye discs of GMR-Gal4; UAS-fussGFP flies the ELAV positive photoreceptor nuclei are strongly disturbed in their patterning. Confocal images in A and B are stacks from three single focal planes. Residual confocal images are from single focal planes. Scale bars: 10 μm.

https://doi.org/10.1371/journal.pone.0262360.g003

Fig 4. fuss overexpression during development disintegrates adult eye structure. (A) Paraffin sections of control heads illuminated with light. (B) Paraffin section of control heads illuminated with 470nm wavelength. (C) Paraffin section of a GMR-Gal4; UAS-fuss head illuminated with light shows small pigment granule containing cells. (D) Paraffin section of a GMR-Gal4; UAS-fuss head illuminated with 470nm wavelength reveals vacuoles (arrows), loss of adult eye structure including photoreceptors and reduced lamina size.

https://doi.org/10.1371/journal.pone.0262360.g004
transformed to a basal pigment cell fate. Therefore, *fuss* overexpression is able to completely inhibit differentiation but, in this context, no striking increase in proliferation was observed.

**Early induction of *fuss* overexpressing clones inhibits eye differentiation and leads to eye disc outgrowths**

The ability of Ski/Sno proteins to function as proto-oncogenes is often linked to their capacity to inhibit the antiproliferative effects of TGF-β signalling. To check for early proliferative defects, we decided to induce *fuss* overexpression clones in first instar larvae. 48 hours after egg laying, we heat-shocked the first instar larvae with the genotype (*P{hsp70-flp}1/+; Fuss-GFP/+; GAL4-Act5C(FRT.CD2).P*) for 12 minutes to induce *fuss* overexpressing clones. Late third instar larvae were dissected, and tissue was analysed for *fuss* overexpressing clones and their effects on development.

Surprisingly, as shown in Fig 5, we detected a completely different behaviour of *fuss* overexpression. *fuss* overexpressing clones in eye imaginal discs result in tissue outgrowths. To check the cellular identity of these outgrowing cells, we first tested for the neuronal differentiation marker Elav, which would be expected in photoreceptor cells. However, these outgrowths lack the late differentiation marker Elav completely and can form big bulbous structures which protrude from the eye imaginal disc (Fig 5A and 5B). All heat shocked larvae die at the latest during pupal stages, therefore we decreased the time of the heat shock, which enabled us to obtain single adult survivors. Some of these survivors had undifferentiated tissue growing out from their eye, which apparently was generated by *fuss* overexpressing clones (Fig 5C). To further understand the *fuss* overexpression defects, we were searching for early retinal differentiation markers. Dpp signalling is assumed to be required for the expression of early retinal differentiation markers *sine oculis*, *dachshund* and *eyes absent* (*eya*) [43, 44]. Previous studies have shown, that overexpression of *fuss* can interfere with Dpp signalling [4] and we could indeed confirm that *fuss* overexpressing clones lack *eya* expression (Fig 5D) and therefore, Fuss might inhibit Dpp signaling in the eye imaginal disc as well. All in all, initiation of photoreceptor differentiation is already impaired in *fuss* overexpressing clones.

**Fuss overexpressing clones exhibit increased proliferation**

However, this does not explain, why *fuss* overexpressing clones lead to tissue outgrowths from the eye. Generally, if eye development is inhibited, this would lead to a transformation from eye to head capsule tissue only and not to additional head capsule tissue as observed [45]. In contrast to *eya* mutant eye discs, where proliferation is strongly reduced [46], the *fuss* overexpressing clones seem to show increased proliferation. To further analyse cell proliferation, we used the Fly-FUCCI system, where degrons of E2F1 and CycB have been fused to GFP and mRFP, respectively, to visualize the cell cycle behaviour of cells [47]. Cells from anaphase to the G1 to S transition are green, S phase cells are red, and cells in G2 and early mitosis are yellow. Expressing the Fly-FUCCI system via the Flipase technique in eye imaginal discs (Fig 6A), we found that many cells posterior to the morphogenetic furrow are green or red, therefore in G1 or S-Phase and only some where yellow, thus in early mitosis. But when we coexpressed *fuss* with the Fly-FUCCI system many more cells where yellow posterior to the morphogenetic furrow and consequently where in G2-Phase or undergoing mitosis (Fig 6B). Interestingly, the shape of *fuss* overexpressing clones were highly different to that of control clones. Whereas control clones integrate into the patterning of the developing eye imaginal discs, *fuss* overexpressing clones might not react to Dpp signalling as shown before, have a rather elliptical shape and are protruding from the eye imaginal disc. Because of this experiment we expected, that *fuss* overexpressing clones have a higher division rate than controls. We chose to use an
antibody against phosphorylated Histone H3, a marker for mitosis, to specifically identify mitotic cells and count them. In this assay we only counted cells behind the second mitotic wave (smw), because after the smw cells start to differentiate instead to proliferate. As controls
Fuss retains pro-oncogenic properties of the Ski/Sno family

