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Interplay between sex determination cascade and major signaling pathways during *Drosophila* eye development: perspectives for future research

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Abstract

Understanding molecular mechanisms of sexually dimorphic organ growth is a fundamental problem of developmental biology. Recent quantitative studies showed that the *Drosophila* compound eye is a convenient model to study the determination of the final organ size. In *Drosophila*, females have larger eyes than males and this is evident even after correction for the larger body size. Moreover, female eyes include more ommatidia (photosensitive units) than male eyes and this difference is specified at the third larval instar in the eye primordia called eye imaginal discs. This may result in different visual capabilities between the two sexes and have behavioral consequences. Despite growing evidence on the genetic bases of eye size variation between different *Drosophila* species and strains, mechanisms responsible for within-species sexual dimorphism still remain elusive. Here, we discuss a presumptive crosstalk between the sex determination cascade and major signaling pathways during dimorphic eye development. Male- and female-specific isoforms of Doublesex (Dsx) protein are known to control sex-specific differentiation in the somatic tissues. However, no data on Dsx function during eye disc growth and patterning are currently available. Remarkably, Sex lethal (Sxl), the sex determination switch protein, was shown to directly affect Hedgehog (Hh) and Notch (N) signaling in the *Drosophila* wing disc. The similarity of signaling pathways involved in the wing and eye disc growth suggests that Sxl might be integrated into regulation of eye development. Dsx role in the eye disc requires further investigation. We discuss currently available data on sex-biased gene expression in the *Drosophila* eye and highlight perspectives for future studies.

Keywords

Eye development; *Drosophila*; organ growth; sexual dimorphism; eye disc; signaling pathways

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Difference in the eye sizes between *Drosophila* males and females

Mechanisms responsible for determination of sexually dimorphic organ size still remain elusive. The developing compound eye is a well-studied model structure. Females have larger eyes than males (Posnien et al., 2012, Hilbrant et al., 2014, Keesey et al., 2019). This dimorphism is clearly evident even after accounting for the larger body size in females by normalization of eye measurements by the tibia length (Posnien et al., 2012, Arif et al., 2013, Ramaekers et al., 2019, Gaspar et al., 2020, Reis et al., 2020). The difference in the eye size between males and females is determined by both the number of photosensitive units (ommatidia) and their size (Posnien et al., 2012, Hilbrant et al., 2014, Gaspar et al., 2020, Casares and McGregor, 2020).

The ommatidia number is specified at the third larval instar when the presumptive eye is an epithelial sac called eye-antennal imaginal disc (eye disc) (Fig. 1A–C). At this stage the indentation in epithelium, called morphogenetic furrow (MF), passes from posterior to anterior of the eye field. This is the wave of differentiation, leaving behind the ordered structure of photoreceptor precursors, which later form ommatidia (Fig. 1A–C, Frankfort and Mardon 2002, Roignant and Treisman, 2009, Kumar 2012).

Examination of *Drosophila* species and strains with different eye sizes demonstrates that females always have more ommatidia than males (Table, Supplementary Table, Cowley and Atchley, 1988, Posnien et al., 2012, Hilbrant et al., 2014, Keesey et al., 2019). Sexual dimorphism in the total number of ommatidia in the eye has been estimated both for closely related species, e.g. *D. melanogaster* and *D. simulans* (Posnien at al., 2012, Hilbrant et al., 2014) and for evolutionary distant species belonging to different subgroups (*D. funebris*, *D. busckii*, *D. suzukii*, *D. pseudotalamancana* and *D. americana*) (Keesey et al., 2019). Average number of ommatidia in these species differed significantly: 1255 ommatidia in adult eye of *D. americana* versus 661 in *D. melanogaster* Canton S. (Table).

The final size of ommatidia is determined after pupariation, this process includes photoreceptor maturation, formation of pigment cells and bristles, and removal of excess cells between ommatidia by programmed cell death (Miller and Cagan, 1998, Cagan and Ready, 1989, Fichelson et al., 2012, Gaspar et al., 2020). Posnien et al. compared ommatidia sizes between males and females in *D. simulans* (YVF) and *D. mauritiana* (TAM16) and the two strains of *D. melanogaster* (M36 and Zi375). In all examined strains females had larger ommatidia than males, although in *D. simulans (YVF)* this difference was small (Posnien et al., 2012, Table S2).

Moreover, male and female eyes have different proportion of ommatidial subtypes. Females have more “pale”+“odd-coupled” ommatidium types (p+OC) due to the higher expression of UV-sensitive Rhodopsin 3 and blue-sensitive Rhodopsin 5 (Hilbrant et al., 2014). Functional consequences of such dimorphism in *Drosophila* require further investigation. It was shown that the number of p+OC ommatidia is positively correlated with the total number of ommatidia in the eye (Hilbrant et al., 2014). The authors suggest a possibility that the dimorphism in ommatidia subtypes could be explained by the difference in total number of

ommatidia between sexes. Interestingly, the number of ommatidia with high polarization sensitivity, located in the evolutionary conserved “dorsal rim area” (DRA) (Homberg et al., 2011), varied very slightly between sexes (Hilbrant et al., 2014). The size and structure of DRA ommatidia also did not vary in a systematic way between males and females (Mathejczyk and Wernet, 2019).

Recent studies were focused on understanding genomic and regulatory bases of the eye size variation between *Drosophila* strains and species (Arif et al., 2013, Norry and Gomez, 2017, Ramaekers et al., 2019, Gaspar et al., 2020, Reis et al., 2020). For example, a single polymorphism in the regulatory intron of the *eyeless/Pax6* gene was shown to explain a large part of variation in eye sizes between *D. melanogaster* strains (Ramaekers et al., 2019). Within the *virilis* phylad, diverged from *D. melanogaster* for 40 million years, GWAS analysis revealed complex genetic architecture underlying eye size differences with the genetic variants located on most chromosomes. Moreover, these species were characterized by a strong association between the eye size and chromosomal inversions (Reis et al., 2020). A recent transcriptomic study identified *pannier (pnr)* gene as a central factor responsible for the variation in progression of eye disc differentiation between *D. melanogaster* and *D. mauritiana* (Buchberger et al., 2021).

Despite the intensive analysis of genetic background underlying the between-species variation in the eye size, little is known about mechanisms which determine the sex-specific variation. Differences in ommatidia number and their size between males and females can affect vision capabilities.

Recently it was shown that the increased number of ommatidia in the *Drosophila* eye leads to narrowing of their diameters and vice versa (Gaspar et al., 2020). High number of narrow ommatidia can increase acuity, while wider ommatidia can increase the inter-ommatidial angles and improve contrast sensitivity, but reduce acuity (Land, 1999, Land and Nilsson, 2012, Gaspar et al., 2020). Examination of visual properties of *Drosophila* with small eyes revealed that they can maintain spatial acuity at the cost of decrease in contrast sensitivity at the optical level. However, contrast sensitivity could be recovered by sacrificing temporal acuity at the level of neural processing (Currea et al., 2018). Moreover, it has been demonstrated that there is a negative correlation between the eye size and the other head capsule traits (Cowley and Atchley, 1990, Norry et al., 2000, Posnien et al., 2012, Arif et al., 2013, Norry and Gomez, 2017, Gaspar et al., 2020) and inverse resource allocation between vision and olfaction (Keesey et al., 2019). Thus, variation in the eye size between sexes may involve other sensory organs and result in behavioral changes.

In this paper, we sought to discuss a possible role of the cross-talk between sex determination genes and the main signaling pathways during *Drosophila* eye development. Currently available molecular data were predominantly obtained for another structure – the developing *Drosophila* wing. Given the similar molecular interactions responsible for growth control of both structures (Romanova-Michaelides, 2015, Vollmer et al., 2016), we discuss the presumptive mechanisms of sexually dimorphic eye development with reference to the published information available for the wing disc and to the preliminary experimental data.

Sex determination genes and dimorphic development of *Drosophila* organ primordia

Sex determination cascade

In *Drosophila*, most somatic sexual differences are controlled by *doublesex (dsx)* gene which encodes a transcription factor and lies at the bottom level of sex determination cascade governed by the master switch gene *Sex lethal (Sxl)*. *Sxl* activation depends on the ratio of X chromosomes to autosomes (X:A ratio). The functional Sxl protein is produced only in females, and maintained by the positive autoregulatory loop (Penalva and Sanchez, 2003, Robinett et al., 2010). Sxl blocks the translation of *male-specific lethal 2 (msl-2)* mRNA to prevent dosage compensation (Fig. 2A, C, Baker and Ridge, 1980, Christiansen et al., 2002, Penalva and Sanchez, 2003, Robinett et al., 2010, Rice et al., 2019).

