Il a été montré que le gène Fsa régule l'homéostasie des lipides ainsi que la différenciation cellulaire dans des cellules humaines et chez Caenorhabditis elegans. Dans ce travail de thèse, le gène orthologue de Fsa chez Drosophila melanogaster a été identifié lors d'un criblé génétique cherchant à identifier des interactants de Sara, un gène impliqué dans le trafic membranaire et la signalisation cellulaire. La caractérisation de Fsa de drosophile confirme le rôle de ce gène dans l'homéostasie des lipides et implique Fsa comme régulateur négatif de la signalisation Hedgehog. Etant donné que la signalisation Hedgehog, de même que Fsa, est impliquée dans l'homéostasie des lipides et la différenciation cellulaire, les effets observés de Fsa sont peut-être dus à son rôle de régulateur de la voie de signalisation Hedgehog. De plus, il a été montré que Sara interagit de manière fonctionnelle avec Fsa chez la drosophile en tant que régulateur négatif de la signalisation Hedgehog.

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Analysis of *Drosophila* Fragile Site-Associated (FSA) in Hedgehog Signalling

**THÈSE**

présentée à la Faculté des sciences de l’Université de Genève
pour obtenir le grade de Docteur ès sciences, mention biochimie

par

**Antje Kuhrs**

de

Helmstedt (Allemagne)

Thèse N° 4143

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1. SUMMARY

In multicellular organisms, cells have to send and receive signals to achieve a coordinated development of the organism and response to environmental influences. One class of signalling molecules that cells use to communicate with each other is the Transforming growth factor β (TGFβ) superfamily. The components of the TGFβ superfamily are conserved among species and are involved in a variety of processes, like cell growth, proliferation and differentiation (reviewed in Massagué et al., 2000; Schmierer and Hill, 2007).

After secretion of a TGFβ superfamily ligand by the signal-sending cell, the ligand binds to its receptors on the surface of the signal-receiving cell. This leads to the internalization of the ligand receptor complex into an endocytic compartment and to the recruitment and activation of the transcription factor of the signalling pathway, which translocates into the nucleus and regulates target gene expression (reviewed in Schmierer and Hill, 2007; Rahimi and Leof, 2007).

The protein Smad anchor for receptor activation (SARA) is an endosome-associated scaffold protein that recruits the transcription factor to the ligand receptor complex (Tsukazaki et al., 1998). For the TGFβ/Activin branch of TGFβ superfamily signalling, it has been shown that endocytosis of the receptors and the recruitment of the transcription factor to the internalized receptors by the protein SARA are required for efficient signal transduction. This suggests that signal transduction occurs from internalized receptor complexes (Itoh et al., 2002; Hayes et al., 2002; Runyan et al., 2005).

In epithelial cells of Drosophila melanogaster, SARA localizes to an apical subpopulation of early endosomal compartments. During cell division, these compartments are targeted to the central spindle of the dividing cell and get equally distributed to the two daughter cells. The equal distribution of the SARA-positive endosomes assures the equal inheritance of the TGFβ superfamily signalling state to the
two daughter cells (Boekel et al., 2006). Furthermore, during asymmetric cell division, the SARA-positive endosomes get unequally distributed to the daughter cells (Coumailleau et al., 2009). The function of the apical localization of the SARA-positive endosomes and the regulation of the movement of these compartments during cell division are unknown.

In the present work, using *Drosophila melanogaster* as a model organism, a genetic screen for interactors of *Sara* was carried out in order to achieve a better understanding of SARA function. In this screen, the ortholog of the human gene *Fragile site-associated* (*Fsa*) was identified as a genetic interactor of *Sara*. The human *Fsa* and its ortholog in *Caenorhabditis elegans* have been shown to be involved in the regulation of lipid homeostasis and cell differentiation, whereas the molecular function is unknown (McKay et al, 2003; Wei et al., 2006; Kuo et al., 2006).

The characterization of *Drosophila Fsa* in the present work confirms the conserved role of *Fsa* in lipid homeostasis. In addition, analysis of the *Drosophila Fsa* mutant phenotype and genetic and physical interactions with components of the Hedgehog signalling pathway imply that *Fsa* acts as a negative regulator of Hedgehog signalling. Since Hedgehog signalling, like *Fsa*, is implicated in lipid homeostasis and cell differentiation (Kim et al., 2006; Suh et al., 2007; Jiang and Hui, 2008), the observed effects of *Fsa* might be mediated by its role as a regulator of the Hedgehog signalling pathway. Furthermore, it was shown that *Sara* interacts with *Drosophila Fsa* in its function as a negative regulator of the Hedgehog signalling pathway.
2. RÉSUMÉ
Translated by Dr. Sylvain Loubéry

Dans les organismes multi-cellulaires, les cellules doivent pouvoir émettre et recevoir des signaux pour permettre le développement coordonné de l’organisme et sa réponse aux changements environnementaux. Un type de molécules de signalisation que les cellules utilisent pour communiquer entre elles est la super-famille du Transforming growth factor β (TGFβ). Les membres de cette famille sont conservés entre les espèces et impliqués dans des processus variés tels que la croissance cellulaire, la prolifération et la différenciation (voir pour revue Massagué et al., 2000 ; Schmierer and Hill, 2007).

Une fois le ligand de la famille du TGFβ sécrété par la cellule émettrice du signal, il lie ses récepteurs à la surface de la cellule qui reçoit le signal. Ceci conduit à l’internalisation du complexe récepteur-ligand, ainsi qu’au recrutement et à l’activation de facteurs de transcription de la voie de signalisation ; ceux-ci vont être transloqués dans le noyau et réguler l’expression de gènes cibles (voir pour revue Schmierer and Hill, 2007; Rahimi and Leof, 2007).

La protéine Smad anchor for receptor activation (SARA) est une protéine d’échafaudage endosomale qui recrute le facteur de transcription sur le complexe récepteur-ligand (Tsukazaki et al., 1998). Pour ce qui est de la branche TGFβ/Activin de la super-famille du TGFβ, il a été montré que l’endocytose des récepteurs et le recrutement par la protéine SARA du facteur de transcription sur les récepteurs internalisés sont nécessaires à la bonne transduction du signal. Ceci suggère que la transduction du signal se produit à partir de récepteurs complexés internalisés (Itoh et al., 2002; Hayes et al., 2002; Runyan et al., 2005).

Dans les cellules épithéliales de Drosophila melanogaster, il a été montré que SARA est localisée sur une population apicale de compartiments d’endocytose précoce. Lors de la division cellulaire, ces compartiments sont adressés au fuseau central de la cellule, avant
d’être équitablement répartis entre les deux cellules-filles. Cette distribution équitable des endosomes positifs pour SARA permet aux deux cellules-filles de conserver le même niveau de signalisation TGFβ (Boekel et al., 2006). Par ailleurs il a été montré que lors d’une division asymétrique, les endosomes positifs pour SARA sont inégalement distribués entre les cellules-filles (Coumailleau et al., 2009). La fonction de la localisation apicale des endosomes positifs pour SARA et la régulation des mouvements de ces compartiments ne sont pas compris.

Dans le cadre de cette étude, utilisant Drosophila melanogaster comme organisme modèle, un crible génétique pour des interactants de Sara a été mené dans le but d’atteindre une meilleure compréhension de la fonction de Sara. Dans ce crible, l’orthologue du gène humain Fragile site-associated (Fsa) a été identifié comme interactant génétique de Sara. Le gène Fsa chez l’homme et son orthologue chez Caenorhabditis elegans ont été montrés comme étant impliqués dans la régulation de l’homéostasie des lipides et la différenciation cellulaire, la fonction moléculaire de la protéine étant quant à elle inconnue (McKay et al, 2003; Wei et al., 2006; Kuo et al., 2006).

La caractérisation de Fsa chez la drosophile dans cette étude confirme la conservation de son rôle dans l’homéostasie des lipides. En outre, l’analyse des phénotypes de drosophiles mutantes pour Fsa et des interactions génétiques et physiques avec des composants de la voie de signalisation Hedgehog montrent que Fsa agit comme un régulateur négatif de cette voie de signalisation. Comme la voie de signalisation Hedgehog, tout comme Fsa, est impliquée dans l’homéostasie des lipides et la différenciation cellulaire (Kim et al., 2006; Suh et al., 2007; Jiang and Hui, 2008), l’action de Fsa dans ce contexte pourrait être médée par son rôle de régulateur de la voie de signalisation Hedgehog. Enfin, il a été montré que Sara interagit avec Fsa chez la drosophile dans sa fonction de régulateur négatif de la voie de signalisation Hedgehog.
3. THESIS OUTLINE

The Introduction gives an overview of the two signalling pathways that are relevant for the work presented, the TGFβ superfamily and Hedgehog signalling pathways. In the chapter about TGFβ superfamily signalling, observations about Sara, the gene of which genetic interactors were searched for in this work, are especially emphasized. The chapter on Hedgehog signalling includes descriptions of processes in which Fsa, which was identified as a genetic interactor of Sara, was analyzed. Afterwards, the literature on FSA is summarized. In the Results, the genetic screen for interaction partners of Sara and the identification of the Drosophila Fsa as an interactor of Sara are described. In addition, the characterization of Drosophila Fsa in lipid homeostasis and Hedgehog signalling is presented. In the Discussion, the results are evaluated and discussed, and experiments for further investigations are suggested.
4. INTRODUCTION

4.1. TGFβ superfamily signalling

4.1.1. Overview of the TGF β signalling pathway

The components of the TGFβ superfamily signalling pathway are conserved among species and are involved in a variety of processes, like cell growth, proliferation, differentiation and apoptosis (Massagué et al., 2000; Siegel and Massagué, 2003). The ligands of the TGFβ superfamily (at least 30 in humans and 7 in Drosophila) are cleaved by proteases after synthesis and secreted as dimeric growth factors (Gentry et al., 1988; Dubois et al., 1995; Constam and Robertson, 1999). They form mainly homodimers, though some heterodimer formation has been reported (Panganiban et al., 1990; Shimmi et al., 2005; Yeo and Whitman, 2001). After secretion, they bind to receptor dimers, which induces the formation of tetramers and the phosphorylation of type I receptor by the constitutively active type II receptor (Yamashita et al., 1994; Mathews and Vale, 1993; Wrana et al., 1994). The receptors are single-pass transmembrane proteins with an intracellular Serin-Threonin kinase domain (Lin et al., 1992; Wrana et al., 1992). Receptor-Smad (R-Smad) proteins are recruited to the receptor complex and phosphorylated by the type I receptor (Kretzschmar et al., 1997; Macias-Silva et al., 1996). The phosphorylated R-Smad proteins form a complex with the Common Smad (Co-Smad), translocate into the nucleus and regulate the expression of target genes (Lagna et al., 1996; Wisotzkey et al., 1998) (Figure 1). TGFβ signalling is negatively regulated by inhibitory Smads, which inhibit the pathway by competing with R-Smads for binding to the type I receptor or targeting of the receptor for degradation (Hayashi et al., 1997; Kavsak et al., 2000, Ebisawa et al., 2001).

The type I receptors of the TGFβ superfamily phosphorylate only a subset of R-Smads, which subdivides the superfamily into two branches, the TGFβ/Activin branch and the Bone morphogenetic protein (BMP) branch. While the type I receptors of the
TGFβ/Activin branch phosphorylate the R-Smads Smad2 and Smad3, the receptors of the BMP family phosphorylate Smad1, Smad3 and Smad8 (Feng and Derynck, 1997; Chen et al., 1998; Persson et al., 1998). However, many ligands and type II receptors of the TGFβ superfamily pathway cannot be clearly assigned to one branch of the pathway, since some type II receptors form complexes with type I receptors of both branches, and several ligands bind to receptor complexes of both the TGFβ/Activin and the BMP branch (Goumans et al., 2003; Macias-Silva et al., 1998; Nishito et al., 1996). Likewise, the single Co-Smad is part of both pathways (Zhang et al., 1997).

Figure 1. TGFβ superfamily signalling pathway. See text for details.
4.1.2. SARA

The protein SARA was identified as a component of the TGFβ/Activin branch of the TGFβ superfamily signalling pathway. It acts as a scaffold protein that recruits R-Smads to the receptor complex (Tsukazaki et al., 1998). The SARA protein contains a FYVE domain, which binds phosphatidyl inositol-3-phosphate and thereby localizes the SARA protein to early endosomes. The endosomal localization of SARA and endocytosis of the Activin receptor have been shown to be required for efficient Activin signalling in mammalian culture cells (Itoh et al., 2002; Hayes et al., 2002; Runyan et al., 2005).

SARA has also been shown to be involved in endosomal membrane trafficking. Overexpression of $\textit{Sara}$ leads to an enlargement of early endosomal compartments and slows down the recycling of Transferrin receptors to the plasma membrane (Panopoulou et al., 2002; Hu et al., 2002).

In $\textit{Drosophila}$, the localization of SARA to a subpopulation of early endosomes and an enlargement of these compartments caused by $\textit{Sara}$ overexpression was confirmed (Boekel et al., 2006). Whereas no link between SARA and the Activin branch of TGFβ superfamily signalling has been reported in $\textit{Drosophila}$, SARA is implicated in BMP signalling. SARA recruits the Protein phosphatase 1c (PP1c), a negative regulator of BMP signalling, to the BMP receptor complex (Bennet and Alphey, 2002), which leads to the dephosphorylation of the type I receptor by PP1c (Shi et al., 2004).

In addition, it was shown in $\textit{Drosophila}$ that the SARA-positive endosomes represent a multivesicular population of early endosomes that are located apically in polarized epithelial cells. These endosomes contain the BMP ligand and receptor complex. During cell division, the SARA-positive endosomes are targeted to the mitotic spindle midzone (Figure 2) and get equally distributed to the daughter cells. This equal segregation of the SARA endosomes is required for the equal inheritance of the BMP signalling state to the two daughter cells. In the absence of SARA, daughter cells inherit unequal BMP signalling states. This is consistent with the observation that SARA mutant flies exhibit
defects in wing vein differentiation (Figure 3), a process that requires BMP signalling
(Boekel et al., 2006).

![Figure 2. SARA localizes to the central spindle during cytokinesis of Drosophila wing imaginal disc cells. Dividing cell expressing GFP-SARA stained for GFP (green) and FasciclinIII (red). Scale bar: 2µm. Picture taken from Boekel et al., 2006.]