A control

B fuss OE clones

C E2F1-GFP

D FussGFP

E wildtype tissue Fuss overexpressing tissue

rH3 histone per pixel

Genotype

***
we used tissue which was not overexpressing \textit{fuss}, therefore we counted the number of pHH3 positive cells inside \textit{fuss} overexpressing clones and outside of it, and measured the area in pixels of the \textit{fuss} overexpressing clonal tissue and the wildtype tissue and divided the number of mitotic cells by the amount of pixels. We found significantly more pHH3 positive cells per pixel in the \textit{fuss} overexpressing clones than in the surrounding wild type tissue (Fig 6C and 6D, S2 File), demonstrating that \textit{fuss} overexpression leads to increased proliferation in developing eye imaginal discs in contrast to controls (Fig 6E).

\textbf{wingless is expressed in \textit{fuss} clonal outgrowths}

Besides eye differentiation, Dpp signalling is also required to inhibit \textit{wg} expression in the eye imaginal discs [36]. In eye imaginal discs of third instar larvae, \textit{wg} expression can normally only be observed at the dorsal and ventral margins of the eye disc, where it is supposed to promote head capsule structures instead of eye tissue [48], but in earlier stages \textit{wg} is expressed in the whole prospective eye [49] and this is around the time we induce the \textit{fuss} overexpressing clones. Loss of Dpp signalling leads to overgrowth and ectopic \textit{wg} expression in the eye imaginal disc [36] and loss of \textit{eya} expression might not only be the result of \textit{fuss} dependent inhibition of Dpp signalling, but also ongoing \textit{Wg} signalling, as it has been shown by ectopic Wingless signalling clones [50]. To test if \textit{fuss} overexpression clones continue expressing \textit{wg} from earlier stages on via a possible inhibition of Dpp signalling, we used a \textit{wg}-LacZ reporter construct to visualise \textit{wg} promotor activity in the eye imaginal disc and as shown in Fig 7A \textit{fuss} overexpressing clones exhibit indeed LacZ expression supporting the hypothesis that the inhibition of Dpp signalling allows continuous \textit{wg} expression in these cells. Wingless signalling is also involved in promoting the proliferation of cells anterior to the morphogenetic furrow and ectopic Wingless signalling leads to increased proliferation [51]. One possible hypothesis for the overproliferation observed with \textit{fuss} overexpressing clones might be that Fuss acts again as an inhibitor of Dpp signalling in the eye imaginal disc allowing continuous \textit{wg} expression, which might lead to the excess proliferation in the eye imaginal disc. To test this hypothesis, we tried to generate clones, which besides \textit{fuss}, also express a knockdown construct for \textit{wg}, but this approach turned out to be highly lethal. In a second approach, we generated clones which expressed \textit{fuss} and a dominant negative form of \textit{Pangolin (dnPan)}, because the effects of \textit{wg} overexpression can be suppressed by dominant negative Pangolin, even when expressed from the same cell [52] and it has been shown, that \textit{wg} expression can be autoregulated endogenously [53, 54] or ectopically in some tissues [55]. \textit{fuss/dnPan} overexpressing clones (Fig 7B) were not only consistently smaller compared to \textit{fuss} overexpressing clones (Fig 7A), but the clonal tissue did also not outgrow anymore. \textit{fuss/dnPan} overexpressing clones did not exhibit \textit{wg} expression anymore, as observed with the absence of LacZ staining, supporting our hypothesis that Fuss, via inhibition of Dpp signaling, might be able to allow \textit{wg} expression from earlier stages to continue and \textit{wg} expression and Wg signalling in these clones might promote the outgrowths from the eye disc. Similarly, overexpressing dominant negative Pan together with nuclear GFP lead to small clones and \textit{wg} expression was also not increased (Fig 7C).
Fig 7. *fuss* overexpressing clones exhibit *wg* expression. (A) *FussGFP* overexpressing clones (green) show LacZ expression (red) under the control of a *wg* promoter. (B) Simultaneous expression of *FussGFP* and *dnPan* (green) inhibits LacZ expression (red) under the control of a *wg* promoter and clonal tissue is strongly reduced in size. (C) *dnPAN* expressed together with nuclear GFP (green) show no expression of LacZ under the control of a *wg* promoter (red). All images are stacks. Scale bars: 50 μm.

https://doi.org/10.1371/journal.pone.0262360.g007
Discussion

In this work, we addressed the question if Skor/Fuss proteins, members of the Ski/Sno family, retained the function of Ski and Sno to induce uncontrolled proliferation as observed in early stages of tumorigenesis.