In females Sxl turns on splicing of *transformer (tra)* gene, and functional Tra protein controls alternative splicing of *dsx* pre-mRNAs. This leads to the expression of protein Dsx^F encoded by female-specific isoform of *dsx* transcript. In males, where functional Tra protein is absent, male-specific Dsx^M isoform is expressed, along with the protein products of *fruitless (fru)* gene. *fru* is responsible for male-specific behavior and has many other functions, including sex-nonspecific (Goodwin et al., 2000, Anand et al., 2001, Ryner et al., 1996, Song et al., 2002, Kimura et al., 2005, Vernes, 2014, Chowdhury et al., 2020). Although some authors mentioned the expression of *fru* transcripts in the eye (Chowdhury et al., 2020), nothing is known about *fru* expression in the eye imaginal disc. Therefore, we do not focus on *fru* and only discuss the presumptive role of *dsx*.

Dsx^F and Dsx^M are transcription factors and their expression controls many somatic dimorphic features (Fig. 2C, Robinett et al, 2010).

dsx expression and regulation in the larval primordia

It has been shown that in *Drosophila* most aspects of sexual differentiation proceed cell-autonomously, i.e. tissues are often the mosaic of sexually dimorphic and monomorphic cells (Rideout et al., 2010, Robinett et al., 2010, Rice et al., 2019). For example, some cells of an organ can express *dsx*, however, it could be absent in neighboring cells (Robinett et al., 2010). Transcriptional regulation of *dsx* results in a highly tissue-specific expression of this gene (Hempel and Oliver, 2007, Lee et al., 2002, Rideout et al., 2010, Robinett et al., 2010, Clough et al., 2014) leading to regulation of many sex-specific traits, including abdominal pigmentation, neuronal development, morphology of genitalia, etc. (Keisman et al., 2001, Goldman and Arbeitman, 2007, Williams et al., 2008, Sanders and Arbeitman, 2008, Luo and Baker, 2015, Rice et al., 2019). Recent analysis uncovered modular organization of the *dsx* cis-regulatory sequences controlling its mosaic expression in the foreleg, a structure containing many sexually dimorphic sensory organs (Rice et al., 2019).

Regulation of somatic tissue development by *dsx* has been intensively investigated. In *Drosophila*, sexual dimorphism of the adult structures is laid down to a large extent during development of their larval primordia. The best studied organ primordium is the genital imaginal disc, which gives rise to the sexually dimorphic genital and anal structures. Dsx

was shown to regulate the dimorphic development of the genital disc by controlling activity of the transcription factor Dachshund (Dac) and by differential deployment of FGF, Decapentaplegic (Dpp) and Wingless (Wg) pathways (Keisman and Baker, 2001, Sanchez et al., 2001, Ahmad and Baker, 2002, Chatterjee et al., 2011).

However, the role of *dsx* in the dimorphic development of eye disc still remains elusive. In the eye-antennal primordium of third instar female larvae, *dsx* expression was detected mostly in the antennal portion and the presumptive head/face area. In the eye domain, *dsx* was expressed ventrally, immediately below the area of differentiated photoreceptor cells (Robinett et al., 2010) and in the anterior proliferation zone (Sammi Ali, personal communication). Only few cells expressing *dsx* colocalized with the proneural marker *neuralized-lacZ (neur-lacZ)* (Boulianne et al., 1991) and thus could be committed toward the photoreceptor fate (Robinett et al., 2010). There is no evidence for *dsx* spatial expression in the adult eye and this is still unknown whether larval *dsx* pattern in the ventral region of the eye disc results in any sexually dimorphic differences. Cells expressing *dsx* were detected in many organ primordia with no anatomic sexual dimorphism in the adult derivatives of these structures (Robinett et al., 2010).

Sxl can act independently of sex determination cascade

Interestingly, many aspects of sexual dimorphism in *Drosophila* develop independently of canonical *Sxl->tra->dsx (fru)* cascade. *Sxl* regulates the difference in body size between males and females (Cline and Meyer, 1996). Remarkably, Sxl target *tra* was reported to control female body growth in concert with the X-linked gene *Myc*, however, without involvement of *dsx* and *fru* regulation (Rideout et al., 2015, Mathews et al., 2017). Recently it has been shown that *Sxl* acts in neurons and controls growth of larval tissues in females (Sawala and Gould, 2017). *Sxl* is involved in the early germline cell differentiation. It directly downregulates translation of the conserved Nanos (Nos) repressor through specific binding sites in the 3ÙTR of *nos* mRNA (Chau et al., 2012, Li et al., 2013). *Sxl* can bypass *tra* in regulation of some aspects of female sexual behavior (Evans and Cline, 2013). The abundance of putative binding sites of Sxl protein in the genome suggests existence of the unknown *Sxl* targets. A computational approach revealed a number of potential Sxl targets within biologically relevant contexts (Robida et al., 2007).

The ability of *dsx* to set sexually dimorphic differentiation by regulating signaling pathways led to the assumption that *Sxl* itself can use a similar strategy (Penn and Schedi, 2007). The results of experiments showed that Sxl can directly interact with components of Hh and N signaling pathways in *Drosophila* (see below). These results raise a fundamental question whether sexual dimorphism in the eye disc development is controlled by *dsx*, or directly by *Sxl*.

The eye and wing discs

Growth and development of the *Drosophila* eye and wing primordia

In order to understand, to what extent the results obtained for the wing disc are applicable for the eye disc, we compare the development of these two primordia.

Imaginal discs are set apart during embryogenesis, grow at the larval stages and terminate proliferation at the early stages of pupal development (Romanova-Michaelides et al., 2015, Beira and Renato Paro, 2016, Kumar, 2018). Interestingly, imaginal discs “know” their final size and stop growing even when they have an additional time (Simpson et al., 1980, Simpson and Morata, 1980, Martin and Morata, 2006). For example, after transplantation into the abdomen of adult flies, the early discs reach their normal size (Bryant and Levinson, 1985, Jursnich et al., 1990). These lines of evidence suggest that the growth of imaginal discs is regulated autonomously via some internal mechanisms (reviewed in Kumar, 2011, Kumar, 2018, Aegerter-Wilmsen et al., 2007, Vollmer et al., 2016).

In the eye disc, growth and patterning are linked and proceed simultaneously. The MF is initiated at the posterior of the disc at the second instar larva. During the third instar larva the MF sweeps from posterior to anterior of the eye field (Fig. 1A–C). Cells within the MF arrest in G1 phase prior to differentiation (Firth and Baker, 2005, Firth et al., 2010). In the anterior portion of the eye field cells continue to divide with a proliferation peak in front of the MF called “first mitotic wave” (Romanova-Michaelides et al., 2015). Posterior to the MF cells differentiate and become organized into the precisely ordered structures of photoreceptor precursors (Fig. 1A–C). The unspecified cells pass through the final mitotic round called “second mitotic wave”, and this leads to the formation of ommatidia (Baker, 2001, Baonza and Freeman, 2005). The growth of the eye disc stops when the MF reaches the anterior end of the eye field and all the photoreceptor progenitors are exhausted. Thus, the size of the eye disc depends on the balance between proliferation and differentiation (Kumar, 2010, Kumar, 2012, Treisman, 2013).

In the wing disc, processes of growth and patterning are separated in time. Growth begins during the first instar larva. The disc goes through 10 rounds of mitosis and reaches about 50000 cells before the end of proliferation during pupariation. Wing disc differentiation starts only after the proliferation arrests in the whole epithelium (Resino et al., 2002, Romanova-Michaelides et al., 2015).

Despite differences in patterning and morphology, the eye and wing disc have many similarities in growth control during the larval stages.

Major signaling pathways involved in growth control of the wing and eye discs

Recent studies detected eQTL, genes and signaling pathways involved in sexual size dimorphism of the *Drosophila* wing based on the proteomic and transcriptomic analysis of the wing disc (Okada et al., 2016, Okada et al., 2019). Transcriptome-wide association study (TWAS) revealed a significant difference in the expression of genes involved in growth signaling pathways such as Wnt and TGF-β between males and females (Okada et al., 2019). However, interconnection between these pathways and sex determination genes has not been comprehensively studied. The TGF-β family member *decapentaplegic (dpp)* is expressed in all *Drosophila* imaginal discs and is one of the major growth regulators (Spencer et al., 1982, Burke et al., 1996, Pignoni and Zipursky, 1997, Martin-Castellanos et al., 2002, Rogulja et al., 2005, Vollmer et al., 2017b). Quantitative studies revealed a correlation between the rates of cell proliferation and the relative change in Dpp signaling in the eye primordium (Wartlick et al., 2014).

