Investigating the evolution and functionality of closely related Wnt ligands in *Drosophila melanogaster*

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Abstract

The Wnt signalling pathway is a fundamental cell-to-cell communication system that plays a pivotal role in orchestrating diverse developmental processes across metazoans. There are thirteen subfamilies of Wnt ligands, seven of which are represented in the model organism *Drosophila melanogaster*. Similar expression of some *Drosophila* Wnt genes has been observed in several tissues, which suggests they may play overlapping roles, however, loss of certain Wnt genes such as *Wnt6* appear to affect only certain tissues. This could suggest that Wnt ligands act in a combinatorial manner in Wnt signalling landscapes in different tissues, despite functional differences at the molecular level. Despite significant advances in our understanding of the Wnt pathway, the functional specificity of these diverse Wnt ligands remains enigmatic. This thesis aimed to address this knowledge gap, using the following different approaches.

Sequences of the seven *Drosophila* Wnt ligands were compared to each other and protein structures for each of the seven Wnt ligands in *Drosophila* were predicted to identify any similarities and differences in the protein structures. Protein structures were modelled using templates from the existing crystallised protein of *Xenopus* Wnt8 and then compared with models created with artificial intelligence. Results from this chapter highlighted the existence of unique domains that vary in positioning across the seven Wnt genes.

The seven Wnt ligands represented in *Drosophila* were then compared to predicted protein models across a range of animals that covers all major animal divisions including: *Nematostella vectensis*, *Platynereis dumerilli*, *Mus Musculus* , *Aides aegypti* , *Tribolium castaneum* *Bicyclus*

anynana , and *Parasteatoda tepidariorum* . This work indicated that insertion predicted in *Drosophila* Wnts appears to be unique and mostly absent from the other orthologs.

Tests were then undertaken to understand whether *Drosophila wg* can be functionally replaced by its ancient paralog *Wnt6,* thus functional evolution within the Wnt ligand family. *Drosophila Wnt6* was found to be unable to functionally replace *Drosophila wg.* This indicates that specific protein domains present in *Drosophila* Wg, but not in *Drosophila* Wnt6*,* are essential for its functionality. To test if specific domains underlie Wg functionality, and thus validate or challenge the above statement, a series of chimeric Wg/Wnt6 proteins, which had domains swapped between these proteins, were tested for their ability to rescue Wg in *Drosophila*. It was observed that these chimerics were unable to rescue.

Finally, the rescue capacity of the Wg orthologs from other species *(Nematostella vectensis* , *Platynereis dumerilli*, *Mus Musculus* , *Aides aegypti* , *Tribolium castaneum Bicyclus anynana* , and *Parasteatoda tepidariorum)* were tested in *Drosophila* development including during wing development. This aimed to map the domains responsible for the functional differences between *wg orthologs*. However, none of the Wg orthologs could rescue indicated the capacity to rescue *Drosophila* Wg function. These results, taken together, suggest that protein domains present in *Drosophila* Wg may confer specific functionality to the ligand.

In summary this thesis will aim to investigate the functional divergence of Wnt genes in *Drosophila*.

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Abbreviations

Wingless	Wg
Hedgehog	Hh
Transforming growth factor	TGF-B
Receptor tyrosine kinase	RTK
Janus kinase	JAK
Signal transducer and activator of transcription	STAT
Bone morphogenetic protein	BMP
Fibroblast growth factor	FGF
Xenopus Wnt8	XWnt8
Coding region determinant	CRD
Frizzled	Fzd
Amino terminal domain	NTD
Carboxyl terminal domain	CTD
Post translational modifications	PTMs
Endoplasmic reticulum	ER
Porcupine	Porc
Wntless	Wls
G-protein-coupled receptor	GPCR
Planar cell polarity	PCP
Low-density lipoprotein- related protein	LRP5/6
Dishevelled	Dvl
Glycogen synthase kinase 3	GSK3

Casein kinase 1 a	CK1a
T cell factor/ lymphoid enhancer binding factor	(TCF/LEF)
Adenomatous polyposis coli	APC
phospholipase C	PLC
Nuclear factor kappa B	NFkB
Phospholipase C-beta	PLC-beta
phosphatidylinositol 4,5-bisphosphate	PIP2
1,2-diacylglycerol	DAG
inositol 1,4,5-triphosphate	IP3
Protein Kinase C	PKC
Clustered Regularly Interspaced Short Palindromic Repeats	CRISPR
RNA-guided Cas9 nuclease	Cas9
Wingless Knock-out	WgKO
No reverse transcriptase	NRT
Cysteine-rich domain	CRD
Nematostella vectensis Wg	NWg
Mus musculus Wg	MWg
Platynereis dumerilii Wg	PlWg
Parasteatoda tepidariorum Wg	PtWg
Tribolium castaneum Wg	TWg
Bicyclus anynana Wg	BWg
Aedes aegypti Wg	AWg
Serine	S

Cysteine	C
Amino acid	AA
Artificial intelligence	AI
Multiple cloning site	MCS
Membrane tethered wingless	Nrt-Wg
Polymerase chain reaction	PCR

1. General Introduction

1.1. Evolution and Development

The development of a multicellular organism from a single-celled zygote involves intricate gene regulatory networks, with a critical role played by signal transduction pathways (Pires-daSilva and Sommer, 2003; Gaiti *et al.,* 2017; Weidemüller *et al.,* 2021). Signalling pathways operate by initiating a series of events after a ligand from one cell or group of cells binds to the transmembrane receptors on target cells. This eventually leads to the activation or repression of transcription factors, that regulate the target cell's gene expression. This results in different outcomes for target cells, such as ensuring efficient growth and division (He *et al.,* 2003; Weidemüller *et al.,* 2021; He *et al.,* 2023).

In animals several signalling pathways play crucial roles during development, including Hedgehog (Hh), Wnt (Wnt), transforming growth factor-β (TGF-β), receptor tyrosine kinase (RTK), Notch, and Janus kinase (JAK)/signal transducer and activator of transcription (STAT). These pathways are utilized individually and in various combinations across different species and tissues, demonstrating remarkable flexibility in generating distinct responses (Lemon and Tjian, 2000; Liu *et al.,* 2022). For example, studies have indicated that the Hh and Wnt signalling pathways are involved in regulating embryogenesis and cellular differentiation (Ding and Wang, 2017). Hh signalling acts upstream of the Wnt signalling pathway, and negatively regulates Wnt activity, and the Wnt/β-catenin pathway downregulates Hh activity, suggesting that Hh and Wnt signaling are required to work in combination, to prevent tumour formation and metastasis (Ding and Wang, 2017).

Despite millions of years of evolution leading to diverse developmental patterns and cell types across the animal kingdom, it is noteworthy that only a limited set of signalling pathways are required to elicit distinct responses in different tissues and across species (Liu *et al.,* 2022). However, signal transduction pathways are not rigidly linear processes involving a fixed set of signalling components, instead, they exhibit remarkable flexibility and versatility through multiple subfamilies of ligands and receptors, exemplified by the Wnt signalling pathway (Eubelen *et al.,* 2018; Qin *et al.,* 2024). The molecular diversity of these ligands allows for specific and combined Wnt ligands to engage with different receptors to engage different intracellular transducers, allowing for the pathway to meet specific requirements in different developmental contexts and species (Pires-daSilva & Sommer, 2003; Gordon & Nusse, 2006; Eubelen *et al.,* 2018; Wiese *et al.,* 2018; Qin *et al.,* 2024).

Investigating the functionality and specificity of signalling ligands is therefore of paramount importance to understand how existing pathways can be adapted to yield new phenotypic outcomes, light on the evolution of signal transduction pathways, and provide critical insights into the intricacies of animal development.

1.2. Wnt Signalling

1.2.1. Wnt Signalling Pathways

Wnt signalling is critical for regulating a wide range of developmental processes and facilitating communication between cells during both animal development and the maintenance of adult tissues (Logan and Nusse, 2004; Amerongen and Nusse, 2009; Willert and Nusse, 2012; Steinhart and Angers, 2018; Majidinia *et al.,* 2018). Importantly, when Wnt signalling goes awry, it can lead to developmental abnormalities and abnormal cell behaviour, a hallmark of many types of cancer (Morgan *et al*., 2017; Deitrick and Pruitt, 2016).

The canonical Wnt pathway, also referred to as the β-catenin dependent pathway, is one of three distinct signalling pathways (Figure 1). The other two 'non-canonical' pathways are the planar cell polarity (PCP) and calcium-dependent pathways. In all three of these pathways, activation occurs when Wnt ligands bind to Frizzled transmembrane receptors found on the surface of target cells (He *et al*., 2004; Komiya and Habas, 2008; Amerongen and Nusse, 2009; Chae and Bothwell, 2018; Akoumianakis *et al.,* 2022).

In the canonical Wnt signalling pathway there is a key interaction between low-density lipoprotein-related protein 5/6 (LRP5/6) and Fzd proteins, which acts as a co-receptor for binding the Wnt ligand (Figure 1). The signal transduction process is regulated by the interaction of the intracellular part of the Fzd receptor with a cytoplasmic protein called Dishevelled (Dvl) (Wallingford & Habas, 2005). When a Wnt ligand is present, Fzd is activated, enabling Dsh to bind to it. This binding event prevents the destruction complex that includes Axin, Protein phosphatase 2A, Glycogen synthase kinase 3 (GSK3), Casein kinase 1 a (CK1a), and Adenomatous polyposis coli (APC) protein from targeting Beta-catenin (Figure

1). As a result, non-phosphorylated Beta-catenin accumulates in the cytoplasm and translocate into the nucleus, where it can bind to transcription factors like T cell factor/lymphoid enhancer binding factor (TCF/LEF) to either activate or repress gene expression, (reviewed in Amerongen and Nusse 2009). In the absence of Wnt ligand binding, the Beta-catenin destruction complex degrades Beta-catenin, preventing its translocation into the nucleus (Figure 1, (Amerongen and Nusse in 2009, and Komiya and Habas in 2008)).

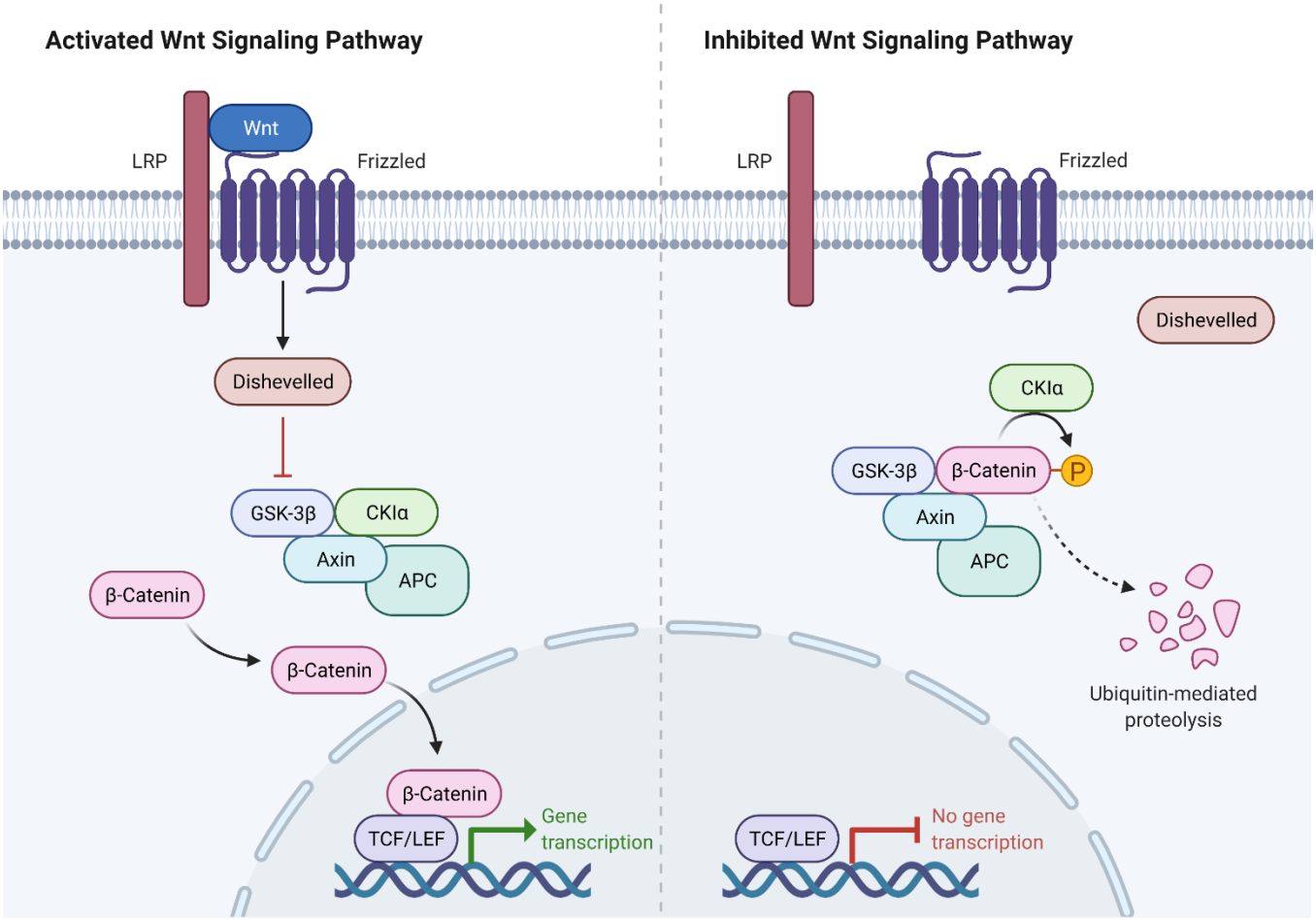


Figure 1: The canonical Wnt signalling pathway of metazoans.