In asymmetrically dividing Sensory Organ Precursor (SOP) cells of Drosophila, the SARA-positive endosomes are unequally distributed during mitosis (Coumailleau et al., 2009). After the division of the SOP, Notch signalling is activated in only one of the two daughter cells and controls its fate (Guo et al., 1996). The majority of SARA-positive endosomes gets targeted to the daughter cell in which Notch signalling is activated after the division. The asymmetrically distributed SARA-positive endosomes contain the ligand and receptor of the Notch signalling pathway. Asymmetric distribution of these endosomes leads to an enhancement of Notch signalling in one daughter cell and a reduction of Notch signalling in the other daughter cell. In contrast to the requirement of SARA for the equal distribution of BMP signalling during symmetric cell division, the presence of SARA is not required for the asymmetric distribution of the Notch signalling ligand and receptor during asymmetric cell division (Coumailleau et al., 2009).
4.2. Hedgehog signalling

4.2.1. Overview of the Hedgehog signalling pathway

Like the TGFβ superfamily and Notch signalling pathways, the two pathways that SARA is implicated in, the Hedgehog signalling pathway is a well conserved signalling pathway in multicellular organisms (Huangfu and Anderson, 2006). Hedgehog signalling regulates many different processes, both during development and also in the adult organism. During development, it regulates the patterning and growth of tissues, such as the Drosophila wing or the vertebrate neural tube. In these tissues, the Hedgehog signalling ligand acts as a morphogen that is released from signal-sending cells into the target tissue, where it forms a concentration gradient that activates target genes in a concentration-dependent manner (Stringini and Cohen, 1997; Briscoe et al., 2001). In the adult organisms, Hedgehog signalling is involved in the regulation of cell division and differentiation. Therefore, misregulation of the pathway can lead to tumour formation (Hatton et al., 2008; Ward et al., 2009; Cretnik et al., 2009).

In the signal-sending cells that produce the ligand Hedgehog (HH), the protein is covalently linked to two lipids. By an autoprocessing mechanism, the C-terminal part of
the HH protein is removed, and cholesterol is linked to the new C-terminus (Lee et al., 1994; Bumcrot et al., 1995). The acyltransferase Skinny Hedgehog adds a palmitate to the N-terminus of the protein (Chamoun et al., 2001). The release of the lipid-modified HH ligand from the signal-sending cells requires the protein Dispatched, which is a membrane protein with similarity to the HH receptor (Burke et al., 1999; Caspary et al., 2002). How Dispatched leads to the release of the ligand is not known.

The ligand HH forms multimers. In mammalian cell culture, it was shown that HH forms a multimer of a size consistent with six HH molecules forming one complex. The multimeric form has a higher signalling activity compared to the HH monomer (Chen et al., 2004; Goetz et al., 2006; Zeng et al., 2001). Both lipid modifications and interactions between the proteins are required for multimer formation (Chen et al., 2004; Callejo et al., 2006; Goetz et al., 2006). In Drosophila, HH is part of bigger complexes (Chen et al., 2004). It was suggested that these complexes represent lipoprotein particles to which the HH protein is associated (Panakova et al., 2005). In addition to their role in multimer formation, the lipid modifications restrict the movement of the HH ligand in tissues by interactions with components of the extracellular matrix, such as heparan sulfate proteoglycans (Han et al., 2004; Takei et al., 2004; Gallet et al., 2008; Callejo et al., 2006).

The signal-receiving cells express Patched (PTC), the receptor of HH. In the absence of the ligand, the 12 transmembrane domain protein PTC represses the signal transducer Smoothened (SMO) (Chen and Struhl, 1996). The mechanism by which PTC represses SMO is unknown. A repression by direct interaction between PTC and SMO seems unlikely, since few PTC molecules can inhibit many SMO molecules (Ingham et al., 2000; Taipale et al., 2002). Several observations suggest that PTC acts as transporter of small lipophilic molecules that regulate SMO activity. Many components similar to steroids can inhibit SMO, among them the drug cyclopamine (Chen et al., 2002; Yang et al., 2009). PTC is similar to bacterial Resistance-Nodulation-Division (RND) transporters, proton antiporters that transport different types of molecules across membranes, and conserved residues required for transporter function are necessary for the activity of PTC.
In addition, a non cell-autonomous repression of Hedgehog signalling by PTC was observed in mammalian cell culture, and PTC overexpression in cells lead to an enrichment of $3\beta$-hydroxysteroids in the medium, which were shown to inhibit Hedgehog signalling (Bijlsma et al., 2006). Furthermore, patients with the disease Smith-Lemli-Opitz syndrome, which is characterized by an accumulation of the $3\beta$-hydroxysteroid 7-dehydrocholesterol, show symptoms that are characteristic of reduced Hedgehog signalling levels (Kelley et al., 1996; Cooper et al., 2003). In addition to its function as a repressor of SMO, PTC restricts the spreading of the HH and thereby limits its signalling range (Chen and Struhl, 1996).

The protein SMO is a seven-transmembrane protein. In the absence of HH, it localizes to the plasma membrane and internal vesicles and is part of a protein complex containing the proteins Costal2 (COS2), Fused (FU) and Cubitus interruptus (CI), the transcription factor of the pathway (Lum et al., 2003; Ruel et al., 2007). This complex recruits the kinases Protein kinase A (PKA), Glycogen synthase kinase 3 and Casein kinase 1, which phosphorylate CI (Smelkinson et al., 2007). The phosphorylated CI is recognized by an E3 ubiquitination complex containing the F-box protein Supernumerary limbs (SLMB) (Jiang and Struhl, 1998; Jia et al., 2005; Smelkinson and Kalderon, 2006). Ubiquitination of CI leads to its targeting to the proteasome and to the partial degradation of CI (Aza-Blanc et al., 1997; Jiang and Struhl, 1998). The tight folding of the Zinc finger domain of CI was shown to serve as a stop signal for the proteasomal degradation (Wang and Price, 2008). The remaining part of CI is the repressor form of CI, which translocates into the nucleus and represses Hedgehog target genes (Aza-Blanc et al., 1997). In the presence of HH, SMO is released from the repression by PTC (Chen and Struhl, 1996). The activation of SMO is associated with conformational changes, phosphorylation and altered localization in the cell (Zhao et al., 2007; Chen et al., 2004). In Drosophila, SMO gets enriched at the plasma membrane, and in vertebrate cells, it localizes to the primary cilium (Zhu et al., 2003; Wilson et al., 2009; Rohatgi et al., 2007). Several components that are involved in primary cilia formation are required for Hedgehog signalling, suggesting that the localization of SMO to the primary cilium is required for signalling (Wilson et al., 2009). The activation of SMO leads, by an unknown mechanism, to the
release of the full-length CI from the complex with COS2 and FU (Ruel et al., 2003; Ruel et al., 2007). The full-length CI represents the activator form of CI and activates Hedgehog target genes. One of the Hedgehog target genes is the receptor ptc (Tabata and Kornberg, 1994). Activation of the expression of the negative regulator ptc by Hedgehog signalling constitutes a negative feedback loop. In addition to the direct interaction of SMO with downstream components of the signalling pathway, SMO acts as a G protein-coupled receptor. It interacts with Galphai, which leads to reduced levels of cytoplasmic cyclic AMP, an activator of PKA. Phosphorylation of CI by PKA is required for the processing of CI into its repressor form (Odgen et al., 2008) (Figure 4).

**Figure 4. Hedgehog signalling pathway.** Hedgehog signalling cascade in the absence (left) or presence (right) of the ligand HH. See text for details.
4.2.2. Hedgehog signalling in Drosophila segmentation

One of the processes during Drosophila development that are regulated by Hedgehog signalling is the segmentation along the anterior-posterior body axis. The segmented pattern, characterized by repeating units of expression of segment polarity genes, is established in a cascade of transient gene expression during the first hours of embryonic development. In this cascade, the embryo gets progressively subdivided into smaller areas (Figure 5). The cascade is initiated by the products of maternal effect genes, which are deposited at the anterior or posterior pole of the embryo (Berleth et al., 1988; Driever and Nuesslein-Volhard, 1988). The proteins encoded by these genes form concentration gradients and activate the expression of gap genes, which subdivide the embryo into broad domains (Tautz 1988; Irish et al., 1989). Gap genes activate the expression of pair-rule genes in seven stripes along the anterior-posterior axis. Each stripe of pair-rule gene expression is regulated by different regulatory elements that respond to different combinations of gap genes (Frasch and Levine, 1987; Carroll and Scott, 1986; Pankratz et al., 1990). The pair-rule genes finally activate the expression of segment polarity genes, such as engrailed (en) and wingless (wg), in 14 stripes along the anterior-posterior body axis (Howard and Ingham, 1986; Ingham et al., 1988). The expression of segment polarity genes in 14 stripes is achieved by their regulation by more than one pair-rule gene. For example, the expression of en is activated by the two pair-rule genes fushi tarazu (ftz) and even skipped (eve), each of which is expressed in seven non-overlapping stripes (Ingham et al., 1988) (Figure 5).
Figure 5. *Drosophila* segmentation. Cascade of gene expression in the *Drosophila* embryo during segmentation. Examples of genes for each class of segmentation genes are shown.

After the initiation of the segment polarity genes *en* and *wg* in adjacent stripes, they transiently regulate each other to stabilize their expression. The *wg* gene is expressed in a stripe anterior to the cells that express *en*. EN activates the expression of the ligand HH, which activates Hedgehog signalling in the neighbouring cells. Hedgehog signalling leads to the expression of the protein WG, which in turn induces EN and thereby HH expression in the neighbouring cells (DiNardo et al., 1988; Hidalgo and Ingham, 1990). The expression of *en* and *wg* does not only depend on their mutual activation, but also on their regulation by other genes, such as sloppy paired (*slp*). Hedgehog signalling can activate *wg* expression only in the presence of the gene product of *slp*. Therefore, the expression pattern of *slp* defines a zone in which *wg* can be activated by Hedgehog signalling (WG competent zone in Figure 6). In addition to its requirement for the activation of *wg*, *slp* inhibits the expression of *en*, thereby restricting the zone in which *en* can be activated by WG (EN competent zone in Figure 6). Because the cells that are anterior to the *wg*-expressing cells are not competent to express *en* and the cells posterior to the *en*-expressing cells are not competent to express *wg*, *wg* is activated only anterior to the *en* expressing cells and *en* only posterior to the *wg*-expressing cells (Cadigan et al.,
Elevated Hedgehog signalling levels, for example in *ptc* mutants, lead to the expression of *wg* in all cells that are competent to express *wg*. This results in the proximity of *wg*-expressing cells with cells competent to express *en* anterior to the *wg* expression domain, leading to ectopic expression of *en* in between the normal *en* stripes (Martinez Arias et al., 1988; Nakano et al., 1989; Hidalgo and Ingham, 1990; Ingham and Hidalgo, 1993) (Figure 6).

**Figure 6.** Broadening of the WG-expressing domain and ectopic EN expression in *ptc* mutant *Drosophila* embryos. Wildtype and *ptc* mutant *Drosophila* embryos stained for WG in red and EN in green. Anterior is left, dorsal up. Scale bar: 100µm. Higher magnifications and a cartoon showing the WG and EN expression are shown to the right of the embryos. Scale bar: 20µm. In the cartoon, WG and EN competent zones are indicated.

### 4.2.3. Hedgehog signalling in *Drosophila* sensory bristle specification

In *Drosophila*, Hedgehog signalling does not only control patterning processes in the embryo, but also during later stages of development. One example is the specification of sensory bristles called macrochaetae on the notum of the adult fly. The number and position of macrochaetae is regulated in a two-step process. In the first step, different combinations of transcription factors, called prepattern genes, activate expression of the
proneural genes *achaete* and *scute* in clusters of 10-20 cells in the wing imaginal disc, the larval tissue that gives rise to the adult wing and notum (Gomez-Skarmeta and Modolell, 1996; Leyns et al., 1996; Skeath and Carroll, 1991; Cubas et al., 1991). The expression of *achaete* and *scute* in each cluster is regulated by a cluster-specific enhancer (Gomez-Skarmeta et al., 1995). In the second step, one or two cells of each proneural cluster develop into SOPs, each of which gives rise to the four cells of the sensory bristles. Selection of the cells that develop into SOPs is regulated by a process called lateral inhibition. Lateral inhibition is mediated by a feedback loop in which the proneural genes *achaete* and *scute* activate the expression of the Notch signalling ligand Delta in the future SOP cell and Notch signalling inhibits the expression of *achaete* and *scute* in the neighbouring cells. By this mechanism, the expression of the proneural genes is stabilized in the future SOP cell and repressed in its neighbouring cells (Skeath and Carroll, 1991; Simpson and Carteret, 1998; Kunisch et al., 1994).

Figure 7. Fate map of macrochaetae of the *Drosophila* notum. Top: wing imaginal disc. Proneural clusters that give rise to macrochaetae in the notum are indicated (dots). The posterior compartment expressing HH and the HH gradient in the anterior compartment are marked in green. Bottom: macrochaetae in the heminotum. Scutellar macrochaetae and proneural clusters are red.
Hedgehog signalling is involved in the prepatterning process. The ligand HH is expressed in the posterior compartment of the wing imaginal disc and forms a concentration gradient in the anterior compartment (Figure 7), where it activates target genes (Chen and Struhl, 1996). Hedgehog signalling regulates the number of bristles in the entire notum, which is subdivided into the scutum and the scutellum. In the scutum, Hedgehog signalling regulates the number of bristles through the activation of the prepattern gene decapentaplegic (dpp). Elevated Hedgehog signalling levels (for example in ptc mutants) lead to an increase in bristle number, whereas reduced Hedgehog signalling decreases the number of bristles (Gomez-Skarmeta and Modolell, 1996; Mullor et al., 1997). Hedgehog signalling exerts the same effect on the number of scutellar bristles (Figure 8), which are specified close to the Hedgehog expression domain (Figure 7). However, this effect is independent of the target gene dpp, and the number of scutellar bristles is more sensitive to changes in Hedgehog signalling levels compared to the bristles in the scutum (Mullor et al., 1997).