In the wing disc, Dpp is secreted by the cells located anteriorly to the anterior-posterior (A-P) boundary and generates a stationary, nearly symmetrical gradient. On the contrary, in the eye disc, Dpp is expressed in the MF and consequently, moves from posterior to anterior over time (Fig. 1B, C, Romanova-Michaelides et al., 2015, Vollmer et al., 2017b).

Both in the wing and eye disc the expression of Dpp is regulated by Hedgehog (Hh) signaling. Hh is a secreted protein which specifies cell fate in many developmental contexts (Varjosalo and Taipale, 2008, Pak and Segal, 2016, Yao et al., 2018). Hh is expressed in the posterior compartments of both primordia. In the wing disc Hh functions as the anterior-posterior (A-P) organizer. In the eye disc, Hh signaling drives MF movement in the posterior-to-anterior direction (Fig. 1B, C). Thus, Hh controls the balance between proliferation and differentiation (Borod and Heberlein, 1998, Spratford and Kumar, 2014). Besides activation of Dpp, Hh regulates cell proliferation directly by inducing Cyclin D and Cyclin E in the eye disc (Duman-Scheel et al., 2002).

The *wg* gene is a founding member of the Wnt family and encodes a secreted signaling protein which contributes to growth both of the eye and the wing discs (Widmann et al., 2009, Legent and Treisman, 2008). Wg signaling organizes the dorso-ventral (D-V) axis of the discs and is involved in regulatory interactions with Notch (N) pathway (Nakagoshi et al., 2002, Giraldez and Cohen, 2003, Legent and Treisman, 2008).

N signaling is required for the specification of the imaginal disc identity, as well as for the cell proliferation, differentiation and survival (Giraldez and Cohen, 2003, Kurata et al., 2000, Rafel and Milán, 2008, Suissa et al., 2010).

In the wing disc, N signaling plays the role of the D-V organizer which induces *wg* expression along the D-V axis. Independently of this function, N is directly involved in cell proliferation (Baonza and Garcia-Bellido, 1999).

N signaling has multiple roles in growth and patterning of the eye. N is implicated in eye disc growth: N activity at the D-V midline leads to the expression of the long-range secreted protein Unpaired (Upd), a ligand of the JAK-STAT pathway that induces cell proliferation (Fig. 4D, Reynolds-Kenneally and Mlodzik, 2005, Luque and Milán, 2007). A recent quantitative study considered the dilution of Upd protein prior to the onset of eye disc differentiation as a possible mechanism of growth control (Fig. 4D, Vollmer et al., 2017a).

Within and posterior to the MF, N signaling is involved in the specification of the first photoreceptors (R8) by selecting R8 cells from the proneural clusters via lateral inhibition (Fig. 4A–C). The selected R8 cells become orderly aligned in the wake of the MF and their positions define the future hexagonal array of ommatidia in the *Drosophila* eye (Frankfort and Mardon, 2002, Kumar, 2012). Subsequently, N signaling plays an important role in cell cycle control during the second mitotic wave (Baonza and Freeman, 2005, Yang and Baker, 2006) and is required for the specification of R3/R4 and R7 cell fates (Fanto and Mlodzik, 1999, Cooper and Bray, 2000). N is also involved in the control of further ommatidia maturation and in apoptosis of the non-committed interommatidial cells. These processes are required for the final size and packing of ommatidia (Miller and Cagan, 1998, Cagan and Ready, 1989, Koca et al., 2019).

Below we review the information on interaction of N and Hh signaling pathways with Sxl protein in the wing disc and other *Drosophila* structures.

Interaction of Sxl with Hh and N signaling pathways

Sxl upregulates Hh signaling and is integrated into the Hh pathway

The Hh signal is transduced through an evolutionary conserved pathway which governs complex developmental processes (Ogden et al., 2004, Varjosalo and Taipale, 2008, Chen and Jiang, 2013, Pak and Segal, 2016, Yao et al., 2018). Fig. 3 summarizes the Hh signaling in *Drosophila*.

The transmembrane protein Smoothened (Smo) is inhibited by the Hh receptor Patched (Ptc). Binding of Hh to Ptc relieves Smo from inhibition and it activates a full-length (155 kDa) isoform of the zinc-finger transcription factor Cubitus interruptus (Ci). This leads to the transcriptional activation of *hh* target genes including *dpp* and *wg* (Horabin et al., 2003, Varjosalo and Taipale, 2008). The expression of the full-length Ci in the eye and wing imaginal discs is shown in Fig. 1D and E.

Without Hh binding, Ci is predominantly present in the 75 kDa isoform and acts as a transcriptional repressor.

Processing and nuclear trafficking of Ci is regulated by the cytoplasmic signals including Costal-2 (Cos2), Fused (Fu) and Suppressor of Fused (Su(fu)) (Robbins et al., 1997, Hepker et al., 1997, Vied and Horabin, 2001, Fu and Baker, 2003, Ogden et al., 2004, Varjosalo and Taipale, 2008, Baker et al., 2009, Míguez et al., 2020).

It was shown that in *Drosophila* early germ cells the expression of Ptc and Fu is similar to that of Sxl. Moreover, in ovaries Hh signaling pathway regulates the degradation and transport of Sxl into the nucleus (Vied and Horabin, 2001, Vierd et al., 2003). As Ci is not expressed in the germ cells, it has been proposed that Sxl may replace Ci in the Hh cytoplasmic complex. In *Drosophila* embryos Sxl is in a complex with Ci, Cos2, Fu and Su(fu) and its localization is predominantly nuclear (Horabin et al., 2003).

On the contrary, analysis of spatial expression of Sxl in the *Drosophila* wing discs showed mostly cytoplasmic localization with low levels detected in the nuclei, which is unusual for the splicing factor (Horabin et al., 2003). However, nuclear/cytoplasmic distribution of Sxl is spatially non-uniform: the nuclear staining was stronger in the posterior compartment and at the A-P boundary of the wing disc. Thus, Sxl has a differential nuclear entry rate along the A-P axis of the *Drosophila* wing disc. It has been shown that the components of the Hh signaling pathway often possess spatially distinct effects on Sxl nuclear entry. Interestingly, the removal of Hh reduced the levels of nuclear Sxl not only in the anterior, but also in the posterior compartment of the wing (Horabin et al., 2003). The subcellular localization of Sxl in the anterior compartment demonstrates a dependence on the Hh signaling components, however, the mechanism affecting Sxl in the posterior remains unclear. The authors suggested a possible role of Fu (Horabin et al., 2003), which was shown to be activated in

the posterior compartment of the wing disc in a Hh-dependent manner (Ramirez- Weber et al., 2000).

Thus, like in germ cells, in somatic cells Hh signaling promotes the entry of Sxl into the nucleus (Fig. 3, Horabin et al., 2003).

To check whether mutations in the Hh signaling pathway affect the process of sex determination, the authors examined forelegs of *Drosophila* females carrying clones, mutant for different genes from the Hh signaling pathway. *Drosophila* foreleg bristles are among the best studied dimorphic organs. Males have sex combs, the arrays of enlarged mechanosensory bristles, and also more chemosensory bristles than females (Rice et al., 2019). Mutations in most genes from Hh complex, including *ptc* and *smo*, resulted in weak sex transformations, namely, slight thickening of the bristles. However, females with *cos2* clones had significantly thickened bristles, resembling male sex combs. A mutation in *cos2* also affected the *Sxl* expression in *Drosophila* embryos, although no effect on *Sxl* was produced by *cos2* mutant clones in the wing discs. The authors suggested that the activation of Hh cytoplasmic complex may slightly change depending on a tissue and developmental stage (Horabin et al., 2003).

Sxl in turn was shown to augment the Hh signal (Fig. 2B). Mature Hh is present in two lipid modifications and the cholesterol modification of Hh allows Sxl to enhance the production of the full-length Ci (Horabin, 2005). Ectopic *Sxl* expression in the *Drosophila* wing discs resulted in higher levels of full-length Ci expression with enhanced nuclear localization both in males and females. It should be noted that the Sxl target *tra* did not provide such increase in Ci expression, suggesting that this effect is provided exclusively by Sxl (Horabin, 2005).