The left panel shows activated Wnt signalling. Wnt ligands bind to two receptors: Fz and LRP, Dishevelled protein is activated at the membrane, and inhibits the β-catenin destruction complex, which includes: GSK-3B, Axin, APC and CKIa. This results in the accumulation of β-catenin in the cytoplasm, which is then translocated into the nucleus, to activate or repress gene expression. The right panel shows inactive Wnt signalling. In the absence of Wnt ligands, β-catenin is phosphorylated by the destruction complex, and ubiquitinated to be targeted for destruction. Figure based upon (Komiya and Habas 2008; MacDonald *et al.* 2009) figure created using BioRender.

1.3. The non-canonical Wnt Pathways

1.3.1. Planar Cell Polarity

In the planar cell polarity pathway, Wnt ligands engage with the Fz receptor and co-receptors Ror or Ryk. This interaction leads to the recruitment and activation of Dvl, followed by the activation of VANGL. Subsequently, Dvl associates with the cytoplasmic protein Daam1, facilitating its binding to the small GTPase Rho. The small GTPases Rac1 and Rho activate Rock and Jnk, respectively (Figure 2) (Yang and Mlodzik, 2015; Butler and Wallingford, 2017). These molecular events result in the reorganization of the cytoskeleton but also trigger transcriptional responses (Butler & Wallingford, 2017) (Figure 2). Recent discoveries by Ewen-Campen *et al.,* (2020) using multiplex CRISPR and dual RNAi against Wnt ligands, have raised the possibility that Wnt ligands may not be imperative for PCP patterning. Yu *et al.,* (2020) were able to show that the secretion and release of Wnts were dispensable for *Drosophila* PCP establishment after showing that a quintuple Wnt mutant left PCP unperturbed (Figure 2) (Yu *et al.*, 2020).

1.3.2. Calcium Dependent Pathway

The Calcium dependent Wnt pathway is also activated by the binding of Wnt ligands to the Fz receptor to activate phospholipase C (PLC) (Figure 2) (Komiya and Habas 2008; Yokoyama *et al.,* 2023). This results in the release of calcium and then several calcium dependent kinases are activated, which subsequently activate downstream transcription factors including factor kappa B (NFκB) (Komiya and Habas 2008; Martin-Orozco *et al.,* 2019; Yokoyama *et al.,* 2023). The calcium dependent pathway is fundamental in the regulation of cell adhesion, cell

migration and embryonic development (Figure 2) (Wallingford *et al.,* 2005; Martin-Orozco *et al.,* 2019; Meško *et al.,* 2020)

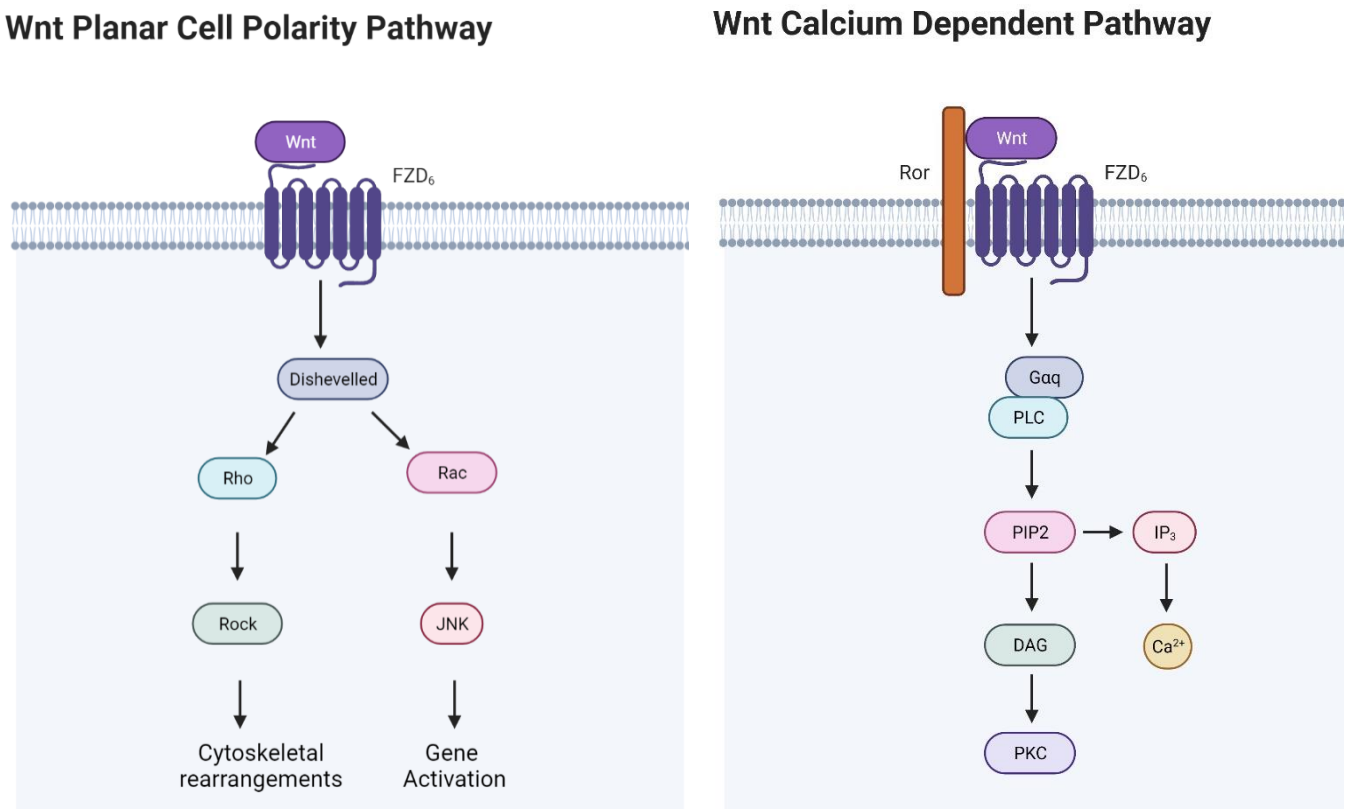


Figure 2: Non-canonical Wnt signalling pathways.

The left panel shows the planar cell polarity pathway. Wnt ligands bind to the Fz (FZD) receptor to allow Dishevelled recruitment. Dishevelled then binds to the small GTPase, which in conjunction with Rac activate Rock and JNK, this leads to cytoskeletal rearrangements and transcriptional responses. The right panel shows the Wnt Calcium dependent pathway. The Wnt Calcium pathway is initiated by the Fz receptor activating a classical G protein-coupled signaling pathway. Frizzled-G protein signaling activates Phospholipase C-beta (PLC-beta), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). Production of DAG and IP3 results in the activation of Protein Kinase C (PKC), Genes activated because of signaling through the Wnt/Ca2+ pathway regulate cell fate and cell migration. Figure based upon (Wallingford *et al.,,* 2005; Komiya and Habas 2008; Yokoyama *et al .,* 2023). Figure created using BioRender.

1.4.Repertoire of Wnt Ligands

Phylogenetic investigations comparing Wnt genes across diverse animal phyla have identified 13 distinct Wnt gene subfamilies: 1-11, 16, and A (Figure 3) (Croce *et al*., 2008; Janssen *et al*., 2010; Prud'homme *et al*., 2002; Bolognesi *et al*., 2008; Garriock *et al*., 2007; Borisenko *et al*., 2016). It appears that the Wnt ligand gene family and the components of the downstream cascade are absent in single-cell organisms (Protozoa), indicating that they likely emerged as an innovation associated with the first multicellular animals (Garriock *et al.,* 2007). Of the thirteen subfamilies of Wnt ligand genes found in metazoans, twelve are regarded as the ancestral set of protostomes (Janssen *et al.,* 2010). This diversity in the Wnt gene repertoire arose early in metazoan evolution (O'Sullivan *et al.,* 2007; Pang *et al.,* 2010). Although there have been lineage specific losses of Wnt gene subfamilies, (e.g. *Wnt3* is not found in protostomes, and *Wnt2* and *Wnt4* have been lost in insects (Janssen *et al.* 2010)), a large Wnt ligand repertoire appears to be essential for the development of most multicellular animals (van Amerongen and Nusse, 2009; Loh *et al.,* 2016; Anderson *et al.,* 2016) (Figure 3). Analysis of the relationships of Wnt ligand genes shows that their diversity likely arose through gene duplication events - for example *wg (Wnt1)* and *Wnt6*, and *Wnt9* and *Wnt10*, are ancient paralogs that have been retained in most metazoans including *Drosophila* where they still form a cluster (Sidow, 1992).

Different metazoan lineages exhibit varying numbers of Wnt genes (Holstein, 2012). The majority of Wnt subfamily diversity is observed in *Cnidaria*, *Ctenophora*, and *Porifera* (Lee *et al*., 2006; Prud'homme *et al*., 2002; Janssen *et al*., 2010; Borisenko *et al*., 2016; Pang *et al*., 2010; Moroz *et al*., 2014). For instance, the demosponge *Amphimedon queenslandica* possesses just three Wnt genes, while the marine sponge *Oscarella carmela* has an eight Wnt

genes repertoire (Reid *et al*., 2018). Surprisingly, the calcareous sponge, *Sycon ciliatum*, stands out with 21 Wnt genes (Reid *et al*., 2018). In the realm of Cnidaria, both the freshwater polyp *Hydra* and the starlet sea anemone, *Nematostella vectensis* , encompass nearly the full spectrum of Wnt subfamilies, with 12 out of the 13 Wnt subfamilies (Figure 3) (Kusserow *et al*., 2005). *Wnt9* is missing from the *Nematostella* genome, and it features two representatives each from the *Wnt7* and *Wnt8* subfamilies (Figure 3) (Kusserow *et al*., 2005. Lee *et al*., 2006; Guder *et al*., 2006; Steele *et al*., 2011).

Many Wnt genes have been isolated from the three main subdivisions of bilaterian animals, the lophotrochozoans, ecdysozoans and deuterostomes (Prud'homme *et al*., 2002). Specific examples within the lophotrochozoan include the annelid *Platynereis dumerlii and the mollusc Lottia gigantea*. *Platynereis dumerilii* is a marine polychaete annelid that possesses twelve Wnt subfamilies lacking only *Wnt3* (Pruit *et al.,* 2014). The mollusc *Lottia gigantea* possesses eleven subfamilies, lacking only *Wnt3* and *Wnt8* (Prud'homme *et al*., 2002; Cho *et al.,* 2010 Pruit *et al.,* 2014). The lophotrochozoan examples presented above retained an almost complete ancestral complement of Wnt genes.

Within the Ecdysozoa, which includes arthropods, nematodes, and related species, the crustacean *Daphnia* magna retains 12 of the 13 subfamilies, whereas the arthropods *Tribolium castaneum* and *Parasteatoda tepidariorum* retain 9 and 10 subfamilies, respectively, with duplication of *Wnt7* and *Wnt11* in the latter (Bolognesi *et al*., 2008; Cho *et al*., 2010; Janssen *et al*., 2010). Some insects, including the mosquito *Anopheles aegypti* and the fruit fly *Drosophila melanogaster* (Diptera), as well as the Western honeybee (*Apis mellifera,*

Hymenoptera), also exhibit Wnt gene loss - retaining only 6 or 7 *Wnt* genes (Figure 3) (Janssen *et al*., 2010).

All thirteen subfamilies are found in deuterostomes, although *WntA* may have been lost in vertebrates and other lineages (Janseen *et al.,* 2010). The deuterostomes, include humans which contains 19 Wnt genes, covering 12 out of 13 subfamilies, excluding only *WntA*, with seven Wnt genes duplicated- *Wnt2*, *Wnt3*, *Wnt5*, *Wnt7*, *Wnt8*, *Wnt9* and *Wnt10* (Figure 3) (Garriock *et al.*, 2007).