First, the overexpression of fuss posterior to the morphogenetic furrow with the GMR-Gal4 driver line resulted in a nearly complete loss of all cell types in the adult eye. During development, photoreceptor axons were not able to target the appropriate layers of the optic lobe anymore and cone cells, primary pigment cells and bristle cells were transformed into a basal pigment cell fate. This transformation was caused by the inhibition of sv expression, which is crucial for accessory cell differentiation. Additionally, increased apoptosis during pupal development lead to the removal of photoreceptors and lastly adult eyes only consisted of cells containing pigment granules. This lack of differentiation cannot be explained by the Dpp inhibiting role Fuss exerts, when overexpressed [4], because inhibiting the Dpp signaling pathway via knockdown of Tkv or Med had no effect. Photoreceptor axon guidance is impaired, if Dpp signaling is disrupted in photoreceptors by the expression of the inhibitory Smad Dad [56]. Thus, the observed photoreceptor axon guidance phenotype, when fuss is overexpressed with GMR, could indeed be a result of Dpp signaling inhibition. However, the loss of nearly all eye cell types is due to other effects (e.g. downregulation of sv and apoptosis) than Dpp signaling repression alone, because loss of Dpp signaling behind the morphogenetic furrow only results in mild patterning defects of the pupal retina [57]. Nonetheless, the inhibition of cell differentiation has already been shown in other cancer models e.g., when two copies of the constitutive active form of the receptor tyrosine kinase dRET_MEN2B are expressed with the GMR-Gal4 line, pupal retinas are devoid of any distinguishable cell types [33]. This phenotype is indistinguishable from the phenotype of the pupal retinas generated by the overexpression of fuss via GMR-Gal4. In a screen for novel oncogenes from breast cancer patients, human transgenes have been overexpressed with the GMR-Gal4 driver line. Overexpression of human RPS12, a subunit of the small ribosomal subunit, whose expression is increased in various cancer types, leads also to a glazed eye phenotype [58]. Therefore, different oncogenes can result in different outcomes when expressed with the GMR-Gal4 driver line and are not always leading to massive tissue overgrowth like the Yorkie overexpression [59]. Most importantly, with this approach to overexpress fuss in cells which already were destined for acquiring a cell fate and have left the cell cycle, we were not able to induce increased proliferation anymore, but could prevent cell differentiation.

Consequently, we switched to a more pluripotent cell type in the eye imaginal disc [60] and induced fuss overexpressing clones prior to the formation of the morphogenetic furrow. These results let us assume that in this context, fuss overexpressing clones do not react to the antiproliferative effects of the Dpp morphogen anymore. Instead, wg expression and thus, proliferation promotion might be maintained. This leads to outgrowths of clonal tissue from the eye imaginal disc of third instar larvae, which showed an increased number of mitotic events. If these flies survived to adulthood, undifferentiated, extra tissue was visible in the complex eye.

An analogous mechanism can be observed in tumors which overexpress Ski or Sno. The TGF-β signaling pathway also acts anti-proliferative, but this action is inhibited by the increased presence of Ski/Sno proteins. Therefore, the molecular mode of action is similar to the human Ski/Sno proteins. The function of Ski and Sno is highly context dependent, as they can fulfill an anti-oncogenic or pro-oncogenic role depending on the cancer type or status of the cancer. We also observed this with fuss overexpressing clones. Only when induced 48h after egg laying, we found additional tissue in late third instar larvae and only in eye imaginal discs, because here, Dpp counteracts the proliferative effects of Wg signaling. When fuss is
overexpressed in the wing disc or after induction of the morphogenetic furrow differentiation is inhibited, this results in a wing with truncated veins [4] or in a smooth eye surface (this work). This is also underlined by RNAseq data from eye and wing imaginal discs, where fuss was overexpressed with the GMR-Gal4 and Nub-Gal4 driver line, respectively. In the eye data-set, wg expression in eye imaginal discs is not significantly different from control eye discs, whereas wg expression in fuss overexpression wing discs is significantly reduced in contrast to control wing discs (S1 Fig).

Thus, we were able to show that the Skor protein Fuss in Drosophila melanogaster still retained the function of the Ski/Sno proteins by inhibiting differentiation but inducing hyper-proliferation. But the hallmarks of real tumorigenesis are lacking, because at some point during pupal development, proliferation stops, and these cells become protruding head tissue as it could be observed in complex eyes of surviving flies. Furthermore, there was no evidence of an epithelial-mesenchymal transition because fuss overexpressing clones maintained their epithelial fate. It will be of high interest if future studies can find similar results in overexpression studies for the vertebrate Skor proteins or detect increased expression of these proteins in specific cancer types.