**Figure 8. Elevated Hedgehog signalling levels increase the number of scutellar bristles in the Drosophila scutellum.** G: wildtype notum showing localization of dorsocentral (DC) bristles in the scutum and scutellar (SC) bristles. K: ptc mutant notum; arrow shows extra bristles in the scutellum. Pictures taken from Mullor et al., 1997

### 4.2.4. Hedgehog signalling in tissue growth control

The regulation of several processes by Hedgehog signalling is conserved in multicellular organisms. One of these processes is the regulation of tissue growth. Examples are the growth of the vertebrate cerebellum (Dahmane and Ruiz i Altaba, 1999; Wallace 1999), cell proliferation during hair follicle morphogenesis in mammals (Chiang et al., 1999) and the regulation of wing size in *Drosophila* (Burke and Basler, 1996; Martin-Castellanos and Edgar, 2002). Furthermore, Hedgehog signalling is required for the maintenance of stem cells in various systems (Zhang and Kalderon, 2001; Balordi and
Many studies indicate that the effect of Hedgehog signalling on tissue growth is mediated by its effect on cell cycle regulators. In vertebrate culture cells, Hedgehog signalling upregulates the expression of Cyclin D, which induces the expression of Cyclin E and thereby promotes entry into the S phase of the cell cycle (Kenney and Rowitch, 2000; Yoon et al., 2002). The same effect of Hedgehog signalling on Cyclin D expression was observed in the *Drosophila* eye. In addition, it was shown in *Drosophila* that Hedgehog signalling also directly induces the expression of Cyclin E (Duman-Scheel et al., 2002). Interestingly, another study in *Drosophila* shows that PTC directly interacts with Cyclin B1 and controls its subcellular localization, thereby directly affecting M phase entry independently of the transcription factor CI. In the presence of the ligand of the Hedgehog signalling pathway, the interaction between PTC and Cyclin B1 is disrupted, which allows B1 to enter into the nucleus and induce M phase entry (Barnes et al., 2001). Recently, it was shown in the mice brain that CI represses the tumour suppressor p53, thereby promoting the proliferation of neural stem cells (Stecca and Ruiz i Altaba, 2009).

In addition to its regulation of cell proliferation, Hedgehog signalling also affects the survival of cells. HH was shown to act as a survival factor in the vertebrate neural tube, and its removal leads to increased cell death (Litingtung and Chiang, 2000; Charrier et al., 2001). Thibert et al. showed that the inhibitory effect of Hedgehog signalling on apoptosis is mediated by the repression of the pro-apoptotic activity of the receptor PTC (Thibert et al., 2003). PTC acts as a dependence receptor, which induce apoptosis in the absence of their ligands. In the absence of ligand, PTC is cleaved by caspases, which leads to the activation of its pro-apoptotic domain and to the recruitment of a caspase-activating complex, leading to cell death (Mille et al., 2009).

Consistent with an important function of Hedgehog signalling in the control of tissue growth and stem cell maintenance, changes of Hedgehog signalling levels are associated with the formation of tumours. In many human tumours, Hedgehog signalling is elevated (Hynes et al., 1997; Dahmane et al., 1997; Dahmane et al., 2001), and mutations in the
gene PTC are associated with several tumours, such as basal cell carcinomas and medulloblastomas (Thomas et al., 2009; Cretnik et al., 2009). Furthermore, mice in which Hedgehog signalling is elevated, for examples in Ptc mutants, show a predisposition to develop tumours (Goodrich et al., 1997; Hahn et al., 1998).

4.2.5. Hedgehog signalling in adipogenesis

Another conserved function of Hedgehog signalling in multicellular organisms is the regulation of adipogenesis. The regulation of adipogenesis by Hedgehog signalling was shown in Drosophila and in the differentiation of mammalian adipocytes (Suh et al., 2006; Kim et al., 2007; Li et al., 2008). Mammalian adipocytes are cells that are derived from mesenchymal stem cells and are specialized in the storage of lipids and the regulation of lipid homeostasis. Adipocyte differentiation is induced by hormones like thyroid hormone, insulin, growth hormone and sex steroids, which lead to the expression of the master regulator of adipogenesis, the orphan nuclear hormone receptor Peroxisome proliferator-activated receptor γ (PPARγ) (reviewed in Rosen and MacDougald 2006; Tontonoz and Spiegelman 2008; Kiess et al., 2008). PPARγ induces the expression of several adipogenic genes, including genes required for lipid uptake, triacylglyceride (TAG) storage, insulin sensitivity and secretion of adipokines (Tontonoz and Spiegelman, 1994; Ren et al., 2002; Mueller et al., 2002). One of the target genes of PPARγ is the transcription factor CCAAT/Enhancer binding protein α (C/EBPα), which in turn activates the expression of PPARγ (Barak et al., 1999; Rosen et al., 2002). PPARγ is not only required for the differentiation of cells into adipocytes, but also for the maintenance of the differentiated state (Tamori et al., 2002).

Several studies have investigated the role of Hedgehog signalling in the differentiation of adipose cells. The delivery of antibodies that inactivate Hedgehog signalling protects mice from diet-induced weight gain, probably caused by a reduced absorption and secretion of TAG by the gut cells (Wang et al., 2002; Buhman et al., 2004). Likewise,
treatment of mice with a recombinant HH ligand increases the body weight and fat mass (Martin et al., 2002). In contrast to these observations, mice mutant for the HH receptor ptc, which is a negative regulator of Hedgehog signalling, show reduced body fat and weight and reduced expression of adipogenic transcription factors like C/EBPα and PPARγ (Sweet et al., 1996; Makino et al., 2001; Li et al., 2008). An inhibiting role of Hedgehog signalling on adipocyte differentiation has also been observed in mammalian pluripotent culture cells stimulated to undergo differentiation into adipocytes. Cells treated with recombinant HH ligand or the drug purmorphamine, which activates Hedgehog signalling, show less lipid droplets, reduced TAG levels and lower expression levels of C/EBPα and PPARγ compared to non-treated cells (Zehentner et al., 2000; Spinella-Jaegle et al., 2001; Wu et al., 2004; Suh et al., 2006; Fontaine et al., 2008). Inhibition of Hedgehog signalling by the drug cyclopamine or expression of a dominant negative transcription factor of the Hedgehog signalling pathway inhibits adipocyte differentiation (Suh et al., 2006; Kim et al., 2007). Reduced expression of PPARγ after Hedgehog signalling activation and the ability to overcome the effect of Hedgehog signalling activation on adipocyte differentiation by overexpression of PPARγ suggest that Hedgehog signalling acts upstream of PPARγ expression (Suh et al., 2006). The effect of Hedgehog signalling on PPARγ and C/EBPα expression might be mediated by the transcription factors GATA and Chicken ovalbumin upstream promotor-transcription factor II (COUP-TFII). GATA factors are induced by HH and inhibit PPARγ expression, and the induction of GATA factors can overcome the HH-dependent inhibition of adipogenesis (Suh et al., 2006). Recently, COUP-TFII has been shown to cooperate with GATA factors to inhibit adipogenesis downstream of Hedgehog signalling (Xu et al., 2008).

An inhibiting role of Hedgehog signalling on fat formation has also been observed in Drosophila melanogaster. Activation of Hedgehog signalling in the larval fat body, the adipose tissue of the larva, decreases fat mass, the size of lipid droplets, TAG content and expression of markers of adipose tissues, whereas inhibition of Hedgehog signalling has the opposite effect (Suh et al., 2006).
4.3. FSA

In the work presented, the gene *Fsa* was identified as a negative regulator of Hedgehog signalling in *Drosophila*. While Hedgehog signalling has an inhibitory effect on fat formation, the gene *Fsa* was shown to acts as a promotor of fat formation. *Fsa* is conserved in different species, like human, mouse, fly and worm (Wei et al., 2006). In mouse and the worm *Ceanorhabditis elegans*, *Fsa* has been shown to be involved in lipid homeostasis. The cells of the fat storage tissue in worms that are depleted for FSA contain less lipid droplets than wildtype worms (McKay et al., 2003). In a mouse cell line that serves as a model for fat cell differentiation, increased levels of *Fsa* expression are observed during differentiation into fat cells (Wei et al., 2006). Depletion of the FSA protein leads to a lower content of lipid droplets in these cells and to a reduced expression of the transcription factor PPARγ, which is involved in fat cell differentiation. Depletion of PPARγ does not have any effect on *Fsa* expression. This suggests that FSA acts upstream of PPARγ expression (McKay et al, 2003).

Originally, *Fsa* was identified as a gene located in the fragile site 1q31 in a Chinese hamster ovary cell line (Wei et al., 2006). Chromosome breakage at this fragile site initiates the amplification of the multiple drug resistance transporter *mdr1*, a locus that is amplified in many drug-resistant cell lines (Kuo et al., 1994). Like many genes that are associated with fragile sites, the hamster *Fsa* gene is large, coding for more than 5000 amino acids (Wei et al., 2006).

The human *Fsa*, which shares > 90% similarity with the hamster *Fsa*, is expressed in many human tissues, with elevated levels of expression in testis and ovaries. In tissues that contain proliferating and non-proliferating compartments, like testis, skin or colon, *Fsa* is strongly expressed in the non-proliferating cells, whereas proliferating cells and tumor cells show low levels of expression (Kuo et al., 2006).
5. RESULTS

5.1. Validation of Drosophila as a model system to study SARA function

The aim of this work was to identify factors that functionally interact with SARA, using Drosophila as a model organism. Many observations about SARA have been made both in Drosophila and in vertebrate systems, which suggests that the function of SARA is conserved among species. In both systems, it was shown that SARA localizes to early endosomes and that overexpression of the SARA protein leads to an enlargement of these compartments (Tsukazaki et al., 1998; Panopoulou et al., 2002; Hu et al., 2002; Boekel et al., 2006). Furthermore, SARA was shown to be involved in TGFβ superfamily signalling both in Drosophila and in vertebrate cells (Itoh et al., 2002; Hayes et al., 2002; Runyan et al., 2005; Bennet and Alphey, 2002; Boekel et al., 2006). However, certain observations about SARA have been reported only in vertebrate systems, while others were shown only for Drosophila. In vertebrates, SARA has a well established role as an adaptor protein that recruits the R-Smad of the TGFβ/Activin branch to the receptor complex (Tsukazaki et al., 1998; Itoh et al., 2002; Hayes et al., 2002). In Drosophila, SARA was shown to be implicated in the BMP branch of TGFβ superfamily signalling, while an involvement of SARA in the TGFβ/Activin branch has not been reported (Bennet and Alphey, 2002; Boekel et al., 2006). Furthermore, the subcellular localization of SARA during cell division was only studied in Drosophila (Boekel et al., 2006; Coumailleau et al., 2009).

To validate Drosophila as a suitable system to study the function of SARA, it was tested whether some of the observations about SARA that were reported for only one model system also apply for the other. Direct binding of SARA to the R-Smads of the TGFβ/Activin branch of TGFβ superfamily signalling was reported for Xenopus laevis and human SARA using different assays, like phage display, GST-pulldown and co-immunoprecipitation (Tsukazaki et al., 1998). In Drosophila, the only ortholog of the TGFβ/Activin R-Smad is the protein Smad on X (SMOX) (Henderson and Andrew,
1998). To test whether SARA and SMOX interact in *Drosophila*, lysates of embryos expressing a MYC-tagged SARA protein and a GFP-tagged SMOX protein were used to test for the interaction between SARA and SMOX in a co-immunoprecipitation assay. GFP-SMOX was detected in immunoprecipitates of MYC-SARA (Figure 9). These results show that *Drosophila* SARA, like vertebrate SARA, binds to the R-Smad of the TGFβ/Activin branch of TGFβ superfamily signalling and suggest that the role of SARA as an adaptor molecule is conserved in *Drosophila*.

**Figure 9. Co-Immunoprecipitation of SARA and SMOX in Drosophila embryos.** Immunoprecipitates (IP) of MYC-SARA (using an anti-MYC antibody) from embryos expressing MYC-SARA and GFP-SMOX or only GFP-SMOX (negative control) were immunoblotted using an anti-GFP antibody to detect GFP-SMOX. The same samples were immunoblotted using an anti-SARA antibody to test for successful immunoprecipitation of the MYC-SARA protein. 3% of the embryo lysate used for the immunoprecipitation (Input) was loaded to confirm presence of the proteins in the embryo lysate.
In mammalian cells, it was shown that the SARA protein is phosphorylated. The phosphorylation state of SARA does not depend on the level of TGFβ/Activin signalling, and the function of the phosphorylation is unknown (Tsukazaki et al., 1998). The immunoprecipitation assay for SARA allowed to test whether the SARA protein is also phosphorylated in *Drosophila*. Tagged SARA proteins were expressed in *Drosophila* embryos and S2 culture cells. Half of the SARA immunoprecipitates were subjected to treatment with Calf Intestinal Phosphatase (CIP), and immunoprecipitates were stained with Pro-Q Diamond phosphoprotein stain (Steinberg et al., 2003) to assay the phosphorylation level of the SARA protein. The tagged SARA proteins were stained by the phosphoprotein stain both in immunoprecipitates from embryos and culture cells, and the staining was strongly reduced after phosphatase treatment (Figure 10). These results show that *Drosophila* SARA, like vertebrate SARA, is phosphorylated.

![Figure 10](image.png)

**Figure 10. Phosphorylation of SARA in *Drosophila* embryos and S2 culture cells.** SARA immunoprecipitates (anti-GFP for S2 cells and anti-MYC for embryos) separated on SDS protein gels stained with coomassie and Pro-Q Diamond phosphoprotein stain. + CIP: treated with CIP, - CIP: non-CIP treated.
In *Drosophila*, the SARA protein localizes to the mitotic spindle midzone during symmetric and asymmetric cell divisions (Boekel et al., 2006; Coumailleau et al., 2009). To test whether SARA shows this localization also in different organisms, human HeLa cells were stained with antibodies against SARA. In dividing HeLa cells, a clear accumulation of endogenous SARA at the mitotic spindle midzone was observed (Figure 11).