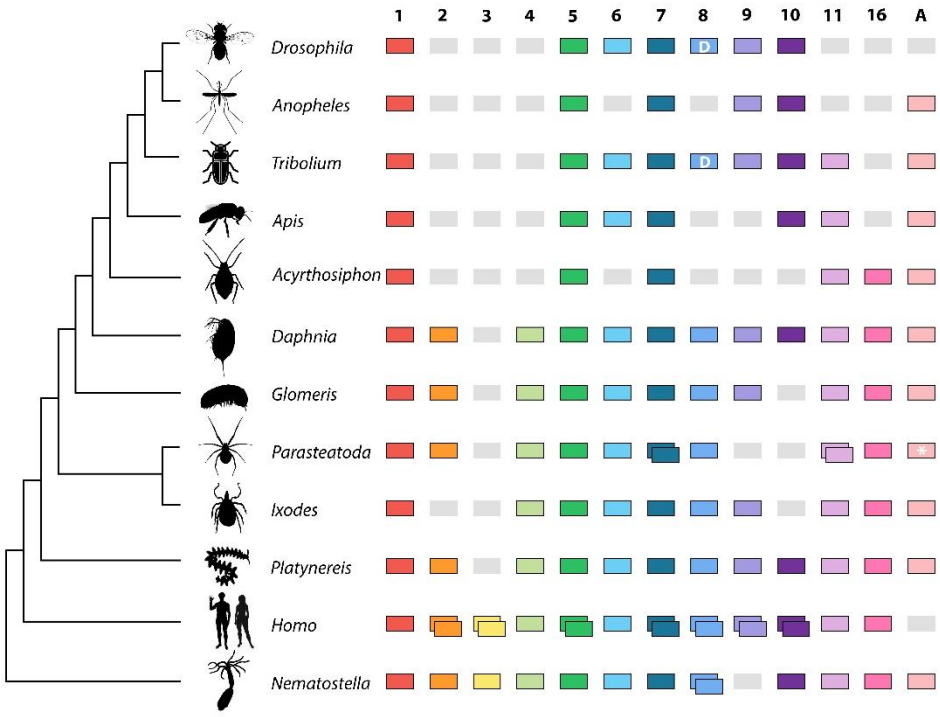


Figure 3: Wnt genes represented in Metazoa.

The Wnt gene subfamilies: 1 to 11, 16 and A are found in the various metazoans, represented by the coloured boxes that code for each Wnt gene subfamily. Grey boxes indicate the loss of Wnt subfamilies. Duplicated Wnts are represented by two overlapping boxes. Note that Wnt8 is also called WntD in *Drosophila* and *Tribolium*. The asterisk indicates that for WntA an orthologue was isolated from another spider, *Cupiennius salei* updated and adapted from Janssen *et al.,* (2010).

1.5. Wnt protein structure

Wnt proteins are about 350 amino acids long and weigh roughly 40 kDa. They are notable for containing 22-24 cysteine amino acids that are highly conserved amongst animal species (Coudreuse and Korswagen, 2007; Lu *et al.,* 2018). These cysteine amino acids play a crucial role in forming disulfide bonds within Wnt proteins, which are necessary for their characteristic globular shape (Tanaka *et al*., 2002; Willert and Nusse, 2012). These disulfide bonds are also vital for the secretion and activity of most Wnt proteins (Janda *et al*., 2012). Additionally, Wnt proteins have regions with many charged amino acids, making them highly hydrophobic. This

makes them more difficult to crystallise. (Coudreuse and Korswagen, 2007; Willert and Nusse, 2012).

Crystallographic studies, such as those involving *Xenopus* Wnt8 (XWnt8) in combination with the coding region determinant (CRD) of mouse Frizzled8 (Fzd8), have provided valuable insights (Janda *et al.,* 2012). These studies have revealed a unique two-domain structure in Wnt proteins, composed of an amino-terminal domain (NTD) and a carboxy-terminal domain (CTD), this structural arrangement enables Wnt8 to interact with Frizzled receptors through a two-part mechanism (Janda *et al.,* 2012). The "index finger" of Wnt proteins, which contains a disulfide bond at its tip, plays a central role in this interaction, and there are an additional eleven pairs of disulfide bonds distributed across the core Wnt protein, index finger, and thumb (Janda *et al.,* 2012) (Figure 4). The NTD consists of a cluster of six alpha-helices and two protruding β-hairpins, with ten conserved cysteine amino acids forming five disulfide bridges (Mikels and Nusse, 2006). The CTD is characterized by two beta-sheets, one alpha helix, and is stabilized by six disulfide bridges. In this structural arrangement, Wnt extends a "thumb" from the NTD and an "index finger" from the CTD to engage with the globular Frizzled CRD (MacDonald *et al*., 2014; Janda *et al*., 2012) (Figure 4).

After their synthesis, Wnt proteins undergo various post-translational modifications (PTMs) in the endoplasmic reticulum (ER) (Yu and Virshup, 2022). These proteins make their way to the ER with the help of amino-terminal signal peptides (Lu *et al.,* 2018). Post-translational acylation and N-glycosylation are common modifications observed in most Wnt proteins. However, an exception is seen in *Drosophila* WntD, which may follow an alternative secretory route. (Coudreuse and Korswagen, 2007; Ching *et al.,* 2008; Yu and Virshup, 2022).

The addition of acyl groups and N-glycosylation to Wnt proteins takes place in the ER and is facilitated by an enzyme called O-acyltransferase Porcupine (Porc). Porcupine catalyzes the transfer of palmitoleic acid onto Wnts, and the presence of palmitoleic acid makes Wnt ligands hydrophobic (Kadowaki *et al*., 1996; Takada *et al*., 2006; MacDonald *et al*., 2014; Routledge & Scholpp, 2019). This palmitoleic acid lipid group is essential for both the secretion and the proper function of Wnt proteins (MacDonald *et al*., 2014). Disruption or inhibition of Porcupine has been shown in several studies to lead to abnormal Wnt signalling and the retention of Wnt proteins within the ER (Barrott *et al.,* 2011; Biechele *et al.,* 2011). The secretion of Wnt proteins relies on a multi-pass transmembrane protein known as Wntless (Wls), also known as Evenness interrupted or Sprinter (Banzinger *et al.,* 2006; Bartscherer *et al.,* 2006; Petko *et al.,* 2019). When Wls is absent, several Wnt proteins become trapped within the secretory pathway of the cells that produce them. This prevents them from reaching the plasma membrane, resulting in significant loss-of-function effects related to Wnt signalling (Banzinger *et al*., 2006; Port *et al.,* 2008; Petko *et al.,* 2019).

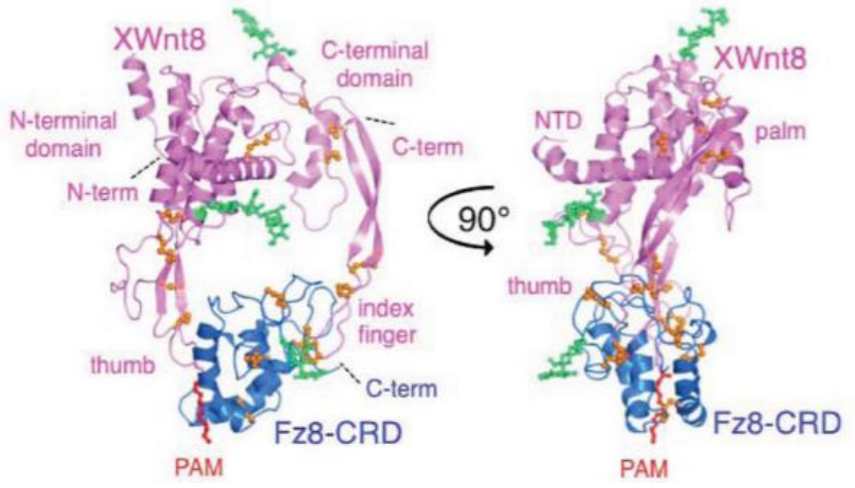


Figure 4: Overall structure of XWnt8 in complex with Fz8-CRD.

Ribbon models of XWnt8 (violet) and Fz8-CRD (blue) as viewed 'face on' (A) and 'side-on' (B). N-linked glycans are drawn as green sticks, disulfide bonds are drawn as orange sticks. (Figure from Janda *et al.,* 2012).

1.6. Wnt Frizzled Interactions

Frizzled (Fzd) genes encode proteins belonging to the G-protein-coupled receptor (GPCR) superfamily (Alrefaei *et al.,* 2021). *Drosophila* has four different Fz proteins, while mice have ten (Hui-Chuan & Klein, 2004; Kikuchi *et al*., 2007; Wang *et al*., 1996). Fz proteins are characterized by specific structural features, including a seven-transmembrane domain, a signal peptide sequence at the amino terminus, and a cysteine-rich ligand-binding domain (Hui-Chuan & Klein, 2004). Additionally, it has been demonstrated that the cysteine-rich domain (CRD) plays a crucial role in the binding of Wnt ligands to the surface of Wnt-expressing cells (Kikuchi *et al*., 2007).

1.7. *Drosophila* Wnt Ligand Loss of Expression Phenotypes

Wnt ligand function and loss of function has been extensively studied in *D. melanogaster,* which contains seven Wnt subfamilies, with the majority of our understanding coming from investigations conducted upon *wg*. Mutations in the *wg* gene lead to various developmental phenotypes in *Drosophila*, including affecting the cuticle pattern and denticle morphologies in embryonic epidermal cells (Nusslein & Wieschaus, 1980; Martinez-Arias & Lawrence, 1985; Swarup & Verheyen, 2012; Bejsovec, 2013). Overexpression of *wg* results in the conversion of ventral epidermis to a naked-cell fate (Swarup & Verheyen, 2012; Bejsovec, 2013). Additionally, *wg* expression during embryogenesis is crucial for specifying neuroblast identity (Bejsovec, 2013). *wg* also plays vital roles in the development of all *Drosophila* imaginal discs, including the wing pouch, which transforms into the adult wing blade during pupal development (Baker, 1988; Couso *et al.*, 1993; Widler & Perrimon, 1995; Legent & Treisman, 2001; Widmann & Dahmann, 2009). Loss of Wg signalling during early larval development can result in the loss of wing structures and their transformation into notal structures (Sharma,

1973; Sharma and Chopra, 1976; Swarup & Verheyen, 2012). Conversely, ectopic *Wg* expression in the notum leads to the formation of wing-like structures (Widmann & Dahmann, 2009; Klein, 2001).

The *Drosophila* adult eye and head capsule develop from the eye-antennal imaginal disc (Legent and Treisman, 2008; Won *et al.,* 2015). In this context, *wg* contributes to the growth, patterning, and differentiation of both the eye and head primordia (Royet & Finkelstein, 1997; Hazelett *et al*., 1998; Lee & Treisman, 2001; Legent & Treisman, 2001). Temperature-sensitive null mutants with reduced *wg* activity led to the expansion of eyes into the dorsal head, while overexpression of *wg*, by removing negative regulators, results in the transformation of eye tissues into head tissue (Lebrenton *et al*., 2008). During the later stages of retinal development, *wg* is required for apoptosis, which removes incomplete and excess ommatidia (Shyamala & Bhat, 2002).

When we compare the range and roles of *wg* to the other six Wnt genes in *Drosophila*, it appears that they have more limited roles in development, although they have not been studied as extensively (Wodarz and Nusse, (1998); Swarup and Verheyen (2012)).

The *Drosophila Wnt5* gene, known as *DWnt3*, was identified by Eisenberg *et al*., (1992). This gene displays complex expression patterns during *Drosophila* embryogenesis. Early on, *Wnt5* is found in limb and other appendage primordia (Fradkin *et al*., in 1995). Later in development, it is expressed in the ventral nerve cord and supraesophageal ganglia (Fradkin *et al*., in 1995). Studies conducted by Yoshikawa *et al.,* (2003) suggested a role for *Wnt5* in the central nervous system, and noted that *Wnt5* nulls lacked the ability to progress axon growth.

During *Drosophila* embryogenesis, *Wnt6* expression is observed in the developing foregut and midgut from around stage 13, and subsequently *Wnt6* expression is observed in the leg, wing, and genital imaginal discs (Janson *et al.,* 2001). It was shown that *Wnt6* is upregulated in response to cell damage and this response declines with larval maturation (Harris *et al.,* 2016). Wang *et al.,* (2018) also found that cap cells express *Wnt6* to regulate germline stem cells, and this gene is necessary for maintaining germline stem cell number. Finally, although Doumpas *et al.,* (2013) associated *Wnt6* loss of function with loss of maxillary palps, recent findings from Holzem *et al.,* (2020), confirmed expression of both *wg* and *Wnt6* in the maxillary palp field, which may reflect shared enhancers in this and other primordia. (Doumpas *et al.,* 2013; Lebreton *et al.,* 2008). Furthermore, in the absence of *Wnt6*, *wg* activity alone in the maxillary palp field allows palps to form (Holzem *et al.,* 2020). Although *Wnt6* loss of function flies are indeed viable, they have delayed pupariation perhaps indicating a role for *Wnt6* in the regulation and coordination of the growth of imaginal discs (Holzem *et al.,* 2020).