Material and methods

Fly husbandry and stocks
Flies were raised at 25˚C under a 12 hr/12 hr light/dark cycle. Fly lines obtained from the Bloomington Drosophila Stock Center were: P{GAL4-ninaE.GMR}12 (BDSC #1104), w1118; P{UAS-Stinger}2 (BDSC #84277), w1118; snaSc/CyO, P[en1]wgen11 (BDSC #1672), y1 w1118; P{UAS-pan.dTCFΔN}4 (BDSC #4784), y[1] w[+]; P[w+] = GAL4-Act5C(FRT. CD2).PJS (BDSC #4780), P[ry+[t.72] = hsFLP1, y[1] w[1118]; Dr[Mio]/TM3, ry[+] Sb[1] (BDSC #7), P{AyGAL4}25 (BDSC #4412), w1118 (BDSC #3605), y1 v1; P{TRiP.HMS05834}attP2 (BDSC #67973), y1 sc v1 sev21; P{TRiP.GL01313}attP40 (BDSC #43961), y1 sc v1 sev21; P{TRiP.HMS04501}attP40 (BDSC #57303), w[1118]; Kr[If-1]/CyO, P[ry+[t.72] = en1] wg[en11]; P[w+] = UAS-GFP.E2f1.1–230]26 P[w+] = UAS-mRFP1.NLS.CycB.1–266]17/TM6B (BDSC #85122), Tb[1], w[; KrIf-1/CyO; P{UAS-GC3Ai}3 (BDSC #84343). Additionally, Nub-Gal4 (J.F. de Celis, Madrid) was employed.

Immunohistochemistry
For analysis of fuss overexpressing clones 48 hrs after egg laying, the larvae were heat shocked at 37˚C for 12 min. Then, for all experiments late third-instar larvae were used for dissection and immunohistochemistry. By pulling the mouth hooks, the anterior mouth part including the eye imaginal discs still attached to the brain were removed from the rest of the larva and then fixed by incubation in 4% PFA in PBS for 20 min. The specimen was washed three times with PBST (PBS with 0.1% Triton-X) for 20 min and incubated in PBST supplemented with 5% normal goat serum and primary antibodies over night at 4˚C. The specimen was washed three times with PBST for 20 min and incubated in PBST supplemented with 5% normal goat serum and secondary antibodies over night at 4˚C. The specimen was washed once with PBST for 20 min, then incubated in PBST supplemented with 1 mg/ml 4’,6-Diamidin-2-phenylindol (DAPI) for 20 min and washed three times with PBST for 20 min. The eye imaginal discs were dissected and mounted using VECTASHIELD Antifade Mounting Medium (Vector Laboratories). Developmental studies Hybridoma Bank (DSHB) antibodies were: LacZ (JIE7, 1:50), Eyes absent (eya10H6, 1:50), Elav (Rat-Elav-7E8A10, 1:50), Cut (2B10, 1:20), Chaoptin (24B10, 1:50). Additional antibodies were: Svpax2 (1:100, gift from Markus Noll), GFP (rabbit 1:1000, ThermoFisher), pHH3 (rabbit 1:2000, Cell signaling technology). Secondary antibodies were
used with a dilution 1:200 overnight at 4˚C. Secondary antibodies were goat anti-mouse, anti-rabbit, anti-rat and anti-guinea pig Alexa Fluor 488, 555 and 594 (ThermoFisher). For anti-Cut and anti-Sv stainings we used the Anti-Mouse / anti-Rabbit HRP-DAB IHC kit (abcam) to increase sensitivity and reduce background.

**Generation of FLP-out clones**

In general, we crossed virgins carrying the P{hsp70-flp}1 allele homozygously to males carrying the GAL4-Act5C(FRT.CD2).P allele. Before the cross was placed for 2h on standard food supplemented with dry yeast at 25˚C, flies were allowed to mate for at least three days. The adult flies were removed from the vial and progeny was allowed to develop for 46h at 25˚C. Progeny was heatshocked for 12 minutes at 37˚C and placed again at 25˚C. Late third instar larvae were then dissected.

**Retina dissection and immunostaining**

White pupae were collected and aged at 25˚C for 40 hrs. The brains with the attached eye discs were dissected in PBS and placed in PBS with 4% PFA on ice until all brains from one genotype were dissected. Afterwards the brains were fixed for another 20 min with 4% PFA in PBS at room temperature. The brains with the attached eye discs were stained with rat-anti-DE-cadherin (DCAD2, 1:50, DSHB) or rat-anti-ELAV (Rat-Elav-7E8A10, 1:50) and goat-anti-rat Alexa Fluor 555 (1:200, ThermoFisher) in PBST 0.1% with 5% normal goat serum (NGS). After staining, the eye discs were removed from the brains directly on the mounting slide in a drop of PBST 0.1% and mounted using VECTASHIELD Antifade Mounting Medium (Vector Laboratories).