Sxl enhances both full-length Ci and its target Dpp, which regulates the wing disc growth. Expression of Dpp downstream targets, Spalt (Sal) and phosphorylated Mothers against Dpp (pMad), is also increased by *Sxl* (Fig. 3). Remarkably, consistent with enhancement of Dpp signal and its transducers, *Sxl* was shown to induce growth of the wing disc. The authors suggest that augmentation of Hh signaling and targets of full-length Ci by *Sxl* could be the mechanism providing sexual dimorphism in body size in *Drosophila* (Horabin, 2005).

Sxl downregulates N signaling

N signaling has many important functions in various developmental processes and misregulation of this pathway results in variety of developmental defects (Andersson et al., 2011, Penton et al., 2012, Mašek and Andersson, 2017, McIntyre et al., 2020).

It has been shown that *N* directly interacts with Sxl, however, contrary to Hh, *N* is downregulated by Sxl (Fig. 2B, Penn and Schedi, 2007).

Loss of *Sxl* from the follicle cells of the *Drosophila* ovaries leads to upregulation of *N*. The experiments revealed that *N* mRNA is in a complex with Sxl in ovaries. Moreover, Sxl protein directly binds to *N* mRNA which has the configuration of Sxl binding sites similar to that in *msl-2*.

As the ability of Sxl to bind *N* mRNA should potentially affect N accumulation in different tissues, this regulatory effect was examined by monitoring the morphology of the *Drosophila* wing. Females heterozygous for strong *N* mutations exhibit notched wing phenotype. This phenotype appeared to be sensitive to *Sxl* dose, the reduction of *Sxl* dose resulted in the suppression of this phenotype and the augmentation of *Sxl* activity made it more severe. Moreover, mutations in *Sxl* gene were capable to rescue the lethal effects of *N* hypomorphic alleles (Penn and Schedi, 2007).

During the wing development, Sxl reduced the *N* expression and *N* signaling. The overexpression of Sxl in the female *Drosophila* wing disc eliminated the expression of *N* target genes *dpn (deadpan)* and *cut* (Suissa et al., 2010).

Analysis of sex-specific morphological consequences of *Sxl* activity alteration was also performed using such dimorphic trait as the number of bristles on *Drosophila* sternite A5. Females have more bristles than males (Kopp et al., 2003). Results of experiments reported in Penn and Schedi, 2007 showed that (1) the difference in bristle number between males and females is independent of *Sxl->tra->dsx (fru)* cascade; (2) *N* downregulates the number of bristles; (3) the number of bristles in females heterozygous for strong *N* mutations could be increased by magnifying Sxl levels. This led to the conclusion that extra A5 bristles in females are formed due to downregulation of *N* by Sxl (Penn and Schedi, 2007).

Hrp48 affects N pathway in Sxl-dependent manner

hrp48 encodes an essential, abundant RNA-binding protein belonging to heterogeneous nuclear ribonucleoprotein (hnRNP) family. It functions as a splicing factor in the nucleus, regulates the mRNA localization, transport, stability and translation. Hrp48 contains two N-terminal RNA-binding domains, necessary for binding with an RNA, and a C-terminal glycine-rich motif, which is essential for protein–protein and RNA–protein interactions (Matunis et al., 1992, Hammond et al., 1997, Dreyfuss et al., 2002, Dutta et al., 2017).

hrp48 was detected as a regulator of the N signaling pathway by the RNAi knockdown screen performed in the wing imaginal disc (Suissa et al., 2010). Significant *hrp48* reduction led to the formation of small and featureless wings, but less strong attenuation resulted in phenotypes resembling mutations in N signaling pathway. Moreover, these wing defects were stronger in females than in males.

Next, it was shown that 1) the knockdown of *hrp48* leads to the substantial reduction of N protein levels; 2) the expression of *cut* and *dpn*, target genes of N signaling pathway, is significantly reduced or absent in the *hrp48* mutant clones. In both cases this reduction was more substantial in females, males were only mildly affected. Conversely, overexpression of *hrp48* led to augmentation in the expression of N protein and its target Dpn, mostly apparent in female wing discs (Suissa et al., 2010).

Sex-specific mode of *hrp48* action on N suggested that it could be linked to the sex determination pathway. As Sxl attenuates *N* activity (Penn and Schedi, 2007), it has been proposed that in females *hrp48* positively influences *N* via downregulation of Sxl. Indeed,

hrp48 overexpression in the wing disc resulted in substantial reduction of *Sxl* expression and Sxl levels were significantly increased in *hrp48* loss-of-function clones (Suissa et al., 2010).

These and other experiments by Suissa et al., 2010 demonstrated that *Sxl* is repressed by *hrp48* in the *Drosophila* wing disc (Fig. 2B). Although Hrp48 is known to be involved in pre-mRNA splicing (Lee and Rio, 2015), this interaction does not affect alternative splicing of *Sxl* mRNA or its stability. The authors suggested that Hrp48 can repress the translation of Sxl mRNA, possibly by the interaction with the components of translational machinery (Suissa et al., 2010).

Remarkably, Hrp48 can interact with the sex determination pathway in some additional contexts. In female embryonic Kc cells, depletion of *hrp48* led to augmentation of Sxl expression, confirming the *hrp48* attenuates Sxl activity. Hrp48 can also act as Sxl cofactor during regulation of X-chromosome dose compensation. Hrp48 binds to the 3` UTR of *msl-2* and is required for its optimal repression by Sxl (Szostak et al., 2018).

Attenuation of Sxl levels by Hrp48 in the female wing imaginal discs have been interpreted as a possible mechanism providing proper N expression in monomorphic contexts. As Sxl represses *N* in females (Penn and Schedi, 2007), they should have lower N levels than males. The suggested function of Hrp48 was to mitigate the repression of N by Sxl and equalize the N levels between males and females.

As described above, recent studies revealed strongly dimorphic expression of genes controlling size and shape of the wing in the wing imaginal disc (Okada et al., 2019). These studies, along with the Sxl-independent function of Hrp48 described below, suggest that interactions between Hrp48, N and the sex determination pathway are more intricate and require further investigation.

Sxl-independent function of Hrp48 in regulation of N

The above described studies showed that Hrp48 regulates *N* in a Sxl-dependent fashion, i.e. the Hrp48 loss-of-function females had more severe defects than males. However, mild effects were also detected in males, suggesting that Hrp48 has some Sxl-independent role in *N* regulation (Suissa et al., 2010).

Recent results showed that Hrp48 and Deltex (Dx), a cytoplasmic modulator of the N signaling (Xu and Artavanis-Tsakonas, 1990), colocalize in cytoplasmic vesicles. The N-binding domain of Dx is responsible for its physical association with Hrp48 (Dutta et al., 2017). It was detected that Dx in combination with Hrp48 can negatively regulate N signaling by affecting the transport of N from the membrane to the cytoplasm in the *Drosophila* wing disc (Dutta et al., 2017, 2020).

The co-expression of Hrp48 and Dx reduces the availability of cytoplasmic N and leads to the downregulation of its target genes *cut* and *wg*. Remarkably, this combinatorial action of Hrp48 and Dx is Sxl-independent (Fig. 2B, D).

Experimental data on sex-biased gene expression in the eye discs

The interaction of Sxl protein with *N* mRNA has not been studied in the *Drosophila* eye. However, loss of *hrp48* function resulted in augmentation in Sxl levels both in the eye and antenna primordia of females (Suissa et al., 2010). *hrp48* knockdown led to the severe reduction of eye sizes and loss of eye pigmentation in females. A moderate reduction in eye sizes was also observed in males (Hammond et al., 1997, Suissa et al., 2010). This suggests that Hrp48 can possess sex-dependent and sex-independent functions in the *Drosophila* eye.

Our recent analysis of wild-type inbred lines of *D. melanogaster* and *D. simulans* revealed a significant variation in the spatial expression of *Delta (Dl)*, the transmembrane ligand of the N receptor (Schweisguth, 2004), between species and sexes (Fig. 5A, Ali et al., 2019).