The *Drosophila* orthologue of *Wnt7*, known as *DWnt2*, is known to play a role in developing the trachea along with *wg* (Llimargas and Lawrence, 2001). *wg* and *DWnt2* work together to form the dorsal trunk, but it is important to note that in the absence of *wg*, the dorsal trunk is still formed (Llimargas and Lawrence, 2001). Wnt7 has a wide functional spectrum, which includes the development of the male reproductive tract and mediating the attachment of direct flight *Mus*cles to the epidermis (Kozopas et al. 1998; Llimargas and Lawrence 2001; Kozopas and Nusse 2002). Male *Wnt7* mutant flies have an abnormal testis morphogenesis, are sterile and hold their wings in an atypical angle to their body (Kozopas *et al.,* 1988). Additionally, it has been shown that *Wnt7* assists *wg* during specification of the main tracheal trunk, (Kozopas

et al., 1998; Llimargas and Lawrence 2001; Kozopas and Nusse 2002; Murat *et al.,* 2010). Mutants of *Wnt7* exhibit abnormal testis morphogenesis and are sterile if they reach adulthood, as observed by Kozopas *et al*., in 1998.

In *Drosophila*, *Wnt8/D* is expressed at both poles of pre-cellular blastoderm embryos (Ganguly *et al*., 2005). Ganguly *et al.,* (2005) demonstrated that *WntD* is a negative regulator of Dorsal/Twist and Snail. When *WntD* is overexpressed, it negatively regulates Dorsal localization, resulting in dorsal cuticle formation. Further studies by Gordon *et al*., in 2006 explored the role of *Wnt8/D* in Toll signalling and found that *Wnt8/D* mutants had compromised immune responses, suggesting a role for *Wnt8/D* in innate immunity.

The *Drosophila Wnt9* orthologue, known as *DWnt4*, was first isolated by Graba *et al*., in 1995. Interestingly, *Wnt9 (DWnt4)* mutants can survive to adulthood but are sterile due to issues with the formation of the epithelium sheath that covers the ovariole, as reported by Cohen *et al*., (2002). *Wnt9* plays a role in specifying the target for synapses by serving as a guidance molecule that actively discourages synapse formation with non-target *Mus*cle cells, furthermore, *Wnt9* mutants have normal *Mus*cle development, but have critically altered innervation patterns (Murat *et al.,* 2010).

Lastly, *Wnt10* has been found to be expressed in the visceral, head and somatic mesoderm, gut and central nervous system (Janson *et al.,* 2001). Han *et al.,* (2020) demonstrated that *DWnt10* is required for the normal development of the medulla, this marks the first demonstration of a loss-of-function phenotype for *DWnt10* to date.

1.8. Combinatorial action of Wnts

To understand how different Wnts may cooperate in specific *Drosophila* tissues, Limargas and Lawrence (2001) investigated the roles of *wg and Wnt7 (DWnt2)* in tracheal development. They found that *wg* was indispensable to the tracheal system, but that its homologue *DWnt2* may assist to specify the tracheal trunk, a loss of both wg and Wnt7 resulted in the absence of the dorsal trunk in approximately 50% of hemi-segments whereas, the other Wnt ligands were dispensable (Limargas and Lawrence, 2001). These findings suggest that the combined action of *wg* and *Wnt7* is necessary for tracheal development, although the presence of the dorsal trunk in the remaining 50% of hemi-segments suggests the involvement of other genes (Limargas & Lawrence, 2001).

wg and *Wnt9/DWnt4* expression also overlaps during embryogenesis, and these genes have similar expression profiles in third instar imaginal discs (Gieseler *et al*., 1995; Graba *et al*., 1995; Gieseler *et al*., 2001). Gieseler *et al.,* (2001) demonstrated that *DWnt4* can rescue the effects of *wg* loss in various developmental contexts including in wing field specification in specific mutant backgrounds, suggesting its ability to fulfil multiple developmental roles. This implies that different Wnt proteins can trigger the same cellular responses during development (Gieseler *et al*., 2001). Furthermore, *DWnt4* exhibits a segment-like polarity pattern in the ectoderm, overlapping with *wg* expression, and its overexpression leads to segmental patterning defects, indicating its involvement in segmentation (Buratovich *et al*., 2000).

Recent research by Yu *et al*., (2020) and Ewen-Campen *et al.*, (2020) revealed that, despite multiple Wnts being expressed in developing wings, the loss of *wg* has a more pronounced impact compared to the loss of *Wnt6/Wnt10* or a double knockout of *Wnt4/Wnt10.*This suggests

that tissues are more sensitive to the absence of *wg*, again emphasizing the distinct role of Wg over other Wnt ligands in *Drosophila*, even when they are co-expressed and potentially working in combination. It is possible that the combinatorial action of multiple Wnt ligands in combination with various different receptors and intracellular components may act to fine tune signalling during developmental processes (Amerongen and Nusse, 2009).

Wnt ligand co-expression in other species

Wnt co-expression has also been explored in contexts beyond *Drosophila.* Ding *et al*., (2019) showed that *wg/Wnt1* and *WntA* share similar expression patterns during silkworm embryogenesis, implying potential synergistic functions. This notion of combinatorial Wnt action is further supported by the expression of multiple Wnt ligands, including Wnt6 and Wg, in the proximal forewing of two *Heliconius* butterfly species and the outgroup species *Agraulis vanilla* (Hanly *et al*., 2019). Within the vertebrate lineage, studies have shown that *Wnt5a* and *Wnt11* can interact and cooperate to promote *Xenopus* axis formation, raising the possibility that Wnt ligand interactions may promote important aspects of vertebrate development (Miller *et al.,* 2012). Finally, Alok *et al., (*2017) found that multiple Wnt combinations can synergistically activate β-catenin signalling in multiple cell types for example Wnt1 and Wnt7B work synergistically, highlighting that through Wnt co- expression it is possible to confer increased combinatorial control over this important regulatory pathway. It should be noted that wg may also be involved (See below).

1.9. Ancient Clustering of Paralogues

The Wnt ligand family is ancient, and underwent much of its expansion before the divergence of arthropod and chordate lineages, reflecting that different extant lineages still have conserved related groups of paralogues (Sidow, 1992; Rubin *et al.,* 2000; Nusse, 2001). This is true for the four Wnt genes: *Wnt9/DWnt4, wg, Wnt6, and Wnt10* exists in many metazoans as an ancient cluster. Analysis of the arrangement of Wnt genes on the *Daphnia* genome scaffolds revealed a syntenic cluster of genes: Wnt9-Wnt1-Wnt6-Wnt10 which is consistent in other metazoans including *Nematostella* and *Drosophila* (Janssen *et al.,* 2010).

1.10. Shared regulatory regions between Wnt genes

The co-expression of clustered Wnt genes suggests that they might be controlled together and could even have common regulatory elements. *wg* and *Wnt6* exhibit overlapping expression in several tissues during development (Janson *et al.,* 2001). Both *wg* and *Wnt6* were previously shown to regulate the development of olfactory organs known as maxillary palps, however, it was never clear as to how *wg* and *Wnt6* work together to functions and the ways in which this is regulated (Holzem *et al.,* 2020). Holzem *et al*., (2020) showed that *Wnt6* does not appear to be necessary for development of maxillary palp formation, but instead alluded to the possibility that cis-regulatory elements contained in *Wnt6* may help to regulate the expression of Wg in the maxillary palp field. This implies that the first exon of *Wnt6* likely contains regulatory sequences that influence the expression of both *wg* and *Wnt6* in this specific region (Holzem *et al*., 2020). Additionally, Harris *et al*., (2016) observed that the expression of *wg* and *Wnt6* is upregulated in response to damage and is likely regulated by the enhancer BRV118, which is positioned between these two genes (Harris *et al*., 2016).

Within the *Drosophila* cluster of *Wnt4*, *wg*, *Wnt6*, and *Wnt10* genes, it has been discovered that two enhancers located within the intron of the *D. guttifera Wnt10* gene are responsible for controlling the transcription of the *wg* gene from a distance of approximately 70 kb, with the *Wnt6* locus in between (Harris *et al*., 2016). The identification of these various cis-regulatory elements associated with Wnt genes suggests that they not only regulate remote regulatory regions but also collaborate to ensure that multiple Wnts are expressed simultaneously and in the same location, facilitating combinatorial functions.

1.11. Wnt Ligands domains confer specificity

The molecular basis for the functional diversification of Wnt proteins is very poorly understood, even though this information is essential to understand the evolution and function of these important signalling ligands in development and disease. This functional diversification could result due to specific protein domain amino acids. For example, in the work of Hayes *et al.,* (1997) they identified a novel 85 amino acid domain that had not been previously described in the literature. This particular domain did not include any cysteine residues and was hydrophobic, it was referred to in the literature as wgΔ85 (Hays *et al.,* 1997). When this insert was deleted there did not appear to be any effect on the protein distribution and wgΔ85 showed normal signalling activity (Hays *et al.,* 1997). It was concluded that wgΔ85 was dispensable for Wg function, however, it should be noted that when rescuing with wgΔ85, the rescue was diminished at 18° C, revealing that this domain had a function and this was indicated by the sensitivity of the fly at lower temperatures in the absence of wild type *wg*. When trying to understand the functional diversification of Wnt proteins, it is important to also understand the molecular interactions that take place, in the case of Wnt ligand the interactions that exist with Frz receptors, as multiple Wnt ligands compete for binding to various Frz receptors. Using a combination of biophysical approaches and ligand-binding assays Eubelen *et al.,* (2018) demonstrated that ligand selectivity is conferred by Reck, which mediates Wnt7-specific binding. Reck is able to discriminate between the different Wnt proteins by binding to the structurally disordered linker of Wnt7 (Eubelen *et al.,* 2018). This highlights that cells are able to distinguish between Wnt ligands, and more importantly there is critical role for the linker domain in Wnt ligand evolution and functionality. These findings suggest that Wnt protein roles are influenced due to differences at the amino acid level, that can lead to diversification of Wnt ligands function as discussed by Janson *et al.,* (2001).

Despite these findings, further investigation is needed to understand the molecular basis for the functional specificity of Wnt proteins. Previous comparative studies have relied on cell culture or overexpression using the GAL4 system, making it challenging to account for specific expression patterns and levels determined by cis-regulatory sequences and chromatin context at the endogenous chromosomal location (Llimargas and Lawrence, 2001; Wu and Nusse, 2002; Doumpas *et al*., 2013).

Through the use of Clustered Regularly Interspaced Short Palindromic Repeats/RNA-guided Cas9 nuclease (CRISPR/Cas9)-mediated homologous recombination, there is an opportunity to comprehensively compare the function of Wnt ligands in their natural chromosomal contexts and understand the molecular basis of their functional differences. This has been successfully demonstrated by modifying the *wg* locus in *Drosophila* to replace endogenous Wg with modified Wg proteins that retain all the cis-regulatory information required by this gene (Baena-Lopez *et al*., 2013; Alexandre *et al*., 2014). This system can now be used to test if Wnts and variants thereof can substitute for each other and to identify the specific amino acids and domains responsible for their functional distinctions, helping to identify distinct classes and shed light on their functional characteristics.

1.12. Aims of this thesis

The primary goal of this project was to increase our knowledge of the evolution of Wnt ligand proteins and to better understand the molecular basis for their specific functions, with a particular focus on Wg in *Drosophila*. I addressed this aim through the following approaches in each of the results chapters:

1. Analysing and modelling the structure of the seven *Drosophila* Wnt ligands and their orthologs in other metazoans to explore how their protein structures have evolved.

2. Testing whether Wg function can be replaced by Wnt6 or specific domains of this protein in *Drosophila*.

3. Investigating the function of Wg orthologs from other metazoans in *Drosophila* to assess their ability to rescue *Drosophila wg* mutants when expressed from the endogenous locus.