**Quantification of pH3 positive cells**

Only eye imaginal discs where the second mitotic wave was clearly detectable via pH3 staining were used. Mitotic cells in *fuss* overexpressing clones and in wildtype tissue were counted. The area of *fuss* overexpressing clones and wildtype tissue was measured with the measurement tool of ImageJ. The number of mitotic cells inside a *fuss* overexpressing clone was divided by its area and the number of mitotic cells inside the wildtype tissue was divided by the wildtype tissue’s area. The acquired data was visualized with Python and the Matplotlib and Seaborn libraries. Statistics were calculated with the SciPy library.

**Paraffin sections**

Paraffin sections were performed from two-day old adult flies. Flies were fixed with carnoy (ethanol:chloroform:acetic acid at a proportion 6:3:1), dehydrated in ethanol, and embedded in paraffin. Paraffin sections (7 μm) from 10 flies of each genotype were analysed under a fluorescence microscope.

**RNA extraction, library generation and sequencing**

Per replicate and genotype 40 eye antennal discs or 30 wing discs from third instar larvae were dissected. RNA was extracted via peqGold MicroSpin Total RNA Kit. Library preparation and RNA-Seq were carried out according to the NEBNext Ultra RNA Library Prep protocol, the Illumina HiSeq 1000 System User Guide, and the KAPA Library Quantification Kit—Illumina/ABI Prism User Guide. Library preparation and RNA-Seq were performed at the Genomics Core Facility "KFB—Center of Excellence for Fluorescent Bioanalytics" (University of Regensburg, Regensburg, Germany).
RNA-Seq analysis
The reads were quantified with the R package Salmon [61] using the release of the Drosophila melanogaster genome BDGP6.22. The data was imported using tximeta [62] and analysed with DESeq2 [63]. Cut-off for significantly dysregulated genes was set with an adjusted p-value < 0.01. Top 21 differentially expressed genes between control and overexpression replicates were visualized with the heatmap.2 package in R.

Supporting information
S1 Fig. Wg is context dependent affected by Fuss overexpression. In wing disc fuss overexpression (Nub-Gal4 > UAS-fuss) leads to decreased wg expression in contrast to controls (Nub-Gal4 > w1118). In eye discs wg expression is unaffected by the overexpression of fuss (GMR-Gal4 > UAS-fuss) if compared to controls (GMR-Gal4 > w1118).
(TIF)

S1 File. Differential expression analysis of fuss overexpressing eye discs and control eye discs.
(XLSX)

S2 File. Data for box plot.
(XLSX)

Author Contributions
Conceptualization: Mathias Rass, Florian Bayersdorfer, Stephan Schneuwly.
Data curation: Mathias Rass, Laura Gizler, Matthias Schramm.
Formal analysis: Mathias Rass, Laura Gizler, Christoph Irlbeck, Matthias Schramm.
Investigation: Mathias Rass, Laura Gizler, Christoph Irlbeck, Matthias Schramm.
Methodology: Mathias Rass, Laura Gizler.
Project administration: Florian Bayersdorfer, Stephan Schneuwly.
Resources: Stephan Schneuwly.
Software: Mathias Rass.
Supervision: Mathias Rass, Florian Bayersdorfer.
Validation: Mathias Rass.
Visualization: Mathias Rass.
Writing – original draft: Mathias Rass, Stephan Schneuwly.
Writing – review & editing: Mathias Rass, Laura Gizler, Stephan Schneuwly.

References
1. Johnson LN, Noble MEM, Barford D, Brown N, Endicott JA, Lawrie A, et al. Signal Transduction Proteins: Structural Basis of Control by Phosphorylation. J Chem Soc Pakistan. 1999; 21: 185–201. https://doi.org/10.1016/S1569-2558(08)60096-X
2. Deheuninck J, Luo K. Ski and SnoN, potent negative regulators of TGF-β signaling. Cell Res. 2009; 19: 47–57. https://doi.org/10.1038/cr.2008.324 PMID: 19114989
3. Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, et al. Smurfl Interacts with Transforming Growth Factor-β Type I Receptor through Smad7 and Induces Receptor Degradation. J Biol Chem. 2001; 276: 12477–12480. https://doi.org/10.1074/jbc.C100082020 PMID: 11278251

4. Fischer S, Bayersdorfer F, Harant E, Reng R, Arndt S, Bosserhoff AK, et al. Fussel (fuss)—A negative regulator of BMP signaling in Drosophila melanogaster. PLoS One. 2012; 7: 1–12. https://doi.org/10.1371/journal.pone.0022948

5. Wilson JJ, Malakhova M, Zhang R, Joachimiak A, Hegde RS. Crystal structure of the Dachshund homology domain of human SKI. Structure. 2004; 12: 785–792. https://doi.org/10.1016/j.str.2004.02.035 PMID: 15130471