To check the possible involvement of *dsx* in the control of *Dl/N* expression levels, we quantified the spatial expression of *Dl* mRNA in pseudo males (*tra* mutants) (Fig. 5B). *tra* mutants express Sxl, but are unable to produce the female-specific *dsx* isoform, Dsx^F (Fig, 2A, C). We compared the *Dl* mRNA expression levels in pseudo males with females and males produced by the same cross in the eye discs of the same age, but did not reveal any statistically significant distinctions (see the legend to Fig. 5B). Thus, Dsx^F presumably has no role in *Dl/N* regulation in the eye discs, or the small differences in expression levels were not captured in this assay.

Sex-biased expression of eye-related genes in the adult flies

Transcriptomic assays on sex differences in *Drosophila* gene expression most frequently utilized complex structures, e.g. whole heads and CNS of the adult flies or the whole pupae, rather than the specific larval primordia (Goldman and Arbeitman, 2007, Lebo et al., 2009, Fear et al., 2015, Arbeitman et al., 2016). Transcriptomic studies of the adult *Drosophila* heads revealed a large number of genes with sex-differential expression (Goldman and Arbeitman, 2007, Chang et al., 2011). Analysis of the expression of *calphotin (cpn)* gene which is involved in the eye disc photoreceptor specification showed that *cpn* is stronger downregulated by Dsx^F than by Dsx^M in the *Drosophila* heads. This leads to higher *cpn* expression in males than females (Goldman and Arbeitman, 2007).

Along with the effects of different Dsx isoforms, the authors detected a large number of genes with a sex-biased expression which was not regulated downstream of *tra*, including eye-related genes (Goldman and Arbeitman, 2007, Chang et al., 2011).

The prospective functions of Sxl and Dsx in the development of *Drosophila* eye

Sxl interaction with the components of the Hh and N signaling pathways in the wing disc leads to the assumption that Sxl may be integrated into regulation of dimorphic development of the eye disc. This can include both protein-protein interactions and Sxl protein binding to the target mRNAs (Horabin et al., 2003, Penn and Schedl, 2007).

Activation of Hh signaling by Sxl in females, discussed in Horabin, 2005, can function as a mechanism of dimorphic eye growth in *Drosophila* (Fig. 2B, Fig. 3). The Hh signaling is implicated in the regulation of proliferation and growth in the eye primordium either directly or via other signals, including a TGF-β family member Dpp (Baker et al., 2009, Duman-Scheel et al., 2002).

Here we considered the major signaling pathways involved in the eye disc growth. In this structure, cell proliferation and cell death are tightly coordinated with cell differentiation (Baker, 2001). Thus, multiple patterning signals could be also implicated in growth control and interact with sex determination genes. Moreover, the final eye disc size should depend on the interplay between different pathways (Legent and Treisman, 2008).

The interconnection between growth and patterning results in multiple functions of signaling pathways, not observed in the wing disc. These functions may be contradictory and differ depending on the spatial position in relation to the MF. For example, Dpp regulates 1) cell proliferation in the anterior; 2) cell cycle arrest within the MF (Wartlick et al., 2014, Horsfield et al., 1998, Firth et al., 2010). Consequently, presumptive interactions of sex determination genes with signaling pathways in the eye disc might be more complex and spatially restricted than in the wing disc.

Moreover, various functions of signaling pathways may be dependent or independent of sex. For example, the size of *Drosophila* eyes is a sexually dimorphic trait, and N is implicated in the eye disc growth. Prior to the eye disc differentiation, the spatially localized N activity promotes the growth of the entire eye field though the induction of secreted protein Upd (Fig. 4D, Chao et al., 2004, Reynolds-Kenneally and Mlodzik, 2005, Luque and Milán, 2007, Vollmer et al., 2017a).

N is also involved in the specification of the first ommatidia founder cells (R8 photoreceptor precursors) by selecting one cell from a proneural cluster via lateral inhibition (Fig. 4A–C). This process has many parallels in neuronal and neural cell fate specification (Lubensky et al., 2011, Formosa-Jordan et al., 2013, Sjöqvist and Andersson, 2019). Precision of this process determines the ordered structure of the future *Drosophila* retina and is critical regardless of sex. Thus, this function of N is presumably sexually independent.

It should be noted that although Sxl is present in each female cell, its level and subcellular localization differ between individual cells and between cell types (Horabin et al., 2003, Horabin, 2005, Suissa et al., 2011). As Hh regulates Sxl nuclear trafficking, Sxl could provide a mechanism for linking the Hh and N signaling pathways (Penn and Schedi, 2007). The Sxl interaction with N and Hh can be cell-specific and inhomogeneous in space. This should resemble an action of Dsx which provides the context-specific mosaic of dimorphic and monomorphic cells (Rice et al., 2019).

The cell-autonomous function of Dsx suggests its role in the specification of spatially restricted traits. Dsx is not expressed in the differentiated area in the posterior of the eye disc, however, the transcriptomic analysis showed that it is involved in sexually dimorphic regulation of *cpn* expressed in photoreceptor cells of adult flies (Goldman and Arbeitman, 2007). This is consistent with the observation that there is no direct connection between the

Dsx expression in the imaginal tissues and sexual dimorphisms in the adult derivatives of these tissues (Robinett et al., 2010). Although the transcriptomic studies of adult flies suggest the dimorphic regulation of gene expression by *dsx*, we still lack the comprehensive analyses of *dsx* function in the eye disc.

Perspectives for future experimental and computational studies

Despite the extensive and long-standing research in the field, the development of the *Drosophila* eye still provides interesting perspectives for future studies. The roles of Dsx and Sxl in the eye disc still need to be clarified. High-resolution transcriptomic data sets acquired for the eye primordium could be utilized to close this gap.

Recently, an atlas of 11,500 wild-type eye disc cells was created using Drop-seq, a microfluidic-based scRNA-seq platform. Along with the transcriptional profiling of wild type cells, this study demonstrated the ability to profile mutant phenotypes (Ariss et al., 2018).

Another publication reports the construction of independent single-cell RNA-seq and single cell ATAC-seq atlases for the eye disc (Bravo González-Blas et al., 2020). This integrates the analysis of gene expression with chromatin accessibility and enables the prediction of cell type-specific enhancers. To assign sex to each cell, the authors estimated a percentage of reads mapped to the X chromosome (Cusanovich et al., 2018, Bravo González-Blas et al., 2020). Finally, the “virtual eye-antennal disc” was created by spatially mapping the single-cell data on a two-dimensional template with virtual cells, enabling to link enhancers with target genes (Bravo González-Blas et al., 2020).

Although these studies were not specifically focused on the analysis of sex-biased gene expression, such data can serve as a valuable resource which enables, for example, to analyze the interactions of sex-specific *dsx* isoforms with their targets at a spatial scale. Given the cell-autonomous nature of the *dsx* expression, single-cell approaches are especially informative. Acquisition of the new datasets enabling joint transcriptomic and proteomic analyses might clarify Sxl interactions with its potential targets.

Along with experimental studies, the *in silico* approaches could provide further perspectives. Mathematical models of the eye development can infer growth control mechanisms based on the expression of major morphogens and quantitative morphometrics. This means that such models can utilize data on dimorphic gene expression and/or sex-specific measurements of the eye disc size.

Wartlick et al. mathematically described the eye disc growth in terms of temporal changes in the levels of the Dpp gradient which moves from the posterior to the anterior over time (Fig. 1B, C, Wartlick et al., 2014). Another spatio-temporal model considered regulatory interactions controlled by Dpp, Hh and the transcription factor Homothorax (Hth). The authors investigated how the model reproduced the initiation and movement of the MF followed by the termination of disc growth (Fried et al., 2016). Quantitative measurements of eye discs and computational modeling identified that the area growth rate in the *Drosophila* eye disc declines inversely proportional to the increase in its area (Vollmer et al.,

2016). Fig. 4D and its legend describe a candidate mechanism of growth control by dilution of the secreted protein Upd, a ligand of the JAK-STAT pathway and the target of N signaling (Vollmer et al., 2017a, b). This mechanism was verified experimentally and is consistent with the model predictions (Vollmer et al., 2016, 2017a).