![Figure 11. Localization of SARA to the mitotic spindle midzone of HeLa cells.](image)

The results described above add to the list of observations about SARA that are common in *Drosophila* and vertebrates: the binding of SARA to the R-Smad of the TGFβ/Activin branch of the TGFβ superfamily, the phosphorylation of SARA and the localization of SARA to the mitotic spindle midzone. Taken together, this strongly suggests that the function of SARA is conserved among species, validating *Drosophila* as a suitable model system to study SARA.
5.2. *Drosophila Fsa* is a genetic interactor of *Sara*

5.2.1. Genetic screen for suppressors of lethality caused by *Sara* overexpression

In order to identify interaction partners of *Sara*, a genetic screen for mutations that suppress the lethal effect of *Sara* overexpression was carried out. The screen was based on a mutagenesis performed by Dr. Christian Boekel to isolate mutations in the *Sara* gene. For the mutagenesis, flies carrying a transposable element that contains an inducible promoter were used. The transposable element is located directly upstream of the endogenous *Sara* gene, and induction of the promoter leads to overexpression of *Sara*. Ubiquitous overexpression of *Sara* during development causes lethality. Flies carrying the transposable element were mutagenized with the methylating agent Ethyl Methane Sulphonate (EMS). In their progeny, overexpression of the *Sara* gene was induced, expecting the survival of flies with mutations in the *Sara* gene that impair *Sara* function. Surviving flies were screened for mutations in the *Sara* gene by sequencing of the coding sequence (Boekel et al., 2006). In addition to flies with mutations in the coding sequence of the *Sara* gene, 18 suppressor lines without mutations in the *Sara* coding sequence were obtained. These flies were analyzed in the present study because they might carry mutations in genes that genetically interact with *Sara* and therefore lead to their survival.

After the mutagenesis, only the second chromosome (the one containing the *Sara* gene) of the mutagenized flies was recovered. To exclude that the rescuing effect of the suppressor lines is due to mutations in regulatory regions of the *Sara* gene, the *Sara* gene and the rescuing mutation were separated from each other. For this, the mutagenized chromosomes were recombined with wildtype chromosomes. Flies carrying recombinant chromosomes without the *Sara* gene of the mutagenized chromosome (marked by a marker gene in the transposable element that is inserted directly upstream of the *Sara* gene) were tested for their ability to rescue the lethal effect of *Sara* overexpression. For 10 chromosomes, a rescuing effect was observed (Figure 12).
5.2.2. Identification of *Drosophila Fsa* as an interactor of *Sara*

The strongest rescue of the lethality caused by *Sara* overexpression was observed for the 16-03 mutant (Figure 12). In addition, this mutation enhances a vein patterning defect of *Sara* mutant wings (see Figure 21, page 40). The suppression of a *Sara* gain-of-function phenotype and the enhancement of a *Sara* loss-of-function phenotype by the 16-03 mutant imply that the 16-03 gene and *Sara* act synergistically. To molecularly identify the 16-03 gene, the region of the mutant chromosome that leads to the rescue of *Sara* overexpression lethality was mapped by determining recombination frequencies between the rescuing mutation and markers at known positions on the chromosome. Recombination frequencies increase with increasing distance between mutation and marker. A mapping procedure developed in the Barry Dickson lab, in which PCR fragment length polymorphisms (PLPs) are used as markers, was followed (Berger et al.,
PLPs are polymorphisms that can be detected by polymerase chain reaction (PCR), leading to different PCR product sizes. The mutant chromosome was recombined with reference chromosomes, and the recombinant chromosomes were tested for their ability to rescue the lethal effect of *Sara* overexpression. In addition, for each recombinant chromosome, a PLP analysis was performed to distinguish regions that were inherited from the mutagenized chromosome from regions that were inherited from the reference chromosome (Figure 13).

**Figure 13. 16-03 mapping procedure.** Schematic representation of the mapping strategy used to identify the region in 16-03 that leads to the rescue of the *Sara* overexpression lethality. Red and black lines represent the mutagenized and the reference chromosome, respectively. The cross on the mutagenized chromosome represents the rescuing mutation.

The results of the PLP analysis (Figure 14) indicated that the rescuing mutation maps in the central region of the second chromosome (cytogenetic map 34A-48E). To see if the rescuing region includes a lethal mutation, the mutant chromosome was crossed to a collection of chromosomes with different deletions that together cover between 50 and 80 percent of the second chromosome (Bloomington deficiency kit, Bloomington *Drosophila* stock centre). Four deletions (*Df*(2L)J2, *Df*(2L)FCK-20, *Df*(2L)cact-255rv64 and *Df*(2R)BSC26) that are lethal in transheterozygosity with 16-03 were identified (Figure 14). The deficiency *Df*(2L)cact-255rv64 uncovers part of the rescuing region identified by the PLP analysis (circled in Figure 14).
Figure 14. PLP analysis and lethality mapping of 16-03. The black line represents the second chromosome; numbering according to cytogenetic map. The blue boxes represent the regions uncovered by deficiencies that do not complement the 16-03 mutant (from left to right: Df(2L)J2, Df(2L)FCK-20, Df(2L)cact-255rv64, Df(2R)BSC26). Deficiencies that are synthetic lethal with 16-03 are represented by empty boxes. The deficiency uncovering part of the rescuing region is circled. Below are the results obtained for the PLP analysis for recombinant chromosomes that rescue the lethal effect of Sara overexpression. Each dot represents the PLP result for one recombinant chromosome. PLP isoforms corresponding to the mutagenized chromosome are marked in red, PLP isoforms corresponding to the reference chromosome are marked in black. An example of a DNA gel showing the PLP analysis for primer pair PLP2L43/44 at the cytogenetic location 36A11 is shown. All chromosomes were analyzed in heterozygosity with the same reference chromosome.

To narrow down the region that includes the lethal mutation, several smaller deletions in this region were tested for complementation of 16-03. The two deficiencies Df(2L)TW137 and Df(2L)TW119 complement 16-03, while the deficiency Df(2L)T317 does not complement 16-03 (Figure 15).
Figure 15. Deficiencies used for the mapping of 16-03. Genes are outlined in blue below the base ruler and the cytologic band. Non-complementing and complementing deficiencies of 16-03 are shown below the genes. Continuous lines represent regions that are present in the chromosome, deleted regions are left blank. Regions for which it is not known if they are present or absent are marked by dashed lines.

The breakpoints of the deficiencies Df(2L)T317 and Df(2L)TW137 were not very well defined (dotted lines in Figure 15). To further map the breakpoints of these deficiencies, DNA fragments of flies heterozygous for the deficiency chromosomes were amplified by PCR and sequenced. PCR fragments amplified from regions present on both homologous chromosomes (regions not uncovered by the deficiency) show single nucleotide polymorphisms (SNPs), which can be detected by sequencing. Genomic regions that are uncovered by the deficiency can be amplified only from one chromosome (the reference chromosome), therefore lacking SNPs (Figure 16). Lack of SNPs in amplified DNA fragments might be due to the absence of the region from the deficiency chromosome or due to the lack of SNPs in the amplified region. Therefore, the deficiency chromosomes were combined with two different reference chromosomes, and PCR products lacking SNPs were only considered for the analysis if the two reference chromosomes showed different SNP isoforms in the amplified regions.
Figure 16. Breakpoint mapping of deficiency chromosomes. Continuous lines represent regions that are present in the chromosome, deleted regions are left blank. SNPs can be detected in DNA fragments amplified from genomic regions covered by the deficiency chromosome (green arrows). SNPs are absent from fragments amplified from regions uncovered by the deficiency chromosome (red arrows).

The results of the breakpoint mapping are shown in Figure 17. Based on the deletions that complement or do not complement 16-03, the location of the lethal mutation was narrowed down to the region 36B2-36C1 (Figure 17). Sequencing of the open reading frames in this region lead to the identification of a splice acceptor site mutation in the gene *CG15134* (encircled in Figure 17).

Figure 17. Mapping of 16-03 to the gene *CG15134*. Genes are outlined in blue below the base ruler and the cytologic band. Non-complementing and complementing deficiencies of 16-03 are shown below the genes. Continuous lines represent regions that are present in the chromosome, deleted regions are left blank. Regions for which it is not known if they are present or absent are marked by dashed lines. Red lines enclose the region that includes the lethal mutation based on mapping with deficiencies. The gene *CG15134* is encircled in red.
BLAST analysis of the predicted protein encoded by the gene CG15134 shows similarity to the central part of the human protein FSA and to its ortholog in other species. The N-terminal and C-terminal parts of FSA are similar to the predicted proteins encoded by the genes CG42556 and CG4841, respectively. These genes are the two flanking genes of CG15134. This suggests that the genes CG42556, CG15134 and CG4841 represent a single gene, which was confirmed by Reverse Transcriptase PCR (RT-PCR) products that show a connection of the three genes at the mRNA level. Therefore, the three genes CG42556, CG15134 and CG4841, as annotated by the FlyBase consortium, represent the Drosophila ortholog of Fsa (Figure 18 and Appendix: FSA protein alignment).

Figure 18. Drosophila Fsa gene structure. Drosophila Fsa gene structure (exons: thick red lines, introns: thin red lines), determined by RT-PCR and rapid amplification of 5’ cDNA ends (5’RACE). Below are the cytologic band and the base ruler. Mutations in Fsa alleles are indicated (cDNA and protein).

In order to find out whether the splice acceptor site mutation in Drosophila Fsa is the lethal mutation in this region, the mutant 16-03 was crossed to a collection of flies that carry mutations in the second chromosome (Winkler et al., 2005). Eight non-complementing alleles were obtained. In addition, the homozygous lethal allele l(2)HT-2 that had been mapped to a region of the second chromosome that includes the gene Fsa (Steward and Nüsslein-Vollhard, 1986) also fails to complement the 16-03 mutant. In five non-complementing fly lines, mutations in the Fsa gene were discovered (Figure 18). This implies that the splice acceptor site mutation in Fsa is a lethal mutation. To confirm that Fsa is an essential gene, the lethality of Fsa mutant flies was rescued by exogenous Fsa expression. For this, a construct containing the coding region of Drosophila Fsa under the control of an inducible promotor was inserted into the genome of flies.
Ubiquitous $Fsa$ expression during development rescued the lethality of all tested combinations of $Fsa$ alleles (Figure 19).

![Figure 19. Exogenous $Fsa$ expression rescues lethality of $Fsa$ mutants. Rescue of lethality of transheterozygous $Fsa$ mutant flies by exogenous expression of $Fsa$. $n=$number of surviving flies.](image)

All identified mutants that do not complement the $16-03$ mutant are also lethal in combination with the deficiency that uncovers $Fsa$. In addition, they fail to complement two of the three other deficiencies that were found to be lethal in combination with the mutant $16-03$ ($Df(2L)FCK-20$ and $Df(2R)BSC26$). Furthermore, the deficiencies $Df(2L)FCK-20$ and $Df(2R)BSC26$ do not complement the deficiency that uncovers $Fsa$. This implies that the chromosome $16-03$ does not carry lethal mutations in the regions that are uncovered by these deficiencies, but that the lethality in combination with the mutant $16-03$ is rather due to synthetic lethality.

For four mutants that fail to complement the mutant $16-03$, no mutation was found in the coding sequence of $Fsa$. To test whether their lethality in transheterozygosity with the $16-03$ mutant is due to a mutation in the $Fsa$ gene, their position in the chromosome was mapped by meiotic recombination. The mutants were recombined with flies that carry Prl$^1$ and amos$^{Tr}$, two visible dominant markers close to $Fsa$ and close to one of the
deficiencies that show synthetic lethality in combination with the mutant 16-03, respectively. Flies carrying the recombinant chromosomes were crossed to the mutant 16-03 to test for the presence or absence of the non-complementing mutation, and recombination frequencies between the non-complementing mutations and the dominant markers were determined. The recombination mapping suggests that all non-complementing mutants carry mutations in the Fsa gene (Figure 20).

![Figure 20. Recombination frequencies between dominant markers and alleles that do not complement mutant 16-03. The black line represents the base ruler of part of the second chromosome; positions of Fsa and dominant markers are indicated. The expected recombination frequencies between Fsa and the dominant markers and recombination frequencies between markers and alleles that do complement mutant 16-03 are shown below the base ruler. n=sample size.]

To test if the new Fsa mutant alleles, like the 16-03 allele, genetically interact with Sara, they were tested for their effect on the vein patterning defect of Sara mutant wings and the lethality caused by Sara overexpression. Seven of eight tested Fsa alleles significantly enhance the vein patterning defect observed in Sara mutant wings (Figure 21). These results confirm that the genes Fsa and Sara interact genetically. However, a rescuing effect on Sara overexpression lethality was only observed for allele 16-03 (Figure 12 and data not shown).
Figure 21. *Fsa* alleles enhance vein patterning defects of *Sara* mutant wings. A: representative wings of wildtype flies and flies mutant for *Sara* and/or 16-03. *Df(2R)PK1* is a deficiency that uncovers the *Sara* gene. Arrows point to extra wing vein material. B: Quantification of extra wing vein material (% of total wing area). Error bars: standard deviation. Asterisks indicate that difference to *Fsa* allele/*Df(2R)PK1* is statistically significant (p≤0.05). Sample size: n=20.
5.3. *Drosophila Fsa* is a negative regulator of Hedgehog signalling

5.3.1. FSA binds to components of the Hedgehog signalling pathway

*Sara* is implicated in TGFβ superfamily and Notch signalling (Tsukazaki et al., 1998; Shi et al., 2004; Coumailleau et al., 2009). The genetic interaction between *Sara* and *Fsa* suggests that *Fsa* is also involved in signalling processes. Interestingly, in a Yeast Two-Hybrid screen for interactors of proteins related to cancer and signalling, *Drosophila* FSA was found to interact with two components of the Hedgehog signalling pathway (Formstecher et al., 2005), a signalling pathway that *Sara* has not been related to. The Yeast Two-Hybrid interaction between FSA and components of the Hedgehog signalling pathway raises the question if *Fsa* and *Sara* are involved in Hedgehog signalling.

The proteins chosen for the Yeast Two-Hybrid screen included the proteins CI and SLMB (see Figure 22). For both proteins, and interaction with FSA was observed (Formstecher et al., 2005). CI is the transcription factor of the Hedgehog signalling pathway (Tabata and Kornberg, 1994; Aza-Blanc et al., 1997), while the protein SLMB is involved in the processing of CI into its repressor form (Jiang and Struhl, 1998; Jia et al., 2005; Smelkinson and Kalderon, 2006).
Figure 22. Protein interactions identified in a Yeast Two-Hybrid assay. Hedgehog signalling cascade in the absence (left) or presence (right) of the ligand HH. Proteins identified as interaction partners of FSA in the Yeast Two-Hybrid assay (Y2H) and FSA are marked in red.