6. Wu JW, Krawitz AR, Chai J, Li W, Zhang F, Luo K, et al. Structural mechanism of Smad4 recognition by the nuclear oncoprotein Ski: Insights on Ski-mediated repression of TGF-β signaling. Cell. 2002; 111: 357–367. https://doi.org/10.1016/s0092-8674(02)01006-1 PMID: 12492146

7. Nomura T, Khan MM, Kaul SC, Dong HD, Wadhwa R, Colmenares C, et al. Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor. Genes Dev. 1999; 13: 412–423. https://doi.org/10.1101/gad.13.4.412 PMID: 10049357

8. Mizuura E, Nakatani T, Minaki Y, Sakamoto Y, Ono Y. Corl1, a novel neuronal lineage-specific transcriptional corepressor for the homeodomain transcription factor Lbx1. J Biol Chem. 2005; 280: 3645–3655. https://doi.org/10.1074/jbc.M41652200 PMID: 15528197

9. Wang B, Harrison W, Overbeeka PA, Zheng H. Transposon mutagenesis with coat color genotyping identifies an essential role for skor2 in sonic hedgehog signaling and cerebellum development. Development. 2011; 138: 4487–4497. https://doi.org/10.1242/dev.067264 PMID: 21937600

10. Arndt S, Poser I, Moser M, Bosserhoff AK. Fussel-15, a novel Ski/Sno homolog protein, antagonizes BMP signaling. Mol Cell Neurosci. 2007; 34: 603–611. https://doi.org/10.1016/j.mcn.2007.01.002 PMID: 17292623

11. Arndt S, Poser I, Schubert T, Moser M, Bosserhoff AK. Cloning and functional characterization of a new Ski homolog, Fussel-18, specifically expressed in neuronal tissues. Lab Investig. 2005; 85: 1330–1341. https://doi.org/10.1038/latinvest.3700344 PMID: 16200078

12. Stegmüller J, Konishi Y, Huynh MA, Yuan Z, DiBacco S, Bonni A. Cell-Intrinsic Regulation of Axonal Morphogenesis by the Cdh1-APC Target SnoN. Neuron. 2006; 50: 389–400. https://doi.org/10.1016/j.neuron.2006.03.034 PMID: 16675394

13. Nakatani T, Minaki Y, Kumai M, Nitta C, Ono Y. The c-Ski family member and transcriptional repressor of the homeodomain transcription factor Lbx1. Dev Biol. 2004; 276: 12477–12480. https://doi.org/10.1074/jbc.C100082020 PMID: 11278251

14. Corl2/Skor2 promotes early differentiation of cerebellar Purkinje cells. Dev Biol. 2014; 388: 68–80. https://doi.org/10.1016/j.ydbio.2014.01.016 PMID: 24491816

15. Kobayashi N, Goto K, Horiguchi K, Nagata M, Kawata M, Miyazawa K, et al. c-Ski activates MyoD in the nucleus of myoblastic cells through suppression of histone deacetylases. Genes to Cells. 2007; 12: 375–385. https://doi.org/10.1011/j.jgc.2006.03.034 PMID: 16675394

16. Jahchan NS, Wang D, BisSELL MJ, Luo K. SnoN regulates mammmary gland alveologenesis and onset of lactation by promoting prolactin/stat5 signaling. Dev. 2012; 139: 3147–3156. https://doi.org/10.1242/dev.079616 PMID: 22833129

17. Stavnezer E, Brodeur D, Brennan LA. The v-ski oncogene encodes a truncated set of c-ski coding exons with limited sequence and structural relatedness to v-myc. Mol Cell Biol. 1989; 9: 4038–4045. https://doi.org/10.1128/mcb.9.9.4038-4045.1989 PMID: 26748685

18. Colmenares C, Stavnezer E. The ski oncogene induces muscle differentiation in quail embryo cells. Cell. 1989; 59: 293–303. https://doi.org/10.1016/0092-8674(89)90291-2 PMID: 2553267

19. Imoto I, Pimkhaokham A, Fukuda Y, Yang ZQ, Shimada Y, Nomura N, et al. SNO is a probable target for gene amplification at 3q26 in squamous-cell carcinomas of the esophagus. Biochim Biophys Res Commun. 2001; 286: 559–565. https://doi.org/10.1006/bbrc.2001.5428 PMID: 11510996

20. Poser I, Rothhammer T, Dooley S, Weiskirchen R, Bosserhoff AK. Characterization of Sno expression in malignant melanoma. Int J Oncol. 2005; 26: 1411–1417. https://doi.org/10.3892/ijo.26.5.1411 PMID: 15809735

21. Bues M, Terracciano L, Reuter J, Bailabeni P, Boulay JL, Laffer U, et al. Amplification of SKI is a prognostic marker in early colorectal cancer. Neoplasia. 2004; 6: 207–212. https://doi.org/10.1593/neo.03442 PMID: 15153322