2. Methods

2.1.Methods used in Chapter 1: Addressing Aim 1

2.1.1. Protein sequence alignment using ESPript

Drosophila Wingless (Wg) protein sequence were aligned to the Wg/Wnt1 orthologues of *Nematostella vectensis* (Cnidaria), *Mus musculus* (Vertebrata), *Platynereis dumerilii* (Annelida), *Parasteatoda tepidariorum* , *Tribolium castaneum* , *Bicyclus anynana* and *Aedes aegypti* using Clustal Omega (UniProt webpage). Protein sequences were obtained from the National centre of biotechnology information database (https://www.ncbi.nlm.nih.gov/protein/) (See Supplementary Table 1 for protein accension numbers) (The Uniprot Consortium, 2021; Janda *et al.,* 2010; Sievers *et al.,*2011). The amino acid sequences of all species included were obtained from NCBI (http://www.ncbi.nlm.nih.gov). Once sequences were aligned using Clustal Omega, the alignments were fed into Easy Sequencing in PostScript 3.0 (ESPript), a program which rendered sequence similarities and secondary structure information from aligned sequences. The beginner mode was selected, and alignment files as well as Protein PDB files were uploaded into the server. The final output harboured an alignment, and quaternary protein structure information. (Robert and Gouet, 2014),

2.1.2. Phylogenetic Analyses

The phylogenetic analysis was carried out using the online tool Phylogeny.fr using the One click mode, which comprised the following steps: Sequences were aligned with *MUS*CLE (v3.8.31) configured for highest accuracy (*MUS*CLE with default settings). After alignment,

ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b) using the following parameters:

- minimum length of a block after gap cleaning: 10
- no gap positions were allowed in the final alignment
- all segments with contiguous non-conserved positions bigger than 8 were rejected
- minimum number of sequences for a flank position: 85%

The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). Reliability for internal branch was assessed using the aLRT test (SH-Like). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3).

2.1.3. Swiss- model Protein Modelling

Swiss-model is a fully automated protein structure homology-modelling server that was used to model all seven *Drosophila* Wnt ligands and Wg orthologs from *Nematostella Mus Platynereis Parasteatoda Tribolium castaneum, Bicyclus* and *Aedes* accessible via the server: https://swissmodel.expasy.org. In order to model these Wnt proteins, *Xenopus* Wnt8 was used as the template structure (P28026 WNT8_XENLA, model ID: AF-P28026-F1). Amino acid sequences of the specific Wnt ligand were pasted into the Swiss-model platform, and the template XWnt8 was (P28026 WNT8_XENLA, model ID: AF-P28026-F1) added. To ensure the settings were correct the sequences were first validated, and then modelled using the template. The predicted 3D models were colour coded to help indicate prediction confidence levels, with blue representing high confidence in the protein structure prediction and red indicating low confidence.

2.1.4. AlphaFold Protein Modelling

AlphaFold v2.3.2 is a novel machine learning approach that incorporates physical and biological knowledge about protein structure to produce predicted 3D protein models (Jumper *et al.,* 2021). The required Wnt ligand structures had not been generated as of yet and were not available on the AlphaFold Protein structure database at the time of conducting this analysis, therefore, I generated required models using DeepMind's Colab notebook (Jumper *et al.,* 2021). The Colab note book source code allows for the generation of required proteins that were not already available on the database. https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb

In comparison to AlphaFold v2.3.2, the Colab notebook uses no templates (homologous structures) and a selected portion of the BFD database. To run the Colab note book code: Firstly, click on the Connect button on the top right to get started. Secondly paste the amino acid sequence of your protein (without any headers) into the "Enter the amino acid sequence to fold". Finally. Run all cells in the Colab, either by running them individually (with the play button on the left side) or via Runtime > Run all (Jumper *et al,.* 2021)

2.1.5. IUPRED & Anchor

Intrinsically disordered proteins (IDPs) have no single well-defined tertiary structure under native conditions (Erdos *et al.,* 2021). IUPred2 is a combined web interface to identify disordered protein regions. IUPred is based on a unique energy estimation approach that provides prediction of disordered regions. Whereas ANCHOR identifies segments that are intrinsically disordered but have the potential to adopt a stable structure upon interaction with a binding partner. (Erdos *et al.,* 2021). IUPred2 supersedes the previous IUPred and ANCHOR servers (Erdos *et al.,* 2021). To run the interface the Wnt ligand sequences were pasted into the "amino acid sequence" field. The parameter "*long disorder protein sequence*" was selected. The primary output of IUPRED is a graph that shows disorder tendency of each residue in a given protein, a value over the 0.5 threshold highlights disorder for that particular residue (Meszaros *et al.,* 2019).

2.2.Methods used in Results Chapters 2 & 3: Addressing Aim 2 & 3

2.2.1. *Drosophila melanogaster* Stocks

All fly stocks were kept at 25 °C on standard yeast extract-sucrose medium at 25℃ under a controlled 12/12 dark to light cycle. Fly food was prepared in house as described by Ish-Horowicz, (1995).

- *D. melanogaster* strain w;WgKO/CyO;TM6B was kindly donated by Cyrille Alexandre, Francis Crick Institute.
- Strain w;:KB19/KB19 (germline flippase) was kindly donated by Luis Alberto Baena-Lopez, Oxford University.

- The following strains were obtained from the Bloomington stock centre: y[1] w[1118]; P{w[+mC]=vgM-GAL4.Exel}3 (8223) and w[*]; P{w[+mC]=UAS-FLP.D}JD2(4540).
- The following strains were also used: *w^{1118}, w−; if/CyO; MKRS/TM6B*. Laboratory stocks were maintained.

2.2.2. *Drosophila melanogaster* Embryo Injections

Genetic transformation of *D. melanogaster* was performed according to Gompel and Schröder, (2015). This differed to previous protocols (e.g., Spradling & Rubin, 1982) because embryos were not dechorionated. *Drosophila* WgKO stock was amplified in advance, to allow for sufficient injection stock (eggs) and for subsequent collection of virgin WgKO females.

Approximately 300 mature flies were transferred to an egg-lay cage 1-3 days prior to injection (Gompel and Schröder, 2015). Needles for the injection process were pulled to ensure they had a short taper and a thin tip to prevent damage to the embryos (Miller *et al.,* 2002). Needles were filled with 1ul of the construct containing FRT-wg-FRT-Desired Wnt-HA-loxP-pax-Cherry-loxP (0.4ug/ul) and mixed with injection dye to be injected into the wg [KO] fly line using phiC31 mediated integration. Successful integration was detected by Cherry expression in the eyes (note that this marker was subsequently removed using Cre mediated recombination between the flanking loxP sites) (Huang *et al.,* 2009).

Embryos were injected using a Leica Axio inverted microscope, equipped with an Eppendorf Femtojet with a hand trigger push button. To inject the embryos, the needle tip was inserted into the posterior fifth of the embryo (close to the germline nuclei). To inject a drop of the mix,

the manual button to inject was pressed and in doing so a small cloud of dye is visible. Injected embryos were transferred into a new vial and left to develop at 25℃ until larvae hatch. Flies were screened to check for Cherry expression in the eye, to confirm successful plasmid DNA integration. All protocols, corresponding targeting vectors and primers are described in Baena-Lopez *et al.,* (2013) and Alexandre *et al.,* (2014).

2.3.General Molecular Biology Techniques used in results chapter 2 and 3

2.3.1. Genomic DNA Extraction

To extract genomic DNA for verification of plasmid integration into WgKO flies, flies were collected and placed at -20℃. Salt DNA extraction was completed according to Miller *et al.,* (1988). This method involves salting out the cellular proteins by dehydration and precipitation with a saturated NaCl solution. Salt extraction was completed for all flies used in this investigation, the WgKO founder line and all transgenics.

2.3.2. PCR primer design and validation

Primers were designed using Primer blast (NCBI) with the following parameters in mind: melting temperature (Tm): 57-63°C, %GC content: 40-60%, product size: 70-150 bp. All reactions were run at annealing temperatures of between 56-63°C to determine their optimal annealing temperature.

Fast SYBR® Green Master Mix (Applied Biosystems) was employed for all reactions formulated as below, with a total reaction volume of 20 ul. All reactions were performed on Applied Biosystmes PCR Machines (Applied Biosystems). All primer sequences can be located in the appendix (Table 2).

2.3.3. Polymerase Chain reaction (PCR) and Gel Extraction

Primers were used to generate PCR products in 50 ul reactions of the following composition:

- up to1,000 ng of template
- forward primer at 1 µM
- reverse primer at 1 µM,
- OneTaq 2X Master Mix Standard Buffer 12.5 µl (New England Biolabs)
- nuclease free water up to 50 µl

The PCR program was as follows:

- Initial denaturation 94℃ 30 s
- Denaturation 94℃ 30 s*
- Annealing (optimal temperature utilised: 45- 68℃ 30 s*
- Extension 68℃ 1min/kb*
- Final extension 68℃ 5 min
- Final hold 10℃

*PCRs was carried out for 30-35 cycles under optimal conditions for each primer pair and product size.

PCR products were loaded on a 1% agarose gel, The specific PCR band of interest was purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel) according to the manufacturer's instructions.

2.3.4. Cloning

PCR products were incorporated into the TOPO pCR4 vector using the TOPO TA kit (cloning of Taq-polymerase amplified PCR products, Invitrogen), according to the manufacturer's guidelines:

The reaction was then transformed into OneShot TOP10 chemically competent cells (Invitrogen) according to the standard heat shock transformation protocol:

- TOP10 cells are thawed on ice
- 2 µl of the TOPO cloning reaction is added to the cells, mixed gently and incubated on ice for up to 30 min
- TOP10 cells are heat-shocked in a water bath at 42°C for 30 sec and immediately transferred to ice
- 250 µl S.O.C. medium (Invitrogen) is added and incubated in a shaking incubator at 200 rpm, at 37 °C for 1 hour
- 50 µl of the transformed cells were plated on Lennox Broth (LB) plates with ampicillin (100 µg/ml) and incubated at 37 ° C over night.

2.3.5. Colony PCR and overnight cultures

Colonies were picked for PCR validation of cloning using the OneTaq 2x Master Mix (NEB). Colonies were grown on Lennox Broth (LB) plates with ampicillin (100 µg/ml) added.The PCR product was loaded on a 1% agarose gel and checked for the correct product size. Colonies with the correct insert size were grown in 5 ml liquid LB cultures with ampicillin (100 µg/ml) at 37 °C in a shaking incubator overnight. Plasmid mini preparations were made from the liquid cultures using the EZNA Plasmid Mini Kit I (VWR) according to the manufacturers guidelines and verified with Sanger sequencing (Eurofins).

2.3.6. Sequencing

PCR products and plasmids were sequenced using Eurofins Genomics (Germany).

Alignments were viewed and assessed using Benchling.

3. Investigating Wnt protein structures across selected metazoans

3.1. Wnt protein alignment and structure modelling

Are Wnt ligand orthologues from the same subfamily functionally conserved throughout metazoans, or do they have lineage-specific differences? Could for example evolved novel domains or receptor specificity, result in differences in their functionality? To begin to shed light on these questions, protein sequence and predicted structures of Wnt ligands from different species were compared and investigated.

Wnt ligands are typically difficult proteins to investigate due to their hydrophobic nature, making them insoluble and difficult to purify and crystallise (Janda et *al.,* 2012). However, the crystal structure of *Xenopus* Wnt8 (XWnt8) in complex with the mouse Frizzled-8 (Fzd8) cysteine-rich domain (CRD) allowed for hydrophobic residues to become masked and this facilitated structural access to Wnt ligands for the first time (Janda *et al.,* 2012). High-resolution structural information on mammalian Wnt proteins still appears to be lacking, using methodology similar to Janda *et al.,* (2012), Hirai *et al.,* (2019) were able to shed further light on structural information around Wnt ligands by providing the crystal structure of Human Wnt3 in complex with mouse Fzd8. By delving further into the structural information of Wnt ligands, this will allow for a greater understanding of how Wnt ligands may work in protein complexes, and the impact different domains can have on receptor binding (Hirai *et al.,* 2019).

The XWnt8 structure served as an important reference point that was used as a template to model Wnt ligand structure across subfamilies and different species (Janda *et al.,* 2012). Therefore, using this structure as a template, Wnt protein structures were modelled using

Swiss-mod- a structural bioinformatics server that models 3D protein structures (Waterhouse *et al*., 2018; Bienert *et al.,* 2017; Guex *et al.,* 2009; Bertoni *et al.,* 2017). These structures were then compared to those predicted via Alpha Deepmind, a more sophisticated protein modelling tool that utilises artificial intelligence rather than a template basis (Jumper *et al.,* 2021). This comparison approach was used, to see if any significant differences among the modelled Wnt ligands could be unlocked. It is important to note that subsequent to the analysis conducted in this chapter, Alpha have now released protein predictions that are available in Swissprot (Varadi *et al.,* 2021).

Using these approaches, *Drosophila* Wg was modelled and compared to Wg from orthologs from seven other metazoans; *Nematostella vectensis* , *Platynereis dumerilli*, *Mus Musculus* , *Aides aegypti* , *Tribolium castaneum* *Bicyclus anynana* , and *Parasteatoda tepidariorum* . Secondly, *Drosophila* Wnt ligands were modelled and compared, and using IUPRED and Anchor analysis, different regions in each *Drosophila* Wnt ligand were investigated to determine the presence of any disordered domains.