Figure 23 shows the regions of the gene Fsa that code for the protein domains interacting with CI and SLMB. The region interacting with CI corresponds to the amino acids 1090 till 1309 in the N-terminal part of FSA (CG42556). The peptide interacting with SLMB is encoded by the central part of Fsa (CG15134), corresponding to the amino acids 2749 till 2905 of FSA (Formstecher et al., 2005, see Appendix: FSA protein alignment, underlined in red).

Figure 23. CI and SLMB binding sites in FSA. Drosophila Fsa gene structure (exons: thick red lines, introns: thin red lines) with cytologic band and the base ruler. The regions of Fsa coding for peptides that interact with CI and SLMB, as determined in the Yeast Two-Hybrid assay, are indicated in black.
To validate the direct interaction of FSA with CI and SLMB, a Yeast Two-Hybrid assay for these proteins was performed based on the assay by Formstecher et al., in which interactions were in addition tested at different stringencies. The genes slmb and ci were fused to a LexA DNA-binding domain, while fragments of Fsa were fused to a Gal4 activation domain. Yeast cells were transformed with combinations of these constructs. The yeast strain that was used lacks the endogenous His3 gene and carries a His3 reporter gene that is driven by a promoter that contains a LexA binding site (see Materials and Methods). Interaction between the protein fused to the LexA DNA-binding domain with the protein fused to the Gal4 activation domain leads to the recruitment of the transcription machinery to the promoter of the His3 reporter gene. Expression of the His3 reporter gene allows growth of the yeast cells on medium lacking histidine. Transformation with the Fsa and slmb constructs or with Fsa and ci constructs allowed growth of the yeast on medium without histidine. To increase the stringency of the growth test, different concentrations of 3-aminotriazole (3-AT), an inhibitor of the His3 gene product, were added to the medium. Yeast transformed with the Fsa and ci constructs grew on medium containing 3-AT up to a concentration of 1mM. Yeast transformed with Fsa and slmb constructs grew on medium containing up to 100mM 3-AT (Figure 24). These results indicate that FSA directly interacts with SLMB and CI.
Figure 24. Yeast Two-Hybrid interactions of FSA with CI and SLMB. Growth of yeast cell culture spotted on media in different dilutions. DO-2: selective medium without tryptophan and leucine, DO-3: selective medium without tryptophan, leucine and histidine, 3-AT: 3-aminotriazole. pB27: empty LexA DNA-binding domain vector, pP7: empty Gal4 activation domain vector. Pos. control: Hybrigenics’ interaction positive control.

5.3.2. Fsa mutants show a phenotype of increased Hedgehog signalling

The interaction of FSA with components of the Hedgehog signalling pathway in the Yeast Two-Hybrid assay suggests an involvement of Fsa in Hedgehog signalling. To further test this, Drosophila embryos mutant for Fsa were analysed for phenotypes characteristic of changes in Hedgehog signalling. Elevated Hedgehog signalling levels lead to broadened expression domains of the gene wg and the ectopic expression of the transcription factor EN in cells anterior to the cells expressing wg. Conversely, reduced Hedgehog levels lead to a reduced expression of the genes wg and en (Martinez Arias et al., 1988; Nakano et al., 1989; Hidalgo and Ingham, 1990; Ingham and Hidalgo, 1993). For most Fsa alleles, no maternal-zygotic mutant embryos were obtained, which suggests a requirement for Fsa during oogenesis. For three Fsa alleles, maternal-zygotic mutant embryos exhibit ectopic expression of EN, characteristic of elevated Hedgehog signalling
levels (Figure 25). The phenotype observed for Fsa mutant embryos suggests that Fsa is a negative regulator of the Hedgehog signalling pathway.

**Figure 25.** Fsa mutant Drosophila embryos show a phenotype characteristic of increased Hedgehog signalling. Examples of wildtype (wt), homozygous patched mutant and Fsa maternal-zygotic mutant embryos stained for the proteins EN in green and WG in red. Maternal contribution of FSA was depleted using the germline clone technique (Perrimon, 1984). Anterior is left, dorsal up. Scale bar: 100µm. Higher magnifications are shown to the right of embryos. Scale bar: 20µm. Graphs show percentages of embryos with different numbers of ectopic EN patches. For the alleles till 05-49 and till 26-54, difference to wildtype is statistically significant (p≤0.05). See Materials and Methods for sample sizes.
5.3.3. Genetic interaction between Fsa and components of the Hedgehog signalling pathway

The phenotype observed for Fsa mutant embryos is characteristic of elevated levels of Hedgehog signalling. To investigate whether the phenotype observed in Fsa mutant embryos is due to interference with the Hedgehog signalling pathway, it was tested if the phenotype can be altered by changing Hedgehog signalling levels. The number of patches of cells ectopically expressing EN in Fsa mutant embryos was increased by two different ptc mutant alleles. Consistently, a hh mutation reduced the phenotype of one Fsa allele (Figure 26). These results suggest that the phenotype observed in Fsa mutant embryos is due to increased Hedgehog signalling levels.
Figure 26. Genetic interaction between Fsa and components of the Hedgehog signalling pathway in Drosophila embryos. Percentage of embryos with different numbers of ectopic EN patches. Left column: Fsa heterozygous mutant embryos derived from homozygous germline clones, where FSA has been maternally depleted. Heterozygous for alleles indicated to the left. Right column: Control. Embryos derived from wildtype mothers, heterozygous for alleles indicated to the left. Enhancement of Fsa mutant phenotype by ptc mutations is statistically significant (p<0.05) for all Fsa alleles. Reduction of Fsa mutant phenotype by hhAC is statistically significant for till 05-49. See Materials and Methods for sample sizes.

To investigate whether the negative regulation of the Hedgehog signalling pathway by Fsa is restricted to the Drosophila embryo, genetic interaction between Fsa and components of the Hedgehog signalling pathway were tested in the specification of the scutellar bristles of the adult fly, which depends on Hedgehog signalling. Wildtype flies have four scutellar bristles. Overactivation of Hedgehog signalling leads to an increase in the number of bristles, whereas downregulation of the pathway reduces the number of bristles (Mullor et al., 1997). Scutellar bristles were counted in flies heterozygous mutant for Fsa and ptc and compared to the number of scutellar bristles in flies heterozygous mutant for either Fsa or ptc. For six out of eight Fsa alleles tested, an increased scutellar bristle number was observed for the flies heterozygous mutant for both Fsa and ptc (Figure 27). The genetic interaction between Fsa and components of the Hedgehog signalling pathway in different tissues of Drosophila suggests that Fsa is a general negative regulator of the Hedgehog signalling pathway.
5.3.4. *Fsa* mutants contain reduced amounts of TAG

Hedgehog signalling has a conserved role in the inhibition of fat formation (Suh et al., 2006; Kim et al., 2007; Li et al., 2008). In *Drosophila*, activation of Hedgehog signalling in the fat body leads to less and smaller lipid droplets and to reduced amounts of triacylglycerides (TAG), which are lipids stored in lipid droplets (Suh et al., 2006). Like the activation of Hedgehog signalling, reduction of FSA levels in worms and human culture cells leads to a reduced number of lipid droplets (McKay et al., 2003; Wei et al., 2006). Hedgehog signalling and *Fsa* act were shown to act upstream of the adipogenic transcription factor PPARγ (Suh et al., 2006; McKay et al., 2003). To test whether the
promoting effect of *Fsa* on fat formation in conserved in *Drosophila*, TAG levels were measured in *Drosophila Fsa* mutants. For this, lipids were isolated from *Drosophila* larvae and analysed by mass spectrometry (see Materials and Methods). In all combinations of *Fsa* alleles that were tested, TAG levels were reduced compared to TAG levels in control larvae (Figure 28). The reduction of TAG levels in *Fsa* mutant larvae is consistent with a negative regulation of Hedgehog signalling by *Fsa*.

![Figure 28. Reduced TAG levels in *Fsa* mutant *Drosophila* larvae.](image)

TAG/phosphatidylcholine (PC) ratios for transheterozygous *Fsa* mutant and control larvae. Asterisk: statistical significance (p≤0.05). See Materials and Methods for sample sizes.

### 5.3.5. *Sara* interacts with *Fsa* in its function as a repressor of Hedgehog signalling

Genetic interactions between *Fsa* and *Sara* and the regulation of Hedgehog signalling by *Fsa* raise the question if *Sara*, together with *Fsa*, is involved in the Hedgehog signalling pathway. To test if the phenotype of *Fsa* mutant embryos can be modified by altered levels of *Sara* expression, the number of EN patches was compared in *Fsa* mutant
embryos and in Fsa mutant embryos carrying a Sara mutant allele. An increased number of cell patches ectopically expressing EN were observed in embryos carrying the Sara mutant allele (Figure 29). These results implicate that Sara, together with Fsa, inhibits Hedgehog signalling.

Figure 29. Genetic interaction between Fsa and Sara in Drosophila embryos. Percentage of embryos with different numbers of ectopic EN patches. Left column: Fsa heterozygous mutant embryos derived from homozygous germline clones, where FSA has been maternally depleted. Heterozygous for alleles indicated to the left. Right column: Control. Embryos derived from wildtype mothers, heterozygous for alleles indicated to the left. Enhancement of Fsa mutant phenotype by Sara\textsuperscript{12} is statistically significant (p\leq0.05) for till 05-49 and till 26-54. See Materials and Methods for sample sizes.
5.4. FSA and SARA affect Drosophila tissue growth

The results presented above show that FSA acts as a negative regulator of Hedgehog signalling in the regulation of patterning and cell fate decisions during Drosophila development. Furthermore, SARA was shown to interact with FSA in its function as a regulator of Hedgehog signalling. Hedgehog signalling does not only control patterning and cell fate decisions, but also plays an important role in the control of tissue growth by promoting cell proliferation and cell survival (Kenney and Rowitch, 2000; Yoon et al., 2002; Duman-Scheel et al., 2002; Stecca and Ruiz i Altaba, 2009; Mille et al., 2009). Furthermore, changes in Hedgehog signalling levels are associated with tumour formation in vertebrates (Hahn et al., 1998; Dahmane et al., 2001; Thomas et al., 2009; Cretnik et al., 2009). The regulation of Hedgehog signalling by FSA and SARA during Drosophila development suggests that these proteins might also be involved in the regulation of tissue growth. Several observations about FSA in mammalian systems support this hypothesis. FSA was shown to be strongly expressed in non-proliferating cells, while proliferating cells and tumour cells show low levels of expression (Kuo et al., 2006). Furthermore, preliminary results of Wei et al. suggest that the expression of FSA in mammalian culture cells inhibits cell growth (Wei et al., 2006).

To test if Fsa mutant Drosophila cells show a growth behavior that is different from wildtype cells, clones of Fsa mutant cells were investigated in Drosophila wings. Site-specific mitotic recombination using the FLP/FRT method (see Materials and Methods) was induced in flies heterozygous for the 16-03 allele to generate clones of cells homozygous mutant for Fsa next to clones of wildtype twin spot cells (scheme in Figure 30). The growth rate of the mutant cells was determined for clones induced at different times during development. The average growth rate of Fsa mutant cells is smaller compared to the growth rate of wildtype cells, which is particularly evident for big clones induced early during development (Figure 30). This behaviour is characteristic of cell populations that are subject to cell competition in Drosophila. Cell competition occurs when two cell populations with different growth rates are present in a tissue, and results in the growth of one cell population at the expense of the other (Johnston, 2009).
slow-growing cells, such as heterozygous *Minute* cells, which have reduced levels of ribosomal proteins, get eliminated by apoptosis in mosaic tissues with wildtype cells, while they are viable in the absence of wildtype cells (Morata and Ripoll, 1975; Simpson and Morata, 1981; Li and Baker, 2007). However, in some cases, it was shown that cells mutant for tumour suppressor genes also get eliminated from mosaic tissues. This was observed for cells mutant for the tumour suppressor genes *scribbled*, *lethal giant larvae* and *discs large* (Brumby and Richardson, 2003; Agrawal et al., 1995; Woods and Bryant, 1991). Therefore, while *Fsa* cells show a growth behaviour different from wildtype cells, it cannot be concluded whether FSA exhibits a growth-promoting or growth-inhibiting function.

**Figure 30. Growth index of *Fsa* mutant cells in *Drosophila* wings.** Left: scheme of a *Drosophila* wing heterozygous mutant for *Fsa* (+ -) carrying a homozygous mutant clone (- -, yellow) and a wildtype twin spot (+ +, green). Below is the expression that was used to calculate the growth index. Right: growth index of *Fsa* mutant (16-03) cells and wildtype cells in clones induced at different times during development. AEL: after egg lay. Asterisks: difference to wildtype is statistically significant (*p* ≤ 0.05). sample size = 10.

The elimination of *Fsa* mutant clones was also observed in the *Drosophila* eye. Clones in the eye were induced in a modified experimental setup compared to the clones in the wing. In the eye, clones were continuously induced during development and twin spot cells died due to the presence of a cell lethal mutation. For most *Fsa* alleles tested, the proportion of the eye tissue composed of homozygous *Fsa* mutant tissue (white in Figure
31) was smaller compared to control flies in which clones of wildtype tissue were induced (Figure 31). For some alleles (till 01-08 in Figure 31), no homozygous mutant tissue was observed. The eyes of these flies, which are composed exclusively of heterozygous mutant cells (dark in Figure 31), are reduced in size, probably due to the failure of the heterozygous tissue to compensate for the reduced growth of the homozygous mutant tissue. Indeed, this phenotype resembles the phenotype of fly eyes in which clones homozygous mutant for a cell lethal mutation were generated (Minute in Figure 31). Taken together, these results show that Fsa mutant cells have a growth behavior different from wildtype cells in Drosophila, which is consistent with the function of FSA as a regulator of Hedgehog signalling.

Figure 31. Reduced growth of Fsa mutant cells in Drosophila eyes. Fsa homozygous mutant cells (white) and heterozygous mutant cells (black) in Drosophila eyes. As controls, wildtype (wt) clones and clones carrying a cell lethal mutation (Minute) were induced.
Interestingly, an effect on tissue growth was also observed for SARA. While *Sara* mutant cells show an increased rate of apoptosis compared to wildtype cells in *Drosophila* (Boekel et al., 2006), overexpression of the SARA protein in *Drosophila* wing imaginal discs lead to a strong overgrowth of the tissue (Figure 32). These results imply that SARA has a growth-promoting function. This is in contrast to expectations for negative regulators of Hedgehog signalling, suggesting that the tissue overgrowth is mediated by SARA functions that are different from its effect on Hedgehog signalling.