22. Zhu Q, Krakowski AR, Dunham EE, Wang L, Bandyopadhyay A, Berdeaux R, et al. Dual Role of SnoN in Mammalian Tumorigenesis. Mol Cell Biol. 2007; 27: 324–339. https://doi.org/10.1128/MCB.01394-06 PMID: 17074815

23. Heider TR, Lyman S, Schoonhoven R, Behrens KE. Ski promotes tumor growth through abrogation of transforming growth factor-β signaling in pancreatic cancer. Ann Surg. 2007; 246: 61–68. https://doi.org/10.1097/SLA.0b013e318070cafa PMID: 17592292
23. Shinagawa T, Dong HD, Xu M, Maekawa T, Ishii S. The sno gene, which encodes a component of the histone deacetylase complex, acts as a tumor suppressor in mice. EMBO J. 2000; 19: 2280–2291. https://doi.org/10.1093/emboj/19.22.2880 PMID: 10811619

24. Shinagawa T, Nomura T, Colmenares C, Ohira M, Nakagawara A, Ishii S. Increased susceptibility to tumorigenesis of ski-deficient heterozygous mice. Oncogene. 2001; 20: 8100–8108. https://doi.org/10.1038/sj.onc.1204987 PMID: 11781823

25. Yang H, Zhan L, Yang T, Wang L, Li C, Zhao J, et al. Ski prevents TGF-β-induced EMT and cell invasion by repressing SMAD-dependent signaling in non-small cell lung cancer. Oncol Rep. 2015; 34: 87–94. https://doi.org/10.3892/or.2015.3961 PMID: 25955797

26. Sarayloo F, Spiegelman D, Rochefort D, Akcimen F, De Barros Oliveira R, Dion PA, et al. SKOR1 has a transcriptional regulatory role on genes involved in pathways related to restless legs syndrome. Eur J Hum Genet. 2020; 28: 1520–1528. https://doi.org/10.1038/s41431-020-0670-4 PMID: 32572201

27. Arndt S, Schmidt J, Wacker E, Karrer S, Bosserhoff AK. Fussel-15, a new player in wound healing, is deregulated in keloid and localized scleroderma. Am J Pathol. 2011; 178: 2622–2631. https://doi.org/10.1016/j.ajpath.2011.02.009 PMID: 21641385

28. Takaesu NT, Hyman-Walsh C, Ye Y, Wisotzkey RG, Stinchfield MJ, O'Connor MB, et al. dSno facilitates baboon signaling in the drosophila brain by switching the affinity of medea away from Mad and toward dSmad2. Genetics. 2006; 174: 1299–1313. https://doi.org/10.1534/genetics.106.064956 PMID: 16951053

29. Takaesu NT, Stinchfield MJ, Shimizu K, Arase M, Quijano JC, Watabe T, et al. Drosophila CORL is required for Smad2-mediated activation of Ecdysone Receptor expression in the mushroom body. Development. 2012; 139: 3392. https://doi.org/10.1242/dev.079442 PMID: 22874913

30. Rass M, Oestreich S, Guettet S, Fischer S, Schneuwly S. The Drosophila fusel gene is required for bitter gustatory neuron differentiation acting within an Rpd3 dependent chromatin modifying complex. PLoS Genet. 2019; 15: e1007940. https://doi.org/10.1371/journal.pgen.1007940 PMID: 30730884

31. Rass M, Oestreich S, Manaj A, Schneuwly S. Loss of fuss in Drosophila melanogaster results in decreased locomotor activity due to an increased number of pauses. microPublication Biol. 2020;2020. https://doi.org/10.17912/micropub.biology.000230 PMID: 32550504

32. Piper MDW, Partridge L. Drosophila as a model for ageing. Biochim Biophys Acta—Mol Basis Dis. 2018; 1864: 2707–2717. https://doi.org/10.1016/j.bbadis.2017.09.016 PMID: 28964875

33. Read RD, Goodfellow PJ, Mardis ER, Novak N, Armstrong JR, Cagan RL. A drosophila model of multiple endocrine neoplasia type 2. Genetics. 2005; 171: 1057–1081. https://doi.org/10.1534/genetics.104.038018 PMID: 15965261

34. Bennett D, Luylecheva E, Cobbe N. Drosophila as a Potential Model for Ocular Tumors. Ocul Oncol Pathol. 2015; 1: 190–199. https://doi.org/10.1159/000370155 PMID: 27172095

35. Freeman M. Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. Cell. 1996; 87: 651–660. https://doi.org/10.1016/s0092-8674(00)81385-9 PMID: 8929534

36. Wiersdorf V, Lecuit T, Cohen SM, Mlodzik M. Mad acts downstream of Dpp receptors, revealing a differential requirement for dpp signaling in initiation and propagation of morphogenesis in the Drosophila eye. Development. 1996; 122: 2153–2162. https://doi.org/10.1242/dev.122.7.2153 PMID: 8681796