Given the involvement of potential Sxl targets as basic regulators in the above described models of growth control, incorporation of sex-specific gene expression into such models may provide more information on mechanisms of dimorphic eye development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- In *Drosophila*, females have larger eyes than males
- Mechanisms of sex-specific regulation of eye growth still remain elusive
- The role of *doublesex* gene in the eye disc needs further investigation
- Sex lethal interacts with Notch and Hedgehog pathways in the wing disc
- We discuss presumptive sex-specific regulation of signaling pathways in the eye disc

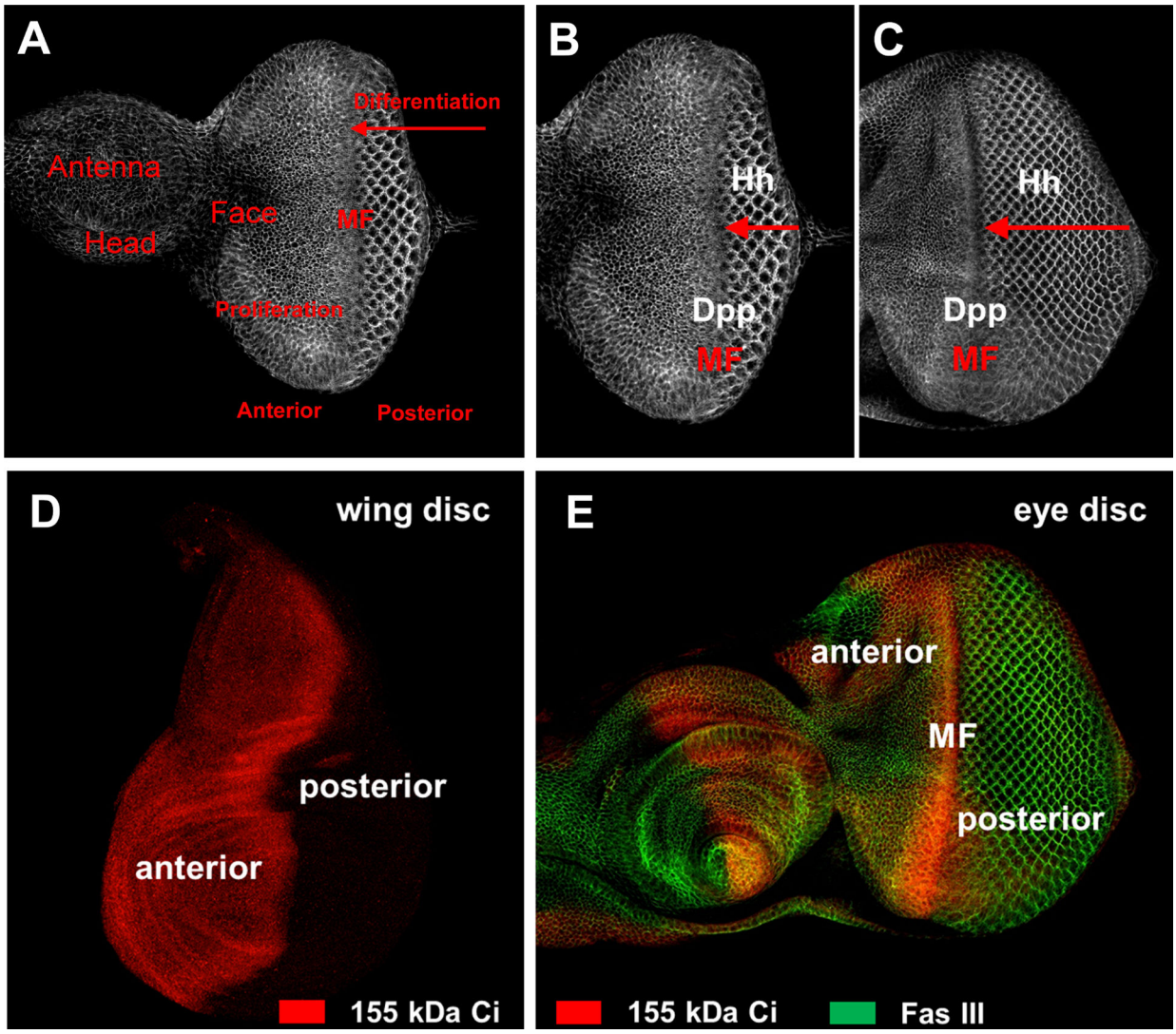

Figure 1.

The eye and wing imaginal discs dissected from wild type *D. melanogaster* (Oregon R) third instar larvae. (A) Eye disc stained with anti-Fasciclin III (FasIII) antibody (Developmental Studies Hybridoma Bank (DSHB)) which marks lateral cell walls. The morphogenetic furrow (MF) moves in the posterior-to-anterior direction of the eye disc (shown with a red arrow). This results in the conversion of undifferentiated cells anterior to MF into the developing photoreceptors in the posterior. The primordium also incorporates the antennal, head and face structures located anterior to the presumptive eye field. (B, C) Hh is responsible for the MF progression (red arrows) and is expressed posterior to the MF (see Ali et al., 2019 for the quantitative expression of *hh* mRNA). Dpp is expressed within the MF (Curtiss et al., 2000). The disc on (B) is younger than the disc on (C) where the MF has made more ommatidia rows. (D, E) The wing and eye imaginal discs stained for the expression of the full-length Ci protein using 2A1 antibody, DSHB. The 155-kb Ci protein is expressed in the anterior area with the elevated levels at the anterior-posterior (A-P)

boundary of the wing disc (D) and ahead of the MF in the eye disc (E). Images were obtained using a Zeiss LSM 700 confocal microscope with 20X objective (TIC USC).

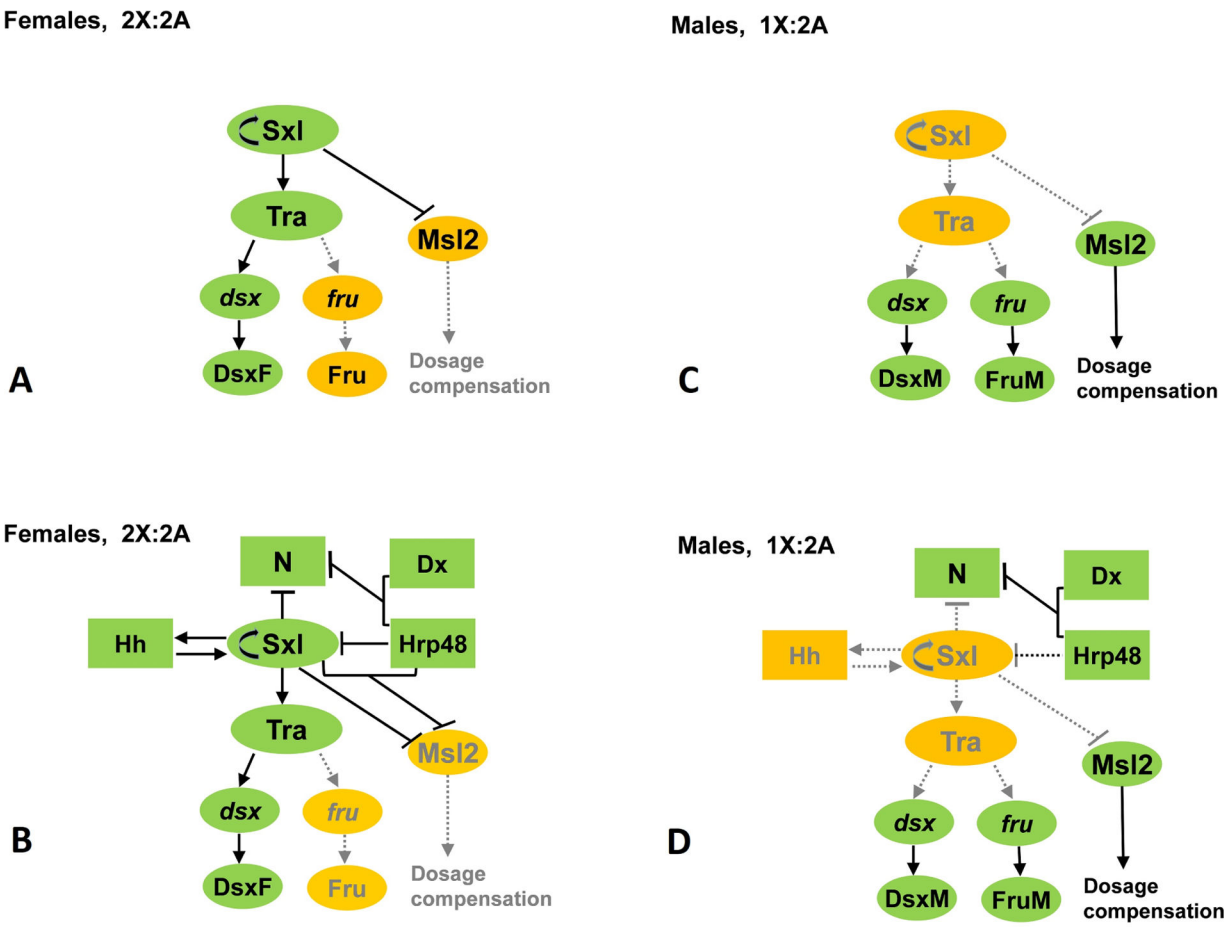

Figure 2.