![Figure 32. Overgrowth of Drosophila wing imaginal discs overexpressing Sara.](image)

Wildtype disc and disc overexpressing *GFP-Sara* stained with DAPI.
6. DISCUSSION

6.1. What is the molecular function of FSA?

In mammalian cells and in Caenorhabditis elegans, FSA was shown to be involved in lipid homeostasis and cell differentiation (McKay et al., 2003; Wei et al., 2006; Kuo et al., 2006). However, the molecular function of the protein is not known, and the protein sequence does not show similarities to proteins with known molecular functions. In the work presented, Drosophila FSA was identified as a negative regulator of Hedgehog signalling. Drosophila Fsa mutant embryos show a phenotype characteristic of elevated levels of Hedgehog signalling (Figure 25), and in embryos and adult flies, Fsa and components of the Hedgehog signalling pathway interact genetically (Figure 26 and 27). Furthermore, in a Yeast Two-Hybrid assay, it was shown that Drosophila FSA physically interacts with the proteins SLMB and CI, which are components of the Hedgehog signalling pathway (Figure 24). SLMB itself is a negative regulator of Hedgehog signalling that directly binds to the transcription factor CI (Jiang and Struhl, 1998; Jia et al., 2005; Smelkinson and Kalderon, 2006). The binding of SLMB to CI leads to the ubiquitination and partial degradation of CI by the proteasome, which results in the formation of the CI repressor form that inhibits Hedgehog target gene expression (Aza-Blanc et al., 1997; Jiang and Struhl, 1998). The direct binding of Drosophila FSA to CI and SLMB suggests that FSA exerts its function as a negative regulator of Hedgehog signalling by mediating the interaction between CI and SLMB, thereby promoting the processing of CI into its repressor form. This raises the question if FSA acts upstream of CI processing, which could be answered by comparing the ratios between the CI repressor and activator forms in wildtype and Fsa mutant flies. Furthermore, it would be interesting to investigate whether FSA enhances the interaction between CI and SLMB, for example by testing if the co-immunoprecipitation of CI and SLMB is impaired in Fsa mutant flies.
6.2. Does FSA regulate lipid homeostasis through repression of Hedgehog signalling?

The Hedgehog signalling pathway has a conserved function in the inhibition of fat formation (Suh et al., 2006; Kim et al., 2007; Li et al., 2008). In mammalian cell culture and in *Drosophila*, Hedgehog signalling inhibits the expression of adipogenic transcription factors, such as C/EBPα and PPARγ. Therefore, elevated Hedgehog signalling levels lead to reduced levels of TAG and to a lower number of lipid droplets, organelles that store TAG (Suh et al., 2006). In contrast to the inhibiting role of Hedgehog signalling on fat formation, FSA was shown to promote fat formation in human culture cells and in *Ceanorhabditis elegans*. In both systems, depletion of the FSA protein leads to a decrease in lipid droplets size and number (McKay et al., 2003; Wei et al., 2006). Interestingly, FSA, like Hedgehog signalling, acts upstream of PPARγ expression in mammalian culture cells (Suh et al., 2006; McKay et al., 2003). In the work presented, it was shown that *Drosophila Fsa* mutants contain reduced amounts of TAG (Figure 28). These results confirm that the effect of FSA on lipid storage is conserved. Hence, Hedgehog signalling and FSA have opposite effects on lipid homeostasis upstream of the adipogenic master regulator PPARγ. Furthermore, FSA was identified as a negative regulator of Hedgehog signalling in *Drosophila* in this work. This suggests that FSA regulates lipid homeostasis via the repression of Hedgehog signalling. To investigate this further, genetic interactions between components of the Hedgehog signalling pathway and *Fsa* in the regulation of lipid homeostasis are tested in ongoing experiments.

6.3. Is FSA a tumour suppressor?

*Fsa* was identified as a gene associated to the fragile site 1q31 in a Chinese hamster ovary cell line (Wei et al., 2006). Fragile sites are loci in the genome that are prone to double strand breaks in response to replication stress (Glover et al., 1984; Sutherland et
Breakage at fragile sites leads to chromosomal rearrangements, which are frequently observed in tumour cells and are often associated with the amplification of oncogenes or genes involved in drug resistance (Coquelle et al., 1997; Corbin et al., 2002; Miller et al., 2006). Breakage of the fragile site 1q31 initiates the amplification of the multiple drug resistance transporter *mdr1*, which is amplified in many drug-resistant cell lines (Wei et al., 2006; Kuo et al., 1994). For several fragile sites, it was shown that they are located inside genes, and chromosome breakage leads to the inactivation of these genes (Zhu et al., 2006; McAvoy et al., 2008). Interestingly, some of the genes that are associated to fragile sites are tumour suppressor genes. For example, the genes *Fragile histidine triad* and *WW domain containing oxidoreductase*, which are associated to the human fragile sites *FRA3B* and *FRA16D*, respectively, are tumour suppressor genes with proapoptotic activity (Ohta et al., 1996; Siprashvili et al., 1997; Aqeilan et al., 2007; Chang et al., 2003). This raises the question if FSA also functions as a tumour suppressor. The observation that *Fsa* is involved in the differentiation of cells into adipocytes and that differentiated cells, in contrast to proliferating cells, express high levels of *Fsa* (Kuo et al., 2006), would be consistent with a role of FSA as a suppressor of cell proliferation. Furthermore, preliminary results of Wei et al. suggest that the expression of *Fsa* in cell culture inhibits cell growth (Wei et al., 2006). Consistently, in the work presented, it was shown that Fsa mutant cells show a growth behaviour that is different from wildtype cells and get eliminated from mosaic tissues in *Drosophila* (Figures 30 and 31). Elimination of mutant cells from mosaic tissues in *Drosophila* was observed for several tumour suppressor genes (Brumby and Richardson, 2003; Agrawal et al., 1995; Woods and Bryant, 1991). In addition, *Drosophila Fsa* was identified as a negative regulator of Hedgehog signalling. Hedgehog signalling regulates tissue growth and the maintenance of stem cells in many tissues (Balordi and Fishell, 2007; Dierks et al., 2008; Palma et al., 2005), and abnormal activation of Hedgehog signalling is linked to tumour formation. In many tumours, such as basal cell carcinoma and medulloblastoma, Hedgehog signalling levels are elevated, and negative regulators of the pathway, like the receptor PTC, act as tumour suppressors (Thomas et al., 2009; Cretnik et al., 2009). Therefore, the identification of FSA as a negative regulator of Hedgehog signalling in this work is consistent with a potential role of FSA as a tumour suppressor.
6.4. How does SARA modulate Hedgehog signalling?

In the work presented, *Drosophila Fsa* was identified as a genetic interactor of *Sara*. *Fsa* mutants suppress the lethality caused by *Sara* overexpression (Figure 12) and enhance the vein patterning defects of *Sara* mutant wings (Figure 21). The suppression of a *Sara* overexpression phenotype and the enhancement of a *Sara* mutant phenotype imply that FSA and SARA act synergistically. Furthermore, FSA was shown to act as a negative regulator of the Hedgehog signalling pathway, which raised the question if SARA is also involved in the Hedgehog signalling pathway. Indeed, it was observed that *Sara* cooperates with *Fsa* in its function as a negative regulator of Hedgehog signalling (Figure 29).

SARA is an early endosome-associated protein involved in the regulation of membrane trafficking. Overexpression of *Sara* leads to an enlargement of early endosomal compartments and slows down the recycling of Transferrin receptors to the plasma membrane (Panopoulou et al., 2002; Hu et al., 2002; Boekel et al., 2006). Membrane trafficking regulates signalling processes in various ways. For example, endocytosis of the Epidermal growth factor receptor (EGFR) leads to the degradation of the receptor and thereby to a downregulation of EGFR signalling (Carpenter and Cohen, 1976). While internalization and degradation of the receptors of the TGFβ signalling pathway through caveolin-dependent endocytosis also lead to the downregulation of signalling, internalization through clathrin-dependent endocytosis is required for efficient signalling (Di Guglielmo et al., 2003). A promoting function of endocytosis in signalling is also observed for the Notch signalling pathway. Interestingly, endocytosis is not only required in the signal-receiving cell, but endocytosis of the ligand in the signal-sending cell enhances Notch signalling in the signal-receiving cell (Seugnet et al., 1997). Furthermore, the asymmetric distribution of endocytic compartments containing the Notch signalling ligand and receptor during the asymmetric division of SOP cells in *Drosophila* enhance Notch signalling in one of the two daughter cells (Coumailleau et al., 2009). Membrane trafficking is also implicated in the regulation of Hedgehog signalling. In the absence of the ligand HH, the signal transducer SMO is repressed by the HH
receptor PTC and localizes to the plasma membrane and to endosomal compartments (Zhu et al., 2003; Stegman et al., 2004). In the presence of HH, SMO gets release from the repression by PTC (Chen and Struhl, 1996). Activation of SMO is associated with a change in SMO localization. In Drosophila, SMO gets enriched at the plasma membrane, and in vertebrate cells, it localizes to the primary cilium. The altered localization of SMO is required for Hedgehog signalling (Zhu et al., 2003; Rohatgi et al., 2007; Wilson et al., 2009). The importance of membrane trafficking for many signalling pathways and the involvement of Sara in the regulation of membrane signalling suggests that Sara might affect several signalling pathways.

Indeed, SARA does not only affect signalling as an adaptor protein in the Activin branch of TGFβ superfamily signalling, but also through the regulation of membrane trafficking. This was shown for the BMP branch of TGFβ superfamily signalling in Drosophila. During cell division, Sara is required for the equal segregation of endosomes that contain signalling components to the two daughter cells. Thereby, Sara assures that the two daughter cells inherit equal BMP signalling states (Boekel et al., 2006). Furthermore, during the asymmetric division of SOP cells in Drosophila, the receptor and ligand of the Notch signalling pathway traffic through the SARA-positive endosomes, which get asymmetrically distributed to the daughter cells (Coumailleau et al., 2009). These observations suggests that Sara mediates Hedgehog signalling, as well as other signalling pathways, through the regulation of membrane trafficking.
7. MATERIALS AND METHODS

7.1. Immunoprecipitation

For each immunoprecipitation, 50µl Protein A-Agarose beads (Roche Diagnostics, 11719408001) were washed in lysis buffer (150mM NaCl, 10mM Tris-HCl, 1mM EDTA, 1% Triton x-100, pH 7.4), incubated with 5µl antibodies (antibodies used: mouse anti-c-MYC 9E10 from Santa Cruz (sc-40), mouse anti-GFP 3E6 from Q-Biogene) in lysis buffer over night at 4°C on a rotating wheel and washed with lysis buffer to remove unbound antibodies. For immunoprecipitations with *Drosophila* embryo lysates, overnight collections of embryos on apple juice plates (85g Agar-Agar, 100g crystal sugar, 1 litre apple juice, 40ml 15% Nipagin (dissolved in ethanol), 3 litres H$_2$O) were dechorionated for two minutes in La Croix Eau de Javel (Colgate-Palmolive AG/SA) and washed with water. Afterwards, ~100µl of embryos were homogenyzed in a douncer containing 500µl of lysis buffer supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche 1836170). After incubation on ice for 15min and centrifugation for 15min at 13000rpm in a microcentrifuge, the supernatant was incubated with the washed Protein A Agarose beads for 3h at 4°C. After several washes in lysis buffer and two short washes in lysis buffer with higher salt concentration (550mM NaCl), the beads were boiled for 5min at 95°C in SDS sample buffer (50mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 0.1M Dithiothreitol, 10% Glycerol, 0.05% bromophenol blue). Samples were separated on 7.5% SDS polyacrylamide gels by gel electrophoresis. Expression of Myc-Sara (UAS-HM-SARA line BC from Daimark Bennet) and GFP-Smox was induced using by crossing flies carrying these constructs under the control of an UAS to flies carrying a daughterless-Gal4 construct. For immunoprecipitations with *Drosophila* S2 cells, cells stably transfected with a GFP-Sara construct under the control of a metallothionein promotor were induced to expressed GFP-SARA with 100mM CuSO$_4$, ~ 200x10$^4$ Cells were washed in PBS and lysed in lysis buffer, and immunoprecipitations were done as for embryo lysates.
7.2. Immunoblotting

After electrophoresis, SDS polyacrylamide gels were incubated for 10min in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, 0.1% SDS). A PVDF membrane (Millipore VSWP 01300) was shortly incubated in methanol, washed in water and incubated in transfer buffer. The transfer was done using a semi-dry blotting system (Trans-Blot SD, Biorad 170-3940) for 1.5h at 15V. After the transfer, the membrane was incubated over night in blocking buffer (0.1% Tween-20, 5% non-fat dried milk in PBS pH7.5). Incubation in the primary antibody (rabbit anti-GFP from Santa Cruz sc-8334 1:200, rabbit anti-SARA-SARA 1:200) in blocking buffer was done for min at room temperature, followed by 3x20min washes in PBT (0.1% Tween-20 in PBS). Afterwards, the membrane was incubated in the secondary antibody (goat anti-rabbit HRP from Dako 1:15.000) in blocking buffer for 45min and washed for 3x20min in PBT. For the detection of the signal, an ECL kit from Amersham Pharmacia Biotech (RPN 2109, 2209, 2106, 2134) was used. For membrane stripping, the membrane was incubated for 30min at 50°C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7).

7.3. Detection of SARA phosphorylation

Immunoprecipitates from Drosophila embryos expressing MYC-SARA (UAS-HM-SARA, daGal4) or S2 cells stably expression GFP-SARA were separated on 7.5% SDS acrylamide gels. For dephosphorylation, the immunoprecipitates were incubated with 0.4 Units/µl Calf Intestinal Phosphatase (CIP, Fermentas) for 1h at 37°C before boiling in SDS sample buffer. Gels were stained with Pro-Q Diamond Phosphoprotein stain (Molecular Probes) according to manufacturer instructions and imaged with a Typhoon 9200 laser scanner (Amersham, 532nm laser, 610BP filter). The same gels were stained with Coomassie afterwards, and the intensities of the phosphostain signals were quantified using ImageJ and normalized to the Coomassie signal. The signal of the phosphostain normalized to the coomassie signal of the sample that was not treated with
CIP was set to 100%.