3.2.Results

3.2.1. Comparison of the seven *Drosophila* Wnt ligands with their orthologs from selected other species

To investigate the sequence evolution of Wnt ligands, protein sequence alignments of the Wnt ligand repertoire found in *Drosophila* were generated with a selection of *wg* orthologs from other certain species (Prud'homme *et al.,* 2002; Sullivan *et al.,* 2007; Janssen *et al.,* 2010). The following species were selected- *Nematostella vectensis (Cnidaria), Mus Musculus (Vertebrata)*, *Platynereis dumerilii (Annelida), Parasteatoda tepidariorum, Tribolium castaneum, Bicyclus anynana* and *Aedes aegypti* (spider/ beetle/butterfly/mosquito, Arthropoda) as they almost all contained the full complement of seven Wnt ligands that are represented in *Drosophila*, and covered a diverse range of animals. Using Clustal omega alignments of each the different Wnt ligands in *Drosophila* and the corresponding Wnt ligand from each of the listed orthologs were aligned (Madeira *et al.,* 2022). However, since the Wnt repertoires have evolved it was not possible to select exactly the same species to compare to all seven *Drosophila* Wnts.

3.2.1.1. Wg/Wnt1 protein alignment

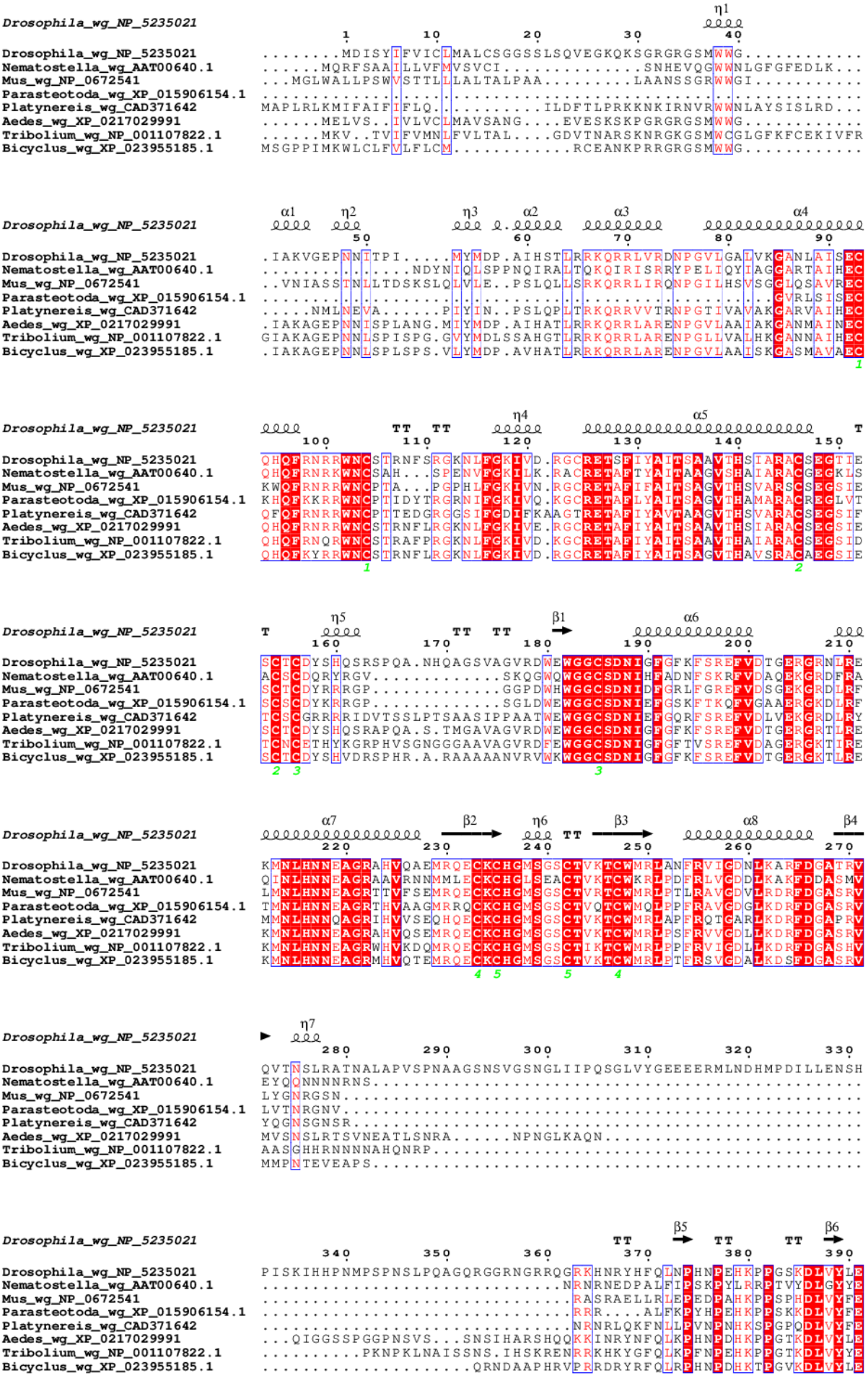
Wg/Wnt1 protein alignments were completed to compare the *Drosophila* Wg protein sequence to the Wg/Wnt1 orthologues: *Nematostella vectensis (Cnidaria), Mus Musculus (Vertebrata), Platynereis dumerilii (Annelida), Parasteatoda tepidariorum , Tribolium castaneum , Bicyclus anynana and Aedes aegypti* (spider/ beetle/butterfly/mosquito, Arthropoda) by aligning the full-length protein sequences using Using Espript 3.0 multiple sequence alignment tool.

Wg amino acid sequences were compared to each other to compare conserved amino acids (amino acid identities). Wg from the dipteran *Aedes* has the greatest level of sequence identity to *Drosophila* Wg with a 76.24% identity match while *Nematostella* only shared 46.35% sequence identity (Table 1). The only sequence pair to have greater sequence identity was *Aedes* and *Bicyclus* sharing 78.29% identities, and the least shared sequence existed between *Nematostella* and *Mus* sharing only 43.42% sequence identity (Table 1).

Table 1: Wg ortholog percentage identity matrix table

***Nematostella*_Wg**	**100.00**	**45.48**	**46.59**	**43.42**	**47.79**	**46.35**	**48.31**	**51.64**
***Platynereis*_Wg**	45.48	100.00	52.72	54.72	52.97	52.38	53.70	57.14
***Parasteotoda*_Wg**	46.59	52.72	100.00	55.12	55.16	54.97	57.14	61.20
***Mus*_Wg**	43.42	54.72	55.12	100.00	54.57	54.72	56.59	62.33
***Tribolium*_Wg**	47.79	52.97	55.16	54.57	100.00	67.81	71.20	68.10
***Drosophila*_Wg**	46.35	52.38	54.97	54.72	67.81	100.00	76.24	72.48
***Aedes*_Wg**	48.21	53.70	57.14	56.59	71.20	76.24	100.00	78.29
***Bicyclus*_Wg**	51.64	57.14	61.20	62.33	68.10	72.48	78.29	100.00

The greatest level of conservation was present in the alpha helices: 4-9 and within the beta pleated sheets: 1-9, identical amino acids within each sequence can be seen in white font and highlighted red, amino acid that were different but have similar properties such as hydrophilic (Figure 5). The alignment highlights that alanine 136 and cysteine 242 are conserved within the alignment, among all species surveyed. The conservation of the following two AA has been reported previously, and that alanine 136 and cysteine 242 are absolutely invariant in Wg across animals (Dierick and Bejsovec, 1998). Furthermore, the number of disulphide bridges are donated in green, within the Wg alignment, there were 20 conserved cysteine residues (Figure 5). It is within these tertiary structures that cysteine conservation was seen. Noticeably, there appears to be a distinct lack of conservation at the start of the alignment in which there is no distinct tertiary protein structure, except for a small alpha helix denoted by n_1. Furthermore, as described previously, *Drosophila* Wg possesses an 85 amino acid insert (position 276AA to 361AA) that is absent from all other ortholog sequences (Figure 5) (Hays *et al.,* 1997). *Aedes* and *Tribolium* appear to be the only orthologs that possess amino acids within positions (276-361) however, there is no amino acid conservation (Figure 5)


Drosophila_wg_NP_5235021
Drosophila_wg_NP_5235021
Nematostella_wg_AAT00640.1
Mus_wg_NP_0672541
Parasteotoda_wg_XP_015906154.1
Platynereis_wg_CAD371642
Aedes_wg_XP_0217029991
Tribolium_wg_NP_001107822.1
Bicyclus_wg_XP_023955185.1

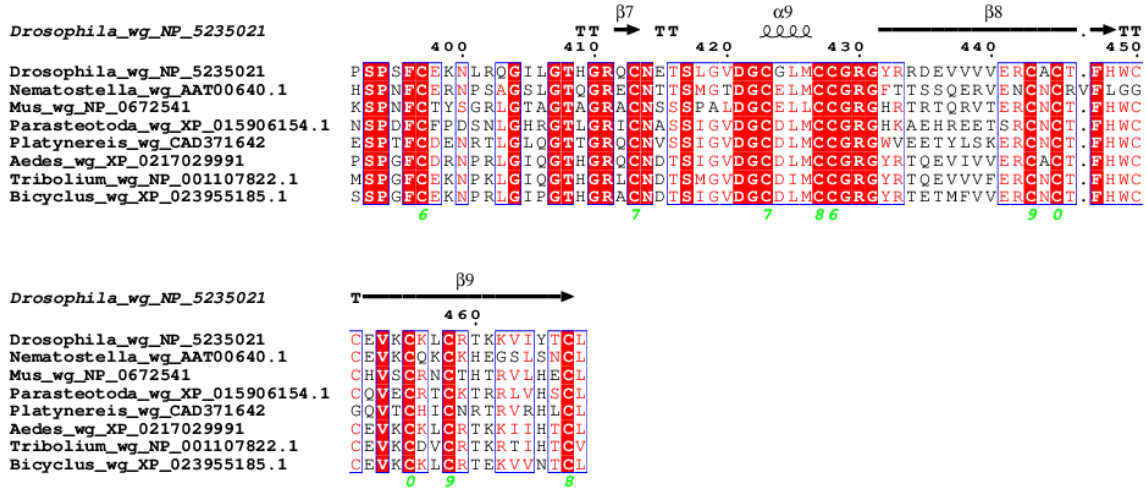


Figure 5:Wg Alignment.

An ESPript output obtained from Wg protein sequences extracted from NCBI data bank, aligned with CLUSTAL. The Wg protein is 468AA long. Sequences are divided into three groups according to similarity. Residues that are strictly conserved have a red background, residues that are well conserved within a group are indicated by red letters and the remaining AA are in regular black font. Residues conserved between groups are boxed. Symbols above blocks of sequences correspond to the secondary structure of the Wg protein. The Wg protein consists of alpha helices depicted as squiggly lines and beta pleated sheets as arrows. The numbers depicted below the alignments in green correspond to the number of disulphide bonds present.

3.2.1.2. Drosophilid Wg with *Musca domestica* & *Aedes* Wg comparison

When comparing Wg orthologs across different animals, it was apparent there was a region of around 85 AA that was present in *Drosophila* Wg but completely absent from the other species surveyed such as in the case of *Nematostella* (Figure 5). However, within the insects *Tribolium* and *Aedes*, the specific 85AA region in question did contain some AA, but there was no conservation, and the inserts in *Tribolium* and *Aedes* were not as prominent.

To explore this further, and to determine if the insert was insect or *Drosophilid* specific, the following species were selected to be investigated further: *Drosophila melanogaster, Drosophila virilis, Drosophila mauritiana, Drosophila pseudoobscura, Musca domestica* and *Aedes aegypti*. The following species were selected due to order of relatedness (Figure 6).