37. Koushika SP, Lisbin MJ, White K. ELAV, a Drosophila neuron-specific protein, mediates the generation of an alternatively spliced neural protein isofrom. Curr Biol. 1996; 6: 1634–1641. https://doi.org/10.1016/s0960-9822(02)70787-2 PMID: 8994828

38. Reinhke R, Krantz DE, Yen D, Lawrence Zipursky S. Chaoptin, a cell surface glycoprotein required for Drosophila photoreceptor cell morphogenesis, contains a repeat motif found in yeast and human. Cell. 1998; 52: 291–301. https://doi.org/10.1016/0092-8674(98)90518-1 PMID: 9655816

39. Brunner E, Brunner D, Fu W, Hafen E, Basler K. The dominant mutation Glazed is a gain-of-function allele of wingless that, similar to loss of APC, interferes with normal eye development. Dev Biol. 1999; 206: 178–188. https://doi.org/10.1006/dbio.1998.9136 PMID: 9986731

40. Fu W, Duan H, Frei E, Noll M. shaven and sparkling are mutations in separate enhancers of the Drosophila Pax2 homolog. Development. 1998; 125: 2943–2950. https://doi.org/10.1242/dev.125.15.2943 PMID: 9655816

41. Fu W, Noll M. The Pax2 homolog sparkling is required for development of cone and pigment cells in the Drosophila eye. Genes Dev. 1997; 11: 2066–2078. https://doi.org/10.1101/gad.11.16.2066 PMID: 9284046

42. Schott S, Ambrosini A, Barbaste A, Benassayag C, Gracia M, Proag A, et al. A fluorescent toolkit for spatiotemporal tracking of apoptotic cells in living Drosophila tissues. Development. 2017; 144: 3840–3846. https://doi.org/10.1242/dev.149807 PMID: 28870988
43. Curtiss J, Mlodzik M. Morphogenetic furrow initiation and progression during eye development in Droso-
phila: The roles of decapentaplegic, hedgehog and eyes absent. Development. 2000; 127: 1325–
1336. https://doi.org/10.1242/dev.127.6.1325 PMID: 10683184
44. Chen R, Halder G, Zhang Z, Mardon G. Signaling by the TGF-beta homolog decapentaplegic functions
reiteratively within the network of genes controlling retinal cell fate determination in Drosophila. Develop-
ment. 1999; 126: 935–943. https://doi.org/10.1242/dev.126.5.935 PMID: 9927595
45. Pappu K, Mardon G. Retinal specification and determination in Drosophila. Results Prob Cell Differ.
2002; 37: 5–20. https://doi.org/10.1007/978-1-60327-469-2_12 PMID: 19109709
46. Weasner BM, Kumar JP. Competition among gene regulatory networks imposes order within the eye-anten-
nal disc of Drosophila. Dev. 2013; 140: 205–215. https://doi.org/10.1242/dev.085423 PMID: 23222441
47. Zielke N, Korzelius J, van Straaten M, Bender K, Schuhknecht GFP, Dutta D, et al. Fly-FUCCI: A Versa-
tile Tool for Studying Cell Proliferation in Complex Tissues. Cell Rep. 2014; 7: 588–598. https://doi.oi-
g/10.1016/j.celrep.2014.03.020 PMID: 24726363
48. Legent K, Treisman JE. Wingless signaling in Drosophila eye development. Methods Mol Biol. 2008;
469: 141–161. https://doi.org/10.1007/978-1-60327-469-2_12 PMID: 19109709
49. Hooper JE. Distinct pathway s for autocrine and paracrine Wingless signalling in Drosophila embryos.
Nat. 1994 3726505. 1994; 372: 461–464. https://doi.org/10.1038/372461a0 PMID: 7984239
50. Yoshida S, Soustelle L, Giangrande A, Umetsu D, Murakami S, Yasugi T, et al. DPP signaling controls
development of the lamina glia required for retinal axon targeting in the visual system of Drosophila.
Development. 2005; 132: 4587–4598. https://doi.org/10.1242/dev.020972 PMID: 16176948
51. Cordero JB, Larson DE, Craig CR, Hays R, Cagan R. Dynamic Decapentaplegic signaling regulates
patterning and adhesion in the Drosophila pupal retina. Development. 2007; 134: 1861–1871. https://
doi.org/10.1242/dev.002972 PMID: 17428827
52. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Reference sequence checksums for provenance identification in RNA-seq. PLoS Comput Biol. 2020;16. https://
doi.org/10.1371/journal.pcbi.1007664 PMID: 32097405
53. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data
with DESeq2. Genome Biol. 2014; 15: 550. https://doi.org/10.1186/s13059-014-0550-8 PMID:
25516281