7.4. Immunostaining of HeLa cells

HeLa cells were grown on coverslips. They were washed in PBS and fixed for 20 min in 4% Paraformaldehyde in PBS. Afterwards, they were incubated for 2x8 min in 0.1M glycine in PBS and for 20 min in blocking buffer (0.2% Bovine Serum Albumin and 0.2% Triton X-100 in PBS). The incubation with the primary antibody in blocking buffer was done overnight at 4°C. After washes in blocking buffer, they were incubated for 1 h in the secondary antibody at room temperature. After washes in blocking buffer and PBS, cells were inbedded in Mowiol. Primary antibodies used: rabbit anti-human SARA sc-9135 from Santa Cruz 1:250, mouse anti-β-Tubulin T4026 from Sigma 1:500. Secondary antibodies used: goat anti-rabbit Alexa 546 A-11010 and goat anti-mouse Alexa 488 A-11001 from Molecular Probes 1:500.

7.5. Mutagenesis

The mutagenesis was performed by Dr. Christian Boekel (BIOTEC, Dresden). For the overexpression of Sara, the GAL4/UAS system was used (Brand and Perrimon, 1993). Flies carrying a transposable element containing an Upstream Activating Sequence (UAS) and a minimal promoter upstream of the endogenous Sara gene (P(EPgy2)Sdc\textsuperscript{EY07208} (Bellen et al., 2004)) were crossed to flies ubiquitously expressing GAL4 (daGal4(G32) (Wodarz et al., 1995)). Co-expression of the transcription factor GAL4 leads to the binding of GAL4 to the UAS, inducing the expression of Sara. For the mutagenesis, male flies of the genotype yw;P(EPgy2)Sdc\textsuperscript{EY07208}/CyO were kept overnight at 25°C in vials containing a paper tissue soaked in water. Afterwards, they were transferred to vials containing paper tissue soaked in a 4% sucrose solution with 125 mM EMS and kept for 24 hours at room temperature. After 30 min in empty vials, they were
crossed to females of the genotype \textit{w;daGal4(G32)}. Surviving single \textit{Cy}+ male progeny flies were crossed to females carrying a \textit{CyO} chromosome. Progeny flies of the genotype \textit{P(EPgy2)Sdc}_{\text{EY07208}} */\textit{CyO} (*=mutagenized) were crossed to each other to establish balanced stocks. The \textit{CyO} balancer chromosome is described in the FlyBase \textit{Drosophila} database.

7.6. Rescue of lethality caused by \textit{Sara} overexpression

After the mutagenesis, the mutagenized chromosomes were recombined with a wildtype chromosome. For this, flies of the genotype \textit{w;P(EPgy2)Sdc}_{\text{EY07208}} */\textit{CyO} were crossed to \textit{w^{1118}} flies. Females carrying the mutagenized chromosome in heterozygosity with a wildtype chromosome were crossed to males of the genotype \textit{w^{1118};noc}^{\text{Sco}}/\textit{CyO}. Single progeny males that did not carry the P-element (detected by the absence of the \textit{white}+ marker) were crossed to female flies of the genotype \textit{P(EPgy2)Sdc}_{\text{EY07208}}/\textit{CyO},\textit{P(tubP-Gal80)};\textit{daGal4} to test their ability to rescue the lethal effect of \textit{Sara} overexpression and to females of the genotype \textit{w^{1118};noc}^{\text{Sco}}/\textit{CyO} to establish stocks. For the calculation of the relative survival of flies carrying the mutation, males of the genotype */\textit{CyO} were crossed to female flies of the genotype \textit{P(EPgy2)Sdc}_{\text{EY07208}}/\textit{CyO},\textit{P(tubP-Gal80)};\textit{daGal4}. The relative survival was calculated as the number of progeny flies of the genotype */\textit{P(EPgy2)Sdc}_{\text{EY07208}};\textit{daGal4}/+ divided by the number of progeny flies of the genotype */\textit{CyO}, \textit{P(tubP-Gal80)};\textit{daGal4}/+. As a control, the same cross was performed in the absence of a mutagenized chromosome. The alleles \textit{noc}^{\text{Sco}} and \textit{P(tubP-Gal80)} are described in the FlyBase database. Number of experiments (\textit{n}) for each fly line: \textit{n}(control)=24, \textit{n}(01-13)=6, \textit{n}(17-22)=10, \textit{n}(16-03)=9, \textit{n}(42-08)=9, \textit{n}(82-23)=9, \textit{n}(10-20)=10, \textit{n}(79-02)=10, \textit{n}(30-29)=9, \textit{n}(34-45)=10, \textit{n}(95-11)=5, \textit{n}(77-53)=10, \textit{n}(55-51)=10, \textit{n}(81-25)=7, \textit{n}(15-24)=10, \textit{n}(09-18)=9, \textit{n}(32-22)=9, \textit{n}(70-40)=5, \textit{n}(61-01)=6.
7.7. PLP analysis

The second chromosome of the 16-03 mutant was recombined with the isogenic reference chromosomes FRT40A and FRT42D (Berger et al., 2001). Single recombinant male flies were crossed to females of the genotype P(EPgy2)Sdc^{EY07208}/CyO,P(tubP-Gal80);daGal4 to test for the presence of the mutation that rescues the lethal effect of Sara overexpression. Recombinant chromosomes (rec) showing relative survival (see rescue of lethality caused by Sara overexpression) rates above 0.2 were considered as rescuing chromosomes. Flies carrying these chromosomes were used for the PLP analysis. For the PLP analysis, flies of the genotype rec/ P(EPgy2)Sdc^{EY07208} were compared to flies of the genotypes FRT40A/ P(EPgy2)Sdc^{EY07208} or FRT42D/ P(EPgy2)Sdc^{EY07208} and 16-03/ P(EPgy2)Sdc^{EY07208} to determine which part of the recombinant chromosome was inherited from the mutagenized chromosome and which part from the reference chromosome. For recombinants with chromosome FRT40A, the following PLP primer pairs were used (Berger et al., 2001):

24D6 (meiotic recombination map): PLP2L5/PLP2L6
30C6: PLP2L35/PLP2L36
34A2: PLP2L15/ PLP2L16
35F12: PLP2L23/ PLP2L24
36A11: PLP2L43/ PLP2L44
37F2: PLP2L47/PLP2L48
48E9: PLP2R39/ PLP2R40

For recombinants with chromosome FRT42D, the following PLP primer pairs were used:
56F8: PLP2R57/PLP2R58
57A3: PLP2R31/ PLP2R32

Preparation of genomic DNA, PCR and agarose gel preparation were performed as described in Berger et al., 2001. Sequencing was done by the sequencing facility of the Max Planck Institute for Molecular Cell Biology and Genetics in Dresden.
7.8. Lethality mapping with deficiencies

To find lethal mutations on the second chromosome of the 16-03 mutant, it was crossed to the Bloomington deficiency kit for the second chromosome (Bloomington Drosophila stock centre). The four deficiencies \( Df(2L)J2 \), \( Df(2L)FCK-20 \), \( Df(2L)cact-255rv64 \) and \( Df(2R)BSC26 \) were found to be lethal in transheterozygosity with 16-03. Deficiencies that were used for further mapping of the lethal mutation uncovered by \( Df(2L)cact-255rv64 \) were \( Df(2L)T317 \), which is lethal in combination with 16-03, and \( Df(2L)TW137 \) and \( Df(2L)TW119 \), which are not lethal in combination with 16-03.

7.9. Mapping of deficiency breakpoints

For breakpoint mapping, PCR products were amplified and sequenced from different genomic regions from flies carrying the deficiency and a CyO balancer chromosome. In case of absence of SNPs, both the deficiency chromosome and the CyO balancer chromosome were crossed over a reference chromosome (carrying the Sco\(^-\) mutation) and PCR products were investigated for SNPs. The region was considered absent in the deficiency chromosome only when SNPs were detected for PCR products amplified from flies of the genotype CyO/ Sco\(^-\), but not for flies carrying the deficiency chromosome over CyO or Sco\(^-\). For \( Df(2L)TW317 \), breakpoint mapping shows that the gene CG6307 is not covered by the deficiency (non-complementation of Fsa mutant alleles by the deficiency indicates that the left breakpoint of the deficiency is to the left of the Fsa gene). For \( Df(2L)TW137 \), breakpoint mapping shows that gene beatIII\(_c\) is covered by the deficiency, while gene clip190 is uncovered.
7.10. PCR/RT-PCR

For the annotation of *Drosophila Fsa*, the following primer pairs were used to amplify and sequence *Drosophila Fsa* fragments, using a cDNA library from Drosophila embryos (Clontech):

11up/11low: GGAGATCTGCGAAACGGAAGTCT/CCCGCCAACAAGAGTAGA
10up/10low: GGCCAATTATCGACTCACC/TCCCAATATCGAGCGTTATGACC
7up/7low: GGCCTGGAGTTGGAAGTCTTA/CGCTTTGGAAAGGCTTGAATAGAG
6up/6low: ACGAACCCGCTGATAGTTT/TATCGCCACTCTCTTCCGTCACC
5up/5low: GCCTGACCACAGATCTCTCAAAC/CCTCCATTTGCCACTTGACAT
2up/2low: GCCTGGGAGACTCTGTACCATC/TCACAAAAATGTGGTATCGAAT

For RT-PCR, RNA was isolated from isogenic flies used for generating the Tilling library (Winkler et al., 2005) using the Qiagen RNAeasy kit (74104). The following primers were used for cDNA synthesis and amplification:

cDNA synthesis: oligodT,
PCR: dFSA3up/dFSA3low:
ACACAGCCCGGGTGACAGGAGTCTCGCTTAAGC/ACACAGCGGCCCGCCTAACT
CGTTGGGGGTACG

cDNA synthesis: 4841-2low:
CAATGTAAGAATGCGAGCGACC,
PCR: dFSA2up/dFSA2low:
ACACAGCCCGGGGACTCTGCTTCTCAGC/ACACAGGGCGGCCGAGAAGAGG
TGCGCTTAAGC

cDNA synthesis: 15134-1low:
GCGATCGCTTTGACTATCT,
PCR: dFSA1up2/dFSA1low1:
ACACACGTCGACAGCGGCCGCTTGGGAAGTGGG/CATAGACATCGCTGAACG

The PCR results confirmed the annotated intron exon structure, as annotated by the
FlyBase consortium, of the genes *CG42556*, *CG15134* and *CG4841*, except for the following differences:

Connection of the last exon of *CG42556* with the first exon of *CG15134*: the last exon of *CG42556* ends 4 base pairs (bp) before the annotated end, while the first exon of *CG15134* starts 1 bp before the annotated start (TGGAGGGACTACAAAG|AATGGTGGAGGCCGCCGT).

Connection of the last exon of *CG15134* with the first exon of *CG4841*: the last exon of *CG15134* ends 21 bp before the annotated end, while the first exon of *CG4841* starts 6 bp before the annotated start (TAATACCAAAATTCAG|AATGAGATGAACCTTCCA).

### 7.11. 5’RACE

To determine the start of the *Drosophila Fsa* transcript, 5’RACE was performed using the FirstChoice RLM-RACE kit (Ambion, AM1700). RNA was isolated from *w*1118 flies using the Qiagen RNAeasy kit. Gene specific primers used were:

A outer: CGCATCCAGATTCATGTCGTTC
A inner: CTGTTCCAGGTGCCCACTTC

The results imply that the transcript starts 649 bp upstream of the annotated translation start of *CG42556* (ATTATTATTCAGATGACAGCTGT…). 89 bp upstream of the annotated translation start is an in-frame stop codon.

### 7.12. *Drosophila Fsa* alleles

The splice acceptor site *Drosophila Fsa* mutation found in the mutant 16-03 is the following: genome: 2L:16902931, cDNA: 5623-1G>A

The following mutations were found in the alleles of the Tilling library that do not complement 16-03:

Till 05-95: genome: 2L:16912802, cDNA: 11659A>T, protein: K3887X
genome: 2L:16914935, cDNA: 13792G>T, protein: D4598Y

7.13. Cloning of the UAS-Fsa construct

The cloning of the UAS-Fsa construct was performed by Dr. Carole Seum (University of Geneva, Biochemistry Department). RNA was isolated from isogenic adult flies (from isogenic fly line used for generating the Tilling library (Winkler et al., 2005)) using the Qiagen RNAeasy kit. The cDNA of Drosophila Fsa was amplified in three pieces using Superscript II reverse transcriptase (Invitrogen 18064-014). The following primers were used for the amplification of the Fsa fragments:

Fsa3 fragment:
cDNA synthesis: oligodT, PCR: dFSA3up/dFSA3low:
ACACAGCCCGGCTACAGGAGTCTCGGCTTAAGC/ACACAGCGGCCGCTTTAATT
CGTTGGGGGTCGATG

Fsa2 fragment:
cDNA synthesis: 4841-2low: CAATGTAAGAATGCGAGCGACC, PCR:
dFSA2up/dFSA2low:
ACACAGCCCGGACTCGTTTCCTCAGC/ACACAGCGGCCGAGAGAGGA
TGCCTTAAGC

Fsa1 fragment:
cDNA synthesis: 15134-1low: GCGATCGCTTGGACTATCT, PCR:
dFSA1up2/dFSA1low1:
ACACACGTCGACAGCGGCCGAGGTATGGAGGCGTGGGAAAGTGG/CATAGA
CATCGCTGAGAAGCG

The Fsa3 fragment was cloned into the sites XmaI/NotI of vector pBst SK(+)
Fsa2 was inserted with Xmal/ArfII, Fsa1 with Sall/Xmal (Enzymes from New England Biolabs). The full Fsa cDNA was excised using NotI and inserted in the NotI site of the vector pUASattB (Bischof et al, 2007). Integration into the genomes of flies was done using the PhiC31 system into the fly line PhiX-86Fa (Bischof et al, 2007).

7.14. Rescue of Fsa lethality

Females of the genotype Fsa<sup>allele</sup>/CyO;UAS-Fsa/TM6B were crossed to males of the genotype Fsa<sup>allele</sup>/CyO;daGal4/TM6B. The rescue was calculated as the number of progeny flies of the genotype Fsa<sup>allele</sup>/Fsa<sup>allele</sup>;UAS-Fsa/daGal4 divided by the number of progeny flies of the genotype Fsa<sup>allele</sup>/CyO;UAS-Fsa/daGal4 multiplied by 200%.