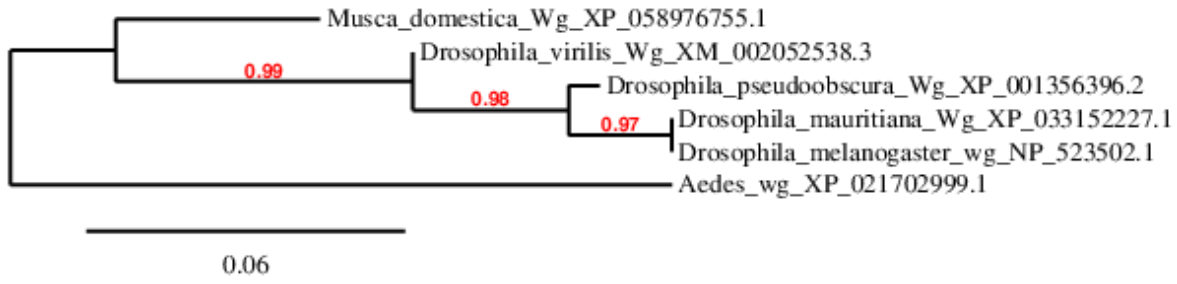
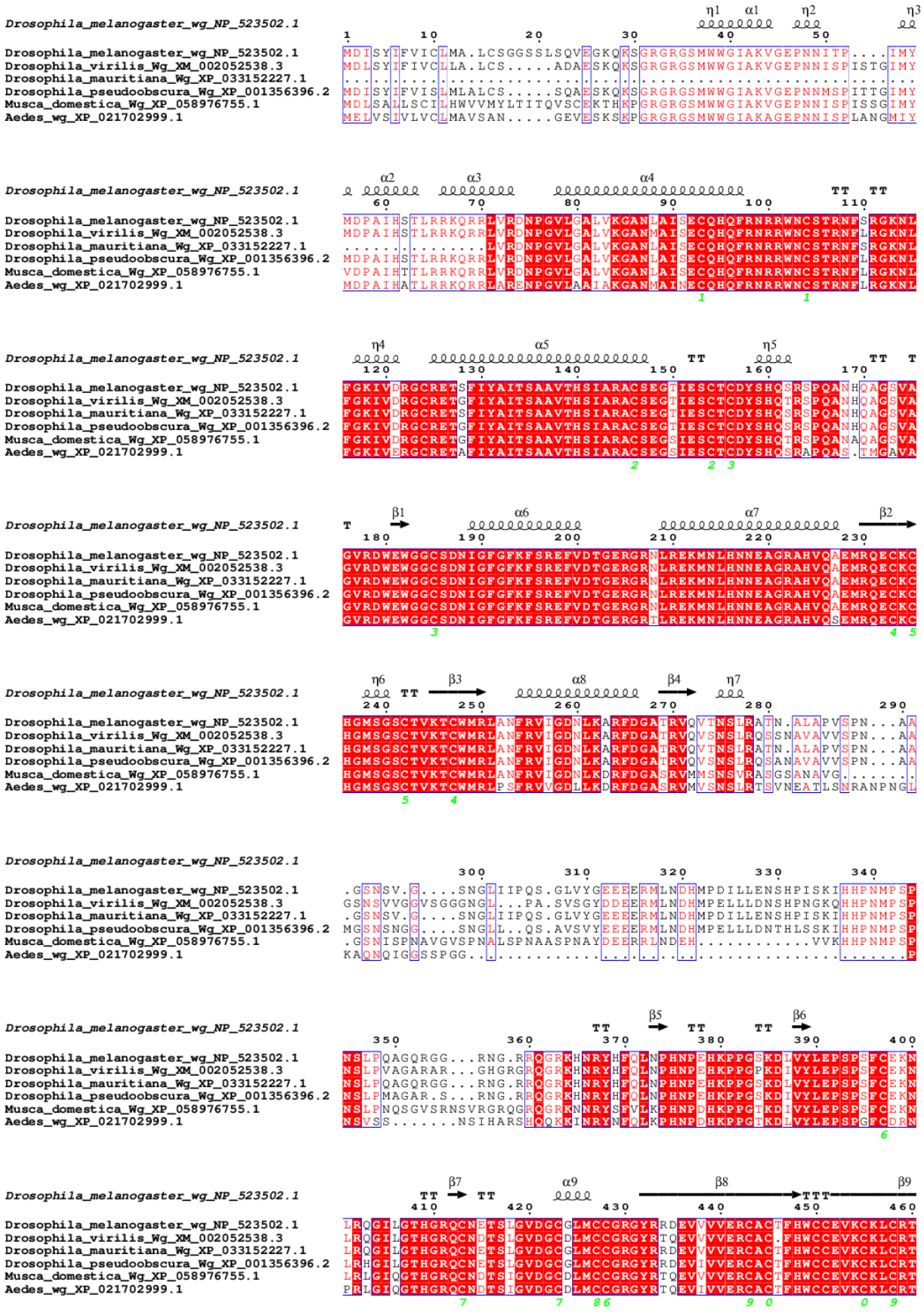


Figure 6: Phylogenetic tree of Wg sequences from different closely related fly species.

The phylogenetic tree shows levels of relatedness between selected Drosophilids, *Musca domestica* and the mosquito *Aedes*. The horizonal lines branches represent evolutionary lineages changing over time. The longer the branch in the horizonal dimension, the larger the amount of change. The bar at the bottom of the figure provides a scale for this. In this case the line segment with the number '0.06' shows the length of branch that represents an amount genetic change of 0.06.

The alignment indicated that the insert is present within *Drosophilids (*280AA- 365AA*),* but does appear to be conserved (Figure 7). The 85 AA insert region did not correspond to any tertiary protein structure (Figure 7). When looking at *M. domestica*, which is part of the *Diptera* order but a member of the *Muscoidea* superfamily, it can be seen that 85 AA region was more highly conserved in *Musca* when compared to *Aedes*. There were two small gap regions in which a total of 20 AA were missing from the *Musca* sequence that are present in *Drosophila*. only 5 AA were identical across the insert region (5%), and 25 AA were conserved residues between groups (29%) indicating a low level of conservation of the insert across closely related species (Figure 7).

Within the *Drosophila* genus the 85 AA region was 57% conserved with four *Drosophila* species surveyed (Figure 8). Interestingly, the region did not contain any cysteine residues (Figure 8). The particular composition of AA within this region, for example a high proportion of alanines highlight that the region could be prone to take on a globular form, and is likely to loop out of the core structure of the protein (Hays *et al.,* 1997).


Drosophila_melanogaster_wg_NP_523502.1
Drosophila_melanogaster_wg_NP_523502.1
Drosophila_virilis_Wg_XM_002052538.3
Drosophila_mauritiana_Wg_XP_033152227.1
Drosophila_pseudoobscura_Wg_XP_001356396.2
Musca_domestica_Wg_XP_058976755.1
Aedes_wg_XP_021702999.1

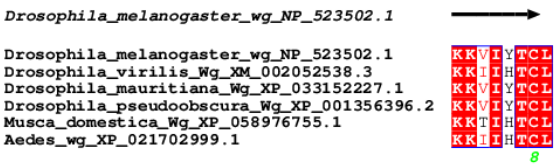


Figure 7: Drosophilid, *Musca* and *Aedes* Alignment.

An ESPript output obtained from Wg protein sequences extracted from NCBI data bank, aligned with CLUSTAL. The Wg protein is 468AA long. Four Drosopholid species were aligned against *Musca* (Horse fly) and the mosquito *Aedes*. Sequences are divided into three groups according to similarity. Residues that are strictly conserved have a red background, residues that are well conserved within a group are indicated by red letters and the remaining AA are in regular black font. Residues conserved between groups are boxed. Symbols above blocks of sequences correspond to the secondary structure of the Wg protein. The Wg protein consists of alpha helices depicted as squiggly lines and beta pleated sheets as arrows. The numbers depicted below the alignments in green correspond to the number of disulphide bonds present.

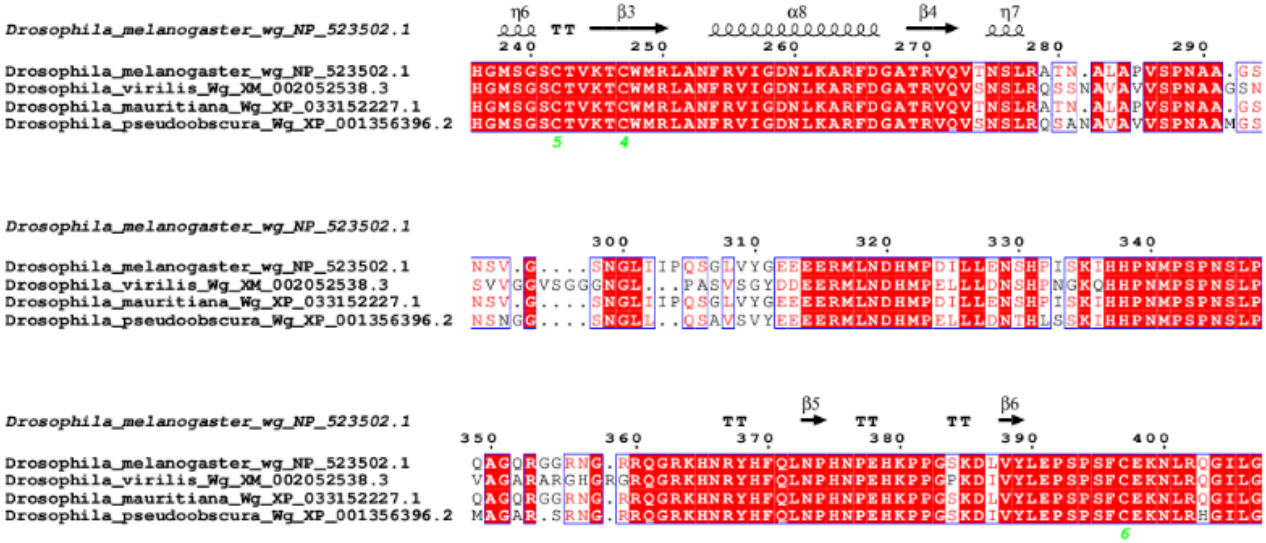
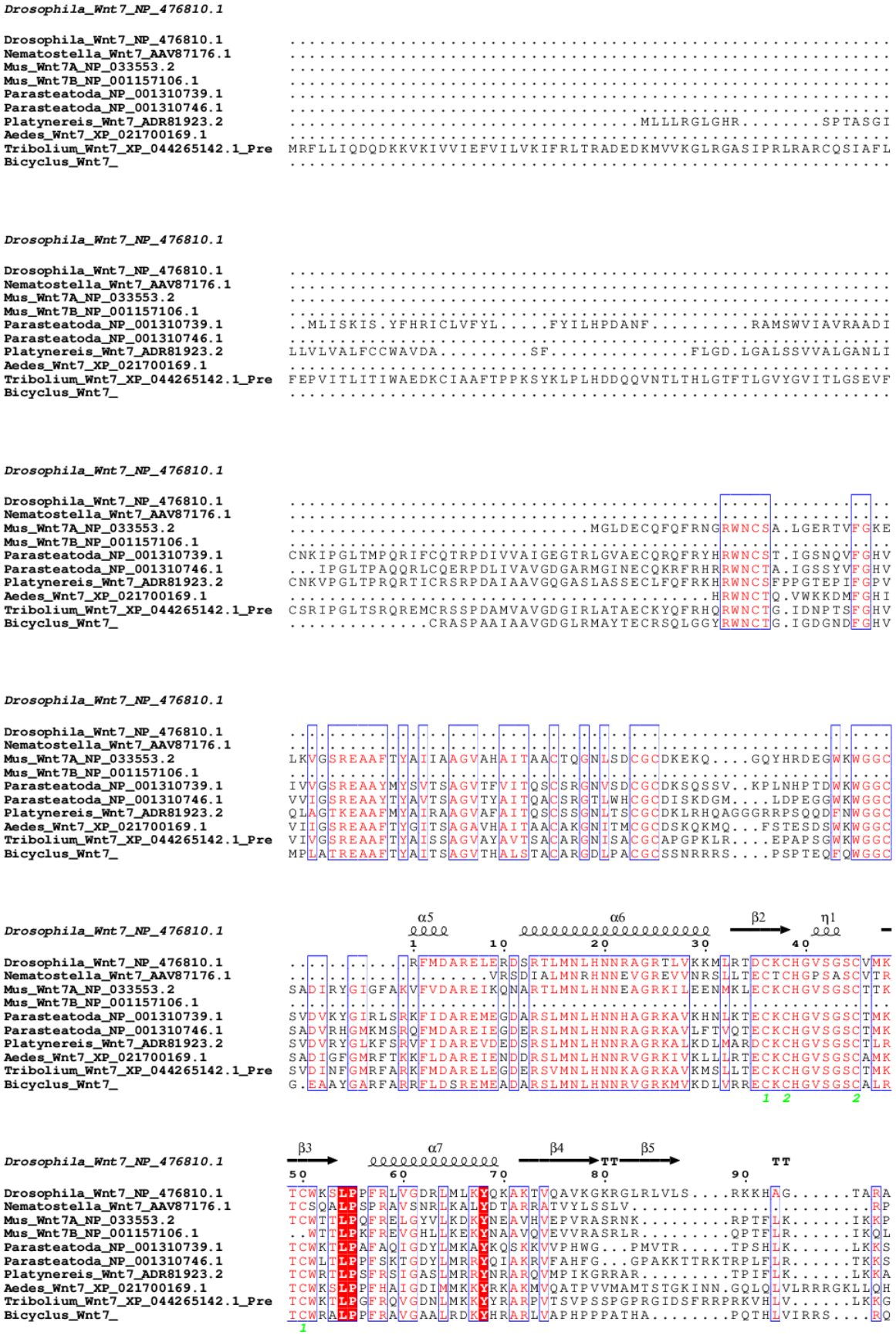


Figure 8: *Drosophila* species Alignment

An ESPript output obtained from Wg protein sequences extracted from NCBI data bank, aligned with CLUSTAL. The Wg protein is 468AA long. Four Drosopholid species. Sequences are divided into three groups according to similarity. Residues that are strictly conserved have a red background, residues that are well conserved within a group are indicated by red letters and the remaining AA are in regular black font. Residues conserved between groups are boxed. Symbols above blocks of sequences correspond to the secondary structure of the Wg protein. The Wg protein consists of alpha helices depicted as squiggly lines and beta pleated sheets as arrows. The numbers depicted below the alignments in green correspond to the number of disulphide bonds present. The above alignment only depicts the insert region of the alignment.