Drosophila sex determination cascade and interactions of Sxl protein with Hh and N signaling pathways. A simplified scheme of the sex determination hierarchy is shown for females (A) and males (C). Active interactions are marked by green ovals and solid black lines, inactive - by gold ovals and dashed grey lines. Arrows and T-bars show positive and negative regulatory interactions respectively. Adapted from Christiansen et al., 2002. (B, D) Schemes from (A) and (C) are supplemented with solid boxes denoting interactions described in the literature (Horabin et al., 2003, 2005, Penn and Schedi, 2007, Suissa et al., 2010).

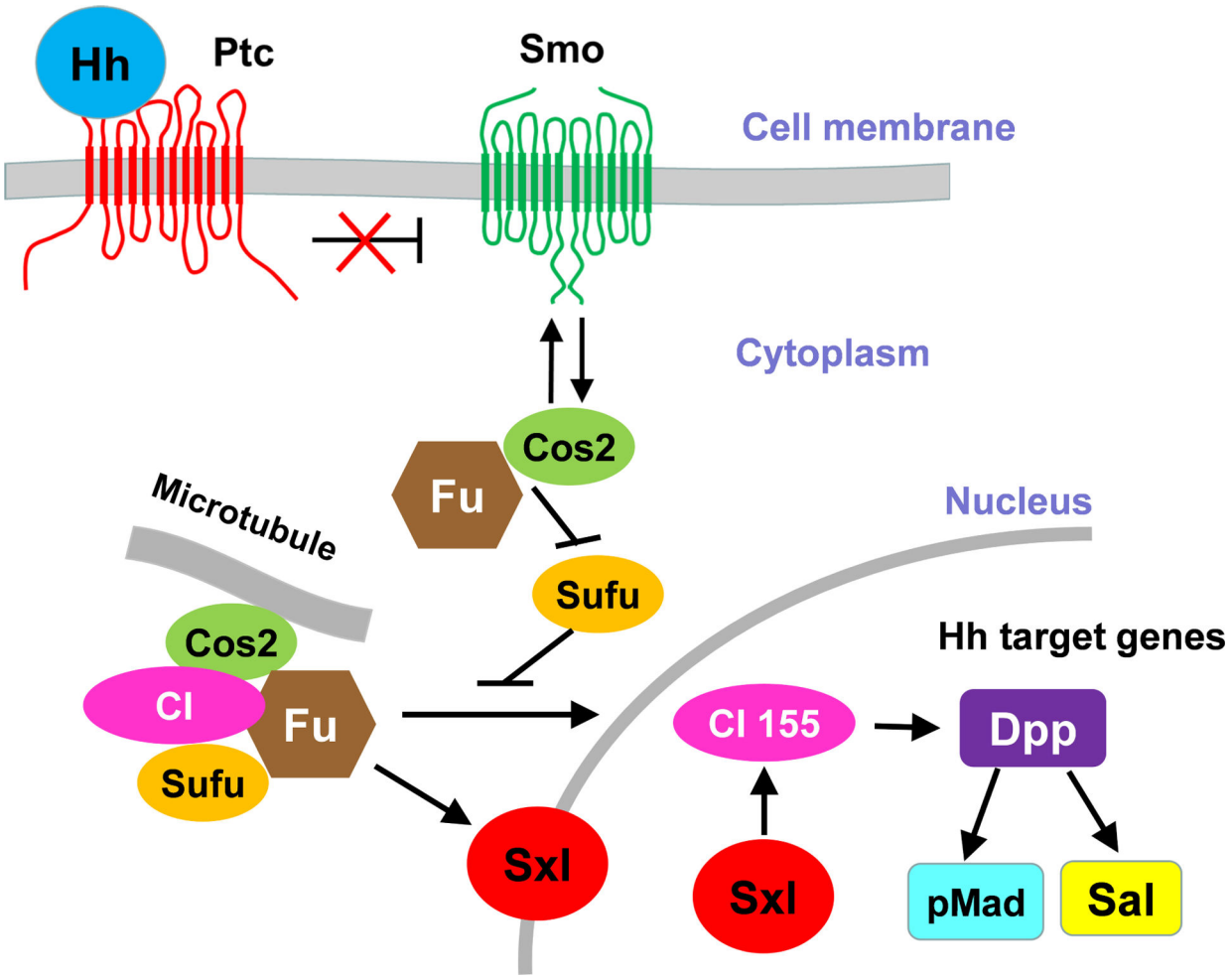

Figure 3.

Sxl is integrated into the Hh signaling pathway. The Hh pathway scheme is modified from Chen et al., 2013. It shows the branch leading to the formation of the transcriptional activator Ci^{155} upon Hh binding to its receptor Ptc. Hh signaling promotes the Sxl entry into the nucleus. Sxl upregulates both Ci^{155} and its target Dpp, as well as Dpp targets pMad and Sal (Horabin, 2005).

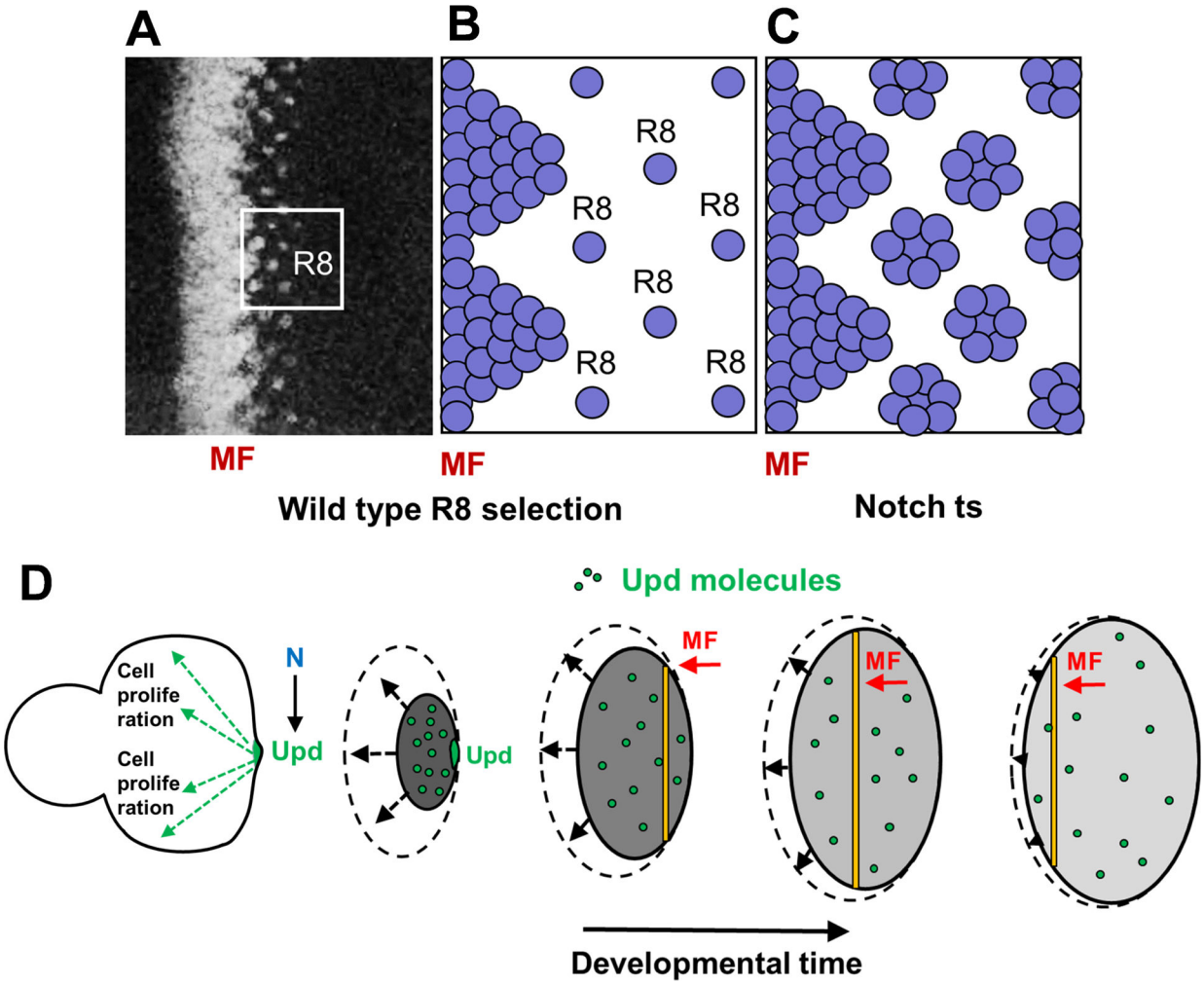

Figure 4.