7.15. Analysis of wing vein phenotype

To test whether Drosophila Fsa alleles enhances the vein patterning phenotype of Sara mutant wings, the Fsa alleles were recombined to chromosomes carrying a Sara<sup>12</sup> allele, in which the entire coding region of Sara is deleted (Boekel et al., 2006), and crossed over the deficiency Df(2R)PK1 (Price et al., 1989), which uncovers the Sara gene. Wings were dissected in ethanol and embedded in 6:5 lactic acid:ethanol. Coverslips were sealed with nail polish. Only the wings of male flies were used for the analysis. The area of extra wing vein material was quantified using ImageJ and calculated as the percentage to the total wing area.

7.16. Yeast Two-Hybrid assay

The Yeast Two-Hybrid assay was performed by Hybrigenics S.A., Paris, France. The coding sequences for amino acids 1-705 of Drosophila melanogaster ci (FlyBase
FBgn0004859) and 1-199 of *Drosophila melanogaster slmb* (GenBank accession number gi 7530434) were PCR-amplified and cloned in frame with the *LexA* DNA binding domain (DBD) into plasmid *pB27*, derived from the original *pBTM116* (Vojtek and Hollenberg, 1995). The DBD constructs were checked by sequencing the entire insert. The fragment corresponding to amino acids 1370-1589 of *CG15133* (FlyBase FBgn0032619) was extracted from the ULTImate Y2H™ screening of *ci* with the *Drosophila* Whole Embryo library. The fragment corresponding to amino acids 987-1143 of *CG15134* (FlyBase FBgn0032621) was extracted from the ULTImate Y2H™ screening of *slmb* with the *Drosophila* Head library. Both prey fragments are cloned in frame with the *Gal4* activation domain (AD) into plasmid *pP6*, derived from the original *pGADGH* (Bartel et al., 1993). The AD constructs were checked by sequencing.

*Ci – CG15133* (now *CG42556*) and *slmb – CG15134* interaction assays are based on the reporter gene *HIS3* (growth assay without histidine). The bait constructs were transformed in *L40ΔGal4* (mata) yeast cells and the prey constructs in *Y187* (mata) yeast strains. The interaction pairs were then tested using a mating approach as previously described (Fromont-Racine et al., 1997).

Interaction pairs were tested in duplicate as two independent clones from each mating reaction were picked for the growth assay. For each interaction, several dilutions (10^-1, 10^-2, 10^-3 and 10^-4) of the diploid yeast cells culture normalized at 5×10^4 cells and expressing both bait and prey constructs were spotted on several selective media. The DO-2 selective medium lacking tryptophan and leucine was used as a growth control. The different dilutions were also spotted on a selective medium without tryptophan, leucine and histidine (DO-3). Four different concentrations of 3-AT, an inhibitor of the *HIS3* gene product, were added to the DO-3 plates to increase stringency and reduce possible autoactivation by the bait proteins. The following 3-AT concentrations were tested: 1, 5, 10 and 100 mM.
7.17. *Fsa* germline clones

For the generation of embryos depleted for the maternal contribution of FSA, the germline clone technique was used (Perrimon, 1984). For this, male flies of the genotype $P(ovoD1-18)2LaP(ovoD1-18)2LbFRT40A/CyO$ (referred to as *ovoD1 FRT40A/CyO*) were crossed to females of the genotype $Fsa^{allele}FRT40A/CyO$ (for the *Fsa* alleles *till 01-65, till 05-49* and *till 26-54*). Progeny larvae were heatshocked at 38°C for two hours 24 to 48 hours and 48 to 72 hours after egg lay. Progeny females of the genotype $Fsa^{allele}FRT40A/ovoD1FRT40A$ were crossed to males of different genotypes (see results). For *Fsa* maternal and transheterozygotic mutant embryos, the $Fsa^{allele}FRT40A/ovoD1FRT40A$ females were crossed to males carrying the *till 09-86* allele. The $P(OvoD1-81)$ allele is described in the FlyBase database. Ectopic EN patches were counted in embryos of the stages 9-11 (Campos-Ortega and Hartenstein, 1985).

Sample sizes for Figure 25: $n$(wt)=68, $n$(till01-65)=18, $n$(till05-49)=40, $n$(till26-54)=51.

Sample sizes for Figure 26: embryos heterozygous mutant for $ptc^{6P43}$: $n$(wt)=52, $n$(till01-65)=19, $n$(till05-49)=19, $n$(till26-54)=40. Embryos heterozygous for $ptc^{9}$: $n$(wt)=49, $n$(till01-65)=19, $n$(till05-49)=46, $n$(till26-54)=54. Embryos heterozygous for $hh^{AC}$: $n$(wt)=34, $n$(till01-65)=30, $n$(till05-49)=65, $n$(till26-54)=36. Wt control: $n$(wt)=68, $n$(till01-65)=18, $n$(till05-49)=40, $n$(till26-54)=51.

Sample sizes for Figure 29: Embryos heterozygous for *Sara*$_{12}$: $n$(wt)=42, $n$(till01-65)=10, $n$(till05-49)=37, $n$(till26-54)=41. Wt control: $n$(wt)=68, $n$(till01-65)=18, $n$(till05-49)=40, $n$(till26-54)=51.

Alleles used for genetic interactions: $hh^{AC}$: deletion removing the start of the open reading frame of *hh* (Ma et al., 1993). $ptc^{6P43}$: unmapped EMS-induced allele, amorph (DiNardo et al., 1988). $ptc^{9}$: EMS-induced point mutation, leads to amino acid replacement S809N, amorph (Strutt et al., 2001). *Sara*$_{12}$: the entire coding region of *Sara* is deleted (Boeikel et al., 2006).
7.18. Antibody staining of embryos

Overnight collections of embryos on apple juice plates (85g Agar-Agar, 100g crystal sugar, 1 litre apple juice, 40ml 15% Nipagin (dissolved in ethanol), 3 litres H₂O) were dechorionated for two minutes in La Croix Eau de Javel (Colgate-Palmolive AG/SA), washed with water and incubated for 20min on a horizontal shaker in vials containing 5ml heptane, 4.5ml fix buffer (100mM HEPES, 2mM MgSO₄, 1mM EGTA, pH 6.9) and 500µl formaldehyde. The lower phase was removed and 5ml methanol added. After vortexing for approx. 30sec, the embryos were collected from the bottom of the vial, washed with methanol and stored at -20°C in methanol.

Fixed embryos in methanol were rehydrated in PBT (PBS (GIBCO 14190), 0.1% Tween-20) for 3x20min on a rotating wheel and incubated overnight in 10% Normal Goat Serum (NGS) in PBT at 4°C. Afterwards, they were incubated in the primary antibody in 10% NGS in PBT for 2 hours at room temperature and washed in PBT for 3x20min. Secondary antibody incubation was done for 1 hour at room temperature in 10% NGS in PBT, followed by 4x20 min washes in PBT. Embryos were washed in 50% PBT, 50% Mowiol (60g Mowiol 4-88 (Hoechst), 150g glycerol, 150ml H₂O, 300ml 0.2M Tris pH8.5) and embedded in Mowiol. Antibodies used:

- rabbit anti-engrailed (Santa Crux Biotechnology, sc-28640) 1:100
- mouse anti-wingless (Developmental Studies Hybridoma Bank, 4D4) 1:100
- goat anti-rabbit Alexa488 (Molecular Probes, A-11008) 1:500 (preadsorbed)
- goat anti-mouse Alexa546 (Molecular Probes, A-11010) 1:500 (preadsorbed)

For the preadsorption of antibodies, 200µl of fixed w1118 embryos from overnight collections were washed on a rotating wheel at room temperature for 1x5min in methanol and 3x20min in BBT (10mM Tris, 55mM NaCl, 40mM KCl, 7mM MgCl₂, 5mM CaCl₂, 20mM glucose, 50mM sucrose, 1% Tween-20, pH6.95). Afterwards, 50µl of antibody was incubated with 450µl BBT and the washed embryos over night at 4°C on a rotating wheel. Embryos were removed and the preadsorbed antibody stored at 4°C.
7.19. Cuticle preparations of nota

Nota were dissected in ethanol. After rinsing with H$_2$O, the nota were incubated for 10min in 10% KOH at 120°C. Afterwards, the nota were washed in H$_2$O and ethanol and embedded in 6:5 lactic acid:ethanol. Coverslips were sealed with nail polish. Genotypes used for the genetic interaction between Fsa alleles and ptc$^{6P43}$ were Fsa$^{\text{allele}}/+$, ref/ptc$^{6P43}$ and Fsa$^{\text{allele}}$/ptc$^{6P43}$ (ref (+ in Figure 27) = isogenic chromosome used for the generation of the tilling collection). Scutellar bristles were counted in nota of female flies. Sample sizes for Figure 27: ptc$^{6P43}$/ref: n=62. Fsa$^{\text{allele}}$/+: n(till18-46)=36, n(till 01-08)=25, n(till14-60)=31, n(till05-49)=36, n(till09-86)=24, n(till01-65)=33, n(till05-95)=26, n(till26-54)=22. Fsa$^{\text{allele}}$/ptc$^{6P43}$: n(till18-46)=40, n(till 01-08)=49, n(till14-60)=35, n(till05-49)=45, n(till09-86)=43, n(till01-65)=60, n(till05-95)=36, n(till26-54)=32.

7.20. TAG measurement

The analysis of TAG was done in collaboration with Dr. Xue Li Guan and Prof. Dr. Markus Wenk (National University of Singapore, Department of Biochemistry). Flies were grown at 25°C in vials containing food (for 15 litres: 90g Agar, 525g yeast, 1200g corn flour, 1050g sugar, 30ml nipagine solution (15% in ethanol), 150ml propionic acid). Samples, containing two third instar larvae each, were extracted as described by modified Bligh-Dyer method (Bligh and Dyer, 1959). Neutral lipids were analyzed using a sensitive HPLC/ESI/MS method (Shui et al., submitted) with an Agilent HPLC 1100 system (Agilent) and an Applied Biosystem 3200Qtrap mass spectrometer (Applied Biosystem). Briefly, separation of diacylglycerides (DAGs) and TAGs from polar lipids was carried out using Agilent Zorbax Eclipse XDB-C18 column (i.d. 4.6X150mm). HPLC conditions were (1) chloroform:methanol:0.1M NH$_4$OAC (100:100:4) as a mobile phase at a flow rate of 0.25ml. min$^{-1}$; (2) column temperature: 30°C; (3) injection volume: 30ml. Mass spectra were recorded in both positive and negative ESI modes using enhanced MS (EMS) scan mode. Turbo spray source voltages were 5000 and -4500 volts for positive and negative, respectively; source temperature, 250°C. A total run time
of 30 min was utilized to elute both polar and neutral lipids. Based on the obtained mass spectra from EMS scan, selective ion monitoring (SIM) scan was further employed to record major TAGs (Shui et al., submitted). For quantification and normalization of the total TAG content, the following formula was used: Σ intensity of all TAG species/Σ intensity of all PC species. Sample sizes for Figure 28: control (isogenic strain used for the generation of the Tilling library (Winkler et al., 2005)): n=5, l(2)HT-2/till05-49: n=4, till01-08/16-03: n=4, till26-54/till05-49: n=2.

7.21. Clone induction in the *Drosophila* wing

Clones were induced in flies of the genotype *f*°6a/hsFlp *f*°6a; ck *P(f*) FRT40A/16-03 FRT40A and as a control in flies of the genotype *f*°6a/hsFlp *f*°6a; ck *P(f*) FRT40A/FRT40A by heatshocking the flies at different times during development (60h, 84h and 108h after egg lay) at 38°C for 1h. The area of Fsa mutant cell clones (marked by *f*) and the area of the twin spot (marked by ck) were measured using ImageJ. The growth rate of the clones was calculated as log₂(area of mutant cells)/log₂(area of twin spot cells).

7.22. Clone induction in the *Drosophila* eye

Flies of the genotype *yw eyFLP; 2L3.1 Pw* 30C FRT40A/ Fsa° allele FRT40A were analysed. Homozygous Fsa mutant tissue is marked by the absence of the *w*° marker (visible by the absence of red pigment in ommatidia). As control, flies of the genotype *yw eyFLP; 2L3.1 Pw+ 30C FRT40A/FRT40A and *yw eyFLP; 2L3.1 Pw* 30C FRT40A/ M(2)z *P(f*) 30B FRT40A were analysed. Minute stock: *f*°6a, M(2)z *P(f*) 30B FRT40A/CyO (Garcia-Bellido stock 195).
7.23. SARA overexpression in Drosophila wing imaginal discs

Wing imaginal discs were dissected from 3. Instar wildtype Drosophila larvae and from larvae carrying two copies of a UAS-GFP-Sara construct and two copies of a vestigial-Gal4 construct. The dissection was done in PEM (80mM Na-Pipes, 5mM EGTA, 1mM MgCl₂ x 6H₂O, pH 7.4), and discs were fixed for 30min in 4% paraformaldehyde in PEM and for 30min in 4% paraformaldehyde in PEMT (0.1% Triton X-100 in PEM). Fixed discs were stained with DAPI, washed in PEM and imbedded in Mowiol.

7.24. FSA protein alignment

For the alignment, the Clustal W alignment tool of the programm MegAlign (DNAStar) was used. The following sequences were aligned:
D. melanogaster: FSA protein sequence translated from cDNA sequenced determined in this work by RT-PCR.
T. castaneum: Tribolium castaneum Similar to fragile site-associated protein (XP_972174)
C. elegans: Ceanorhabditis elegans hypothetical protein Y47G6A.29 (NP_740830) and Lipid-depleted-3 (NP_491182). The two proteins show strong similarity to FSA of other species and are neighbouring genes. It was therefore assumed that the two genes together represent the C. elegans ortholog of FSA.
D. rerio: Danio rerio Fragile site-associated protein (NP_001139056)
H. sapiens: Homo sapiens Fragile site-associated protein (NP_056127)
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9. APPENDIX: FSA protein alignment
Figure 33. FSA protein alignment. Alignment of *Drosophila* melanogaster FSA with FSA orthologs of different species. The consensus sequence is shown above the alignment, and conservation of amino acids is colour-coded (high conservation is red, low conservation black). The protein domains of Drosophila FSA that interact with CI and SLMB are underlined in red (CI binding domain: AA1090-1309, SLMB binding domain: AA2749-2905).
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