Examples of N functions in the early *Drosophila* eye development. (A-C) N is responsible for the selection of the R8 cell from a proneural cluster at the initial stage of photoreceptor specification posterior to the MF. R8 cells are the precursors of first photoreceptors R8 and their positions prefigure the hexagonal pattern of the adult eye. N is responsible for the selection of R8 cells from the clusters by lateral inhibition. (A) Expression of the proneural gene *atonal (ato)* showing segregation of R8 cells from clusters in wild type. The image was obtained using a Zeiss LSM 700 confocal microscope with 20X objective. (B) Enlarged area is marked by a white rectangle frame in (A). (C) Removal of N function by the temperature-sensitive mutation (Notch ts) leads to the failure of lateral inhibition. As a result, clusters resolve into the large groups of R8 precursors instead of single R8 cells. Modified from Pepple et al., 2008 and Frankfort and Mardon, 2002. (D) The left panel: N regulates the growth of the eye disc by the activation of the JAK/STAT ligand cytokine Upd prior to the onset of cell differentiation (modified from Lucue et al., 2007). The right panel: model of the eye disc growth control by dilution of Upd (modified from Vollmer et al., 2017a, b). In the early stages of eye disc development, Upd molecules (green points) are produced at the posterior pole (green oval) and spread through the eye field by diffusion. Upd synthesis

ceases at the onset of MF progression (yellow line). The total eye disc area increases over time, and as a result, the Upd concentration decreases by dilution. The growth rate of the proliferation zone anterior to the MF is directly proportional to the concentration of Upd, which declines over time. The increase of the total area within the next time step (shown by dashed lines) becomes smaller over time. This allows the MF to reach the anterior end of the eye field and terminate growth (Vollmer et al., 2017a).

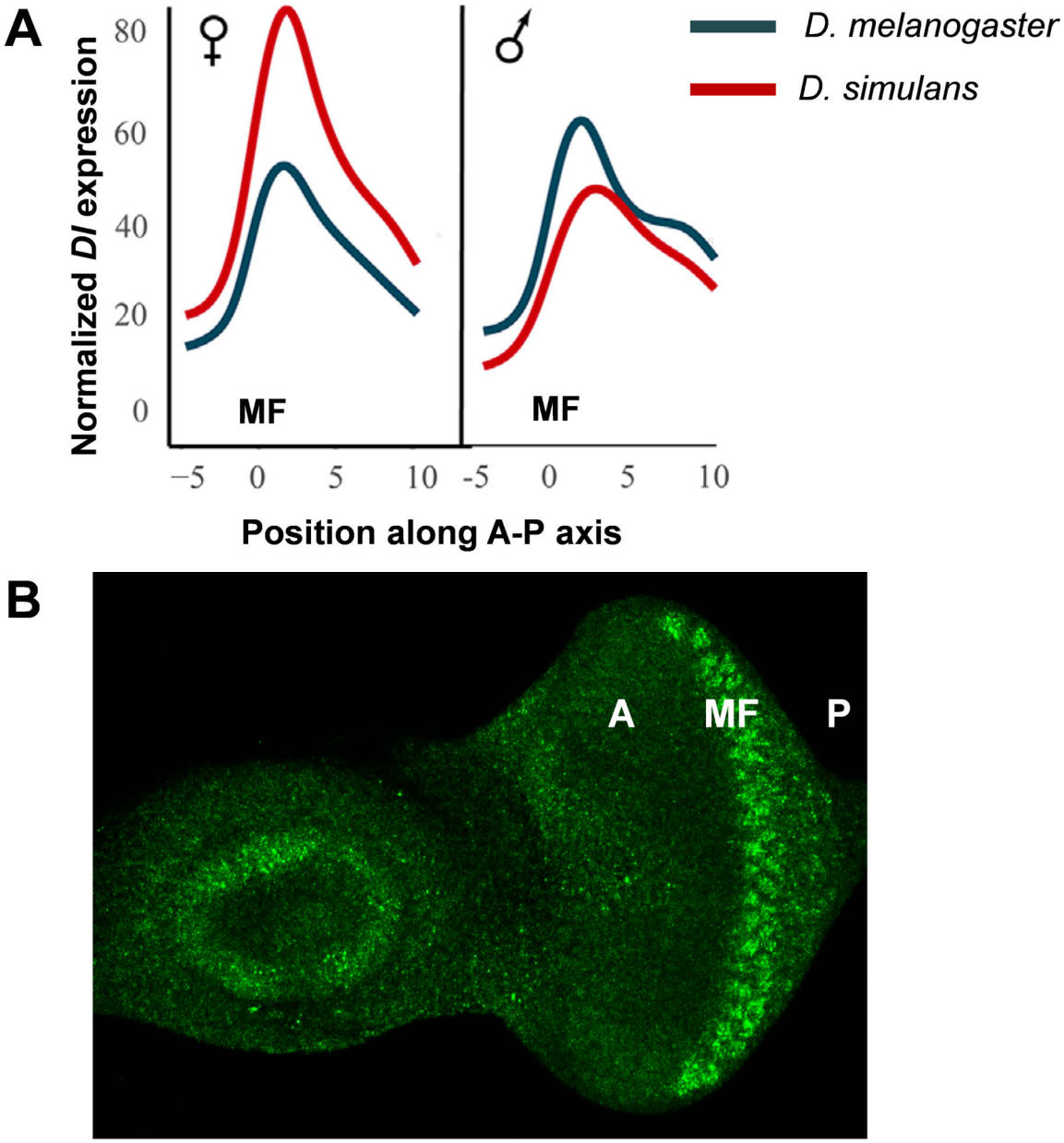

Figure 5.

(A) *Dl* mRNA expression significantly varies between sexes and species in the *Drosophila* wild type inbred lines. Curves are fitted to all examined wild type inbred lines of *D. melanogaster* and *D. simulans*. (reproduced from Ali et al., 2019). (B) An example of *Dl* mRNA expression in pseudo males obtained using the HCR technique (Choi et al., 2014) and Zeiss LSM 780 confocal microscope with 63X objective (TIC USC). Pseudo males are females which carry a mutation in the *tra* gene (*tra1*), express Dsx^M instead of Dsx^F and are phenotypically males. Eye discs were dissected from the cross between *Xw/Xw;tra1/TM6B* females and *Xw ubi–GFP/BsY;dfj7/TM6B* males (gift of Michelle Arbeitman). Quantitative

data on *Dl* expression in pseudo males was statistically compared with males and females from the same cross using ANOVA.

Table.

Total ommatidia number in the eyes of males and females from eight *Drosophila* species and strains. The blue and yellow shading marks the genotypes with maximum and minimum difference in ommatidia number between the sexes respectively (see Supplementary Table for the full list).

Species (strain)	Source	N ommatidia females	N ommatidia males	N ommatidia average	Difference in N ommatidia between males and females	
					N	%
D. melanogaster (Canton S)	Keesey et al., 2019	679	643	661	36	5,4
D. busckii	Keesey et al., 2019	740	651	696	89	12,8
D. melanogaster (Zi372)	Hilbrant et al., 2014	872	761 *	817	111	13,6
D. melanogaster (Zi375)	Posnien et al., 2012	906	815	861	91	10,6
D. funebris	Keesey et al., 2019	953	926	940	27	2,9
D. americana	Keesey et al., 2019	1265	1244	1255	21	1,7
D. suzukii	Keesey et al., 2019	1524	1378	1451	146	10,1
D. pseudotalamancana	Keesey et al., 2019	1535	1489	1512	46	3,0

* One replicate. For sample sizes and error values see Table S2 from Posnien et al., 2012, Table S1 from Hilbrant et al., 2014 and Fig. 2e from Keesey et al., 2019.