3.2.1.3. Wnt7 (DWnt2) Protein alignment

Using the Espript 3.0 multiple sequence alignment tool Wnt7, (Dwnt2) protein sequences were aligned to investigate amino acid sequences conservation for species: *Nematostella*, *Mus*, *Parasteatoda*, *Platynereis*, *Aedes*, *Tribolium* and *Bicyclus* compared to *Drosophila*. For Wnt7 (DWnt2) the greatest level of sequence conservation was seen within the beta pleated sheets 6-11 and alpha helices 7 and 8, with 10 cysteine residues conserved within these tertiary residues (Figure 9). There does appear to be conservation of amino acid properties within alpha helix 5-7 and beta pleated sheets 2 and 3, within these structures although there is not a strict identity match between amino acids within the orthologs, there does appear to be similarity present within the group (Figure 9). Interestingly, there appears to be ~ 120AA present in *Tribolium* that are absent from the other orthologs, although there were amino acids present within this region within *Platynereis* and *Parasteatoda* there is no amino acid conservation among the three orthologs (Figure 9).


Drosophila_Wnt7_NP_476810.1
Drosophila_Wnt7_NP_476810.1
Nematostella_Wnt7_AAV87176.1
Mus_Wnt7A_NP_033553.2
Mus_Wnt7B_NP_001157106.1
Parasteatoda_NP_001310739.1
Parasteatoda_NP_001310746.1
Platynereis_Wnt7_ADR81923.2
Aedes_Wnt7_XP_021700169.1
Tribolium_Wnt7_XP_044265142.1_Pre
Bicyclus_Wnt7_
α5
α6
β2
η1
β3
α7
β4
β5
TT

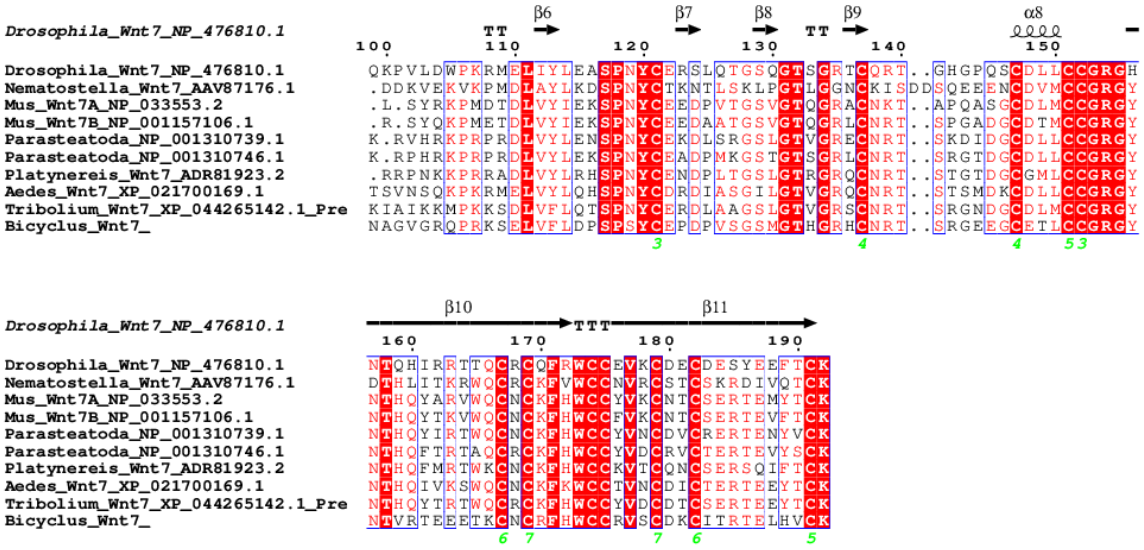
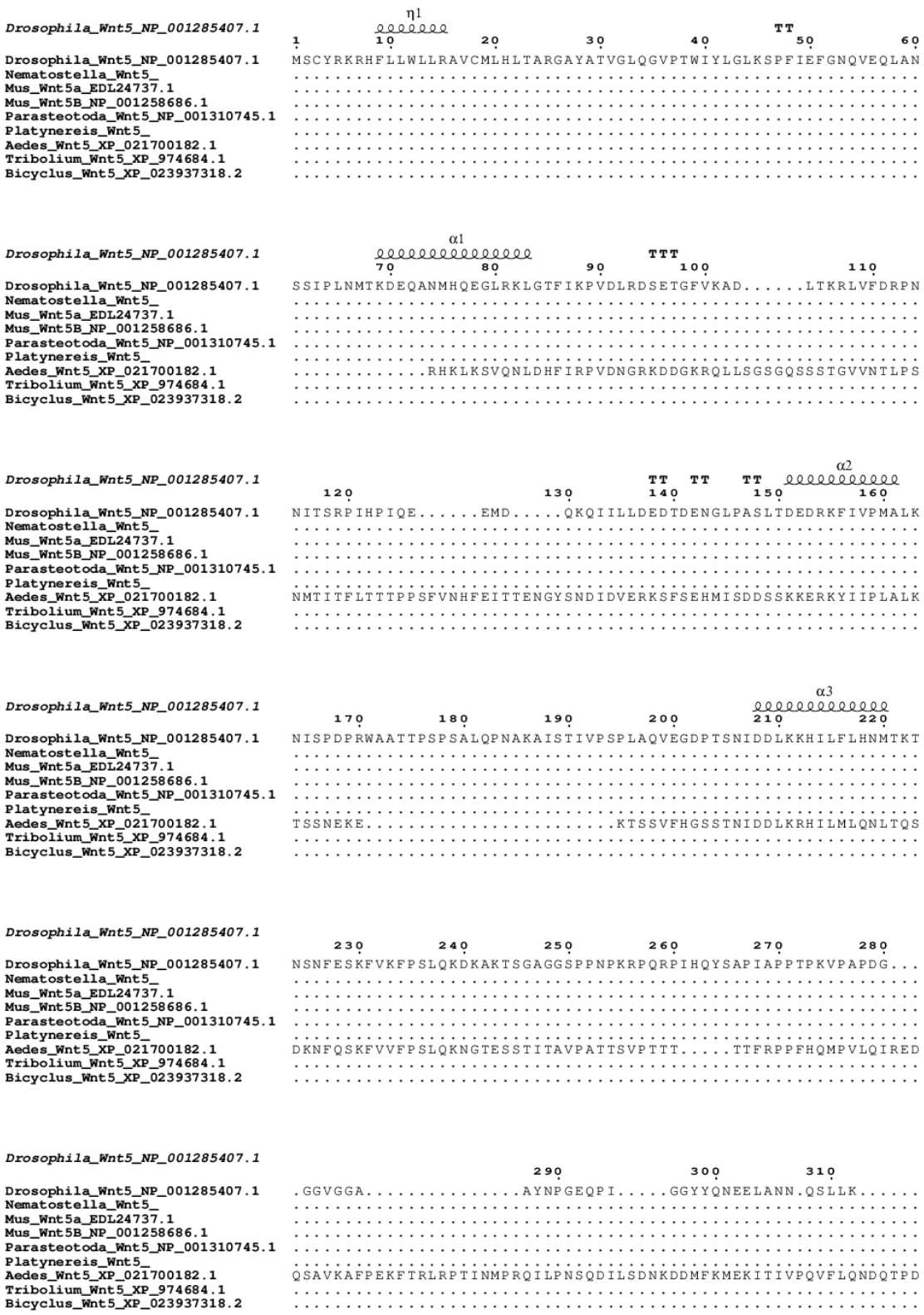


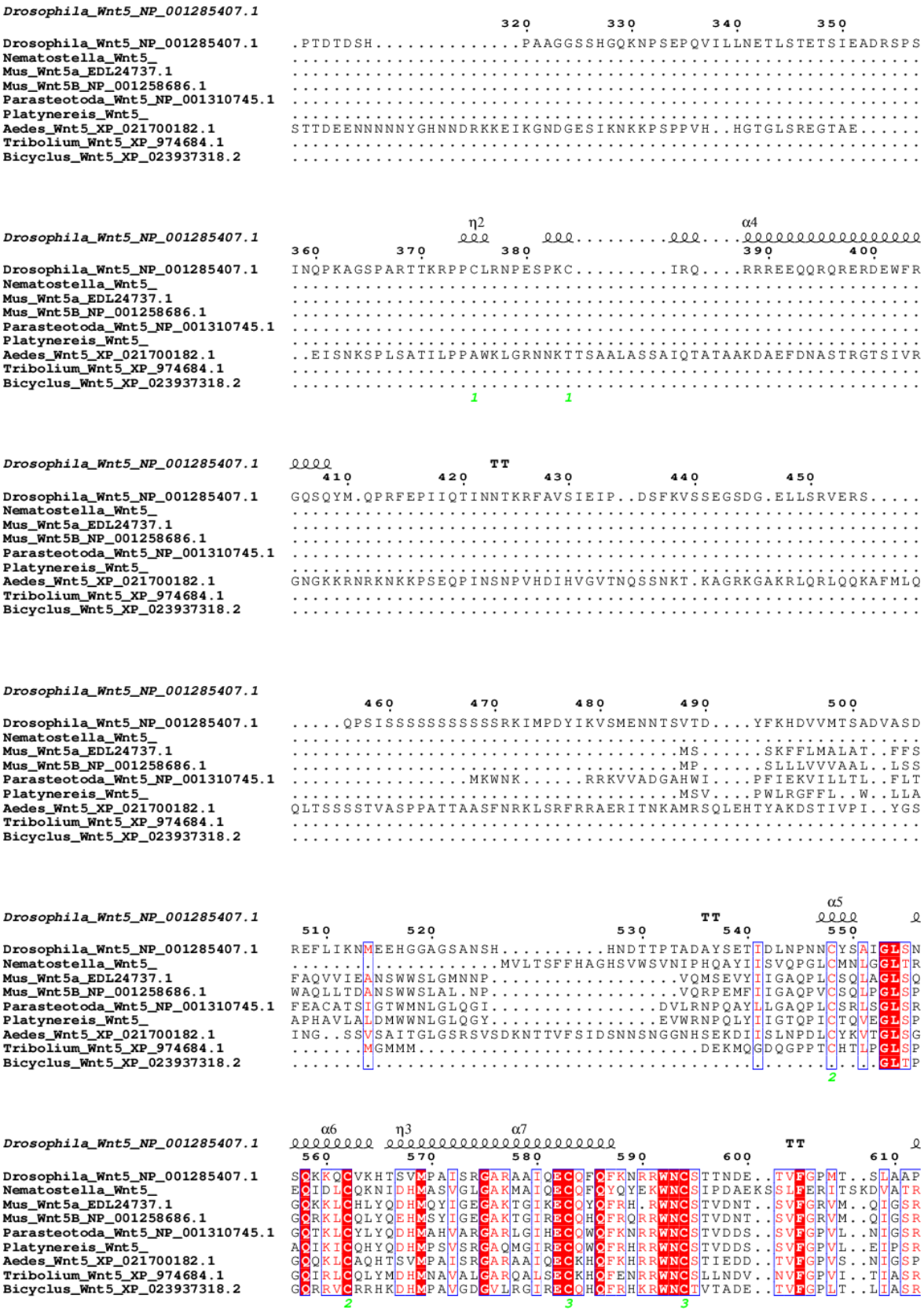
Figure 9: Wnt7 species Alignment

An ESPript output obtained from Wnt7 protein sequences extracted from NCBI data bank, aligned with CLUSTAL. The Wnt7 protein is 192AA long. Sequences are divided into three groups according to similarity. Residues that are strictly conserved have a red background, residues that are well conserved within a group are indicated by red letters and the remaining AA are in regular black font. Residues conserved between groups are boxed. Symbols above blocks of sequences correspond to the secondary structure of the Wnt7 protein. The Wnt7 protein consists of alpha helices depicted as squiggly lines and beta pleated sheets as arrows. The numbers depicted below the alignments in green correspond to the number of disulphide bonds present.

3.2.1.4. Wnt5(DWnt3) protein alignment

Using Espript 3.0 multiple sequence alignment tool Wnt5 (Dwnt3) protein sequences were aligned to investigate amino acid sequences conservation within *Nematostella*, *Mus*, *Parasteatoda*, *Platynereis*, *Aedes*, *Tribolium* and *Bicyclus* compared to *Drosophila*. Noticeably, Wnt5 (Dwnt3) was a much larger protein compared to the other Wnt paralogs and orthologs examined, with just over 950 AA (Figure 10). In particular, *Drosophila* and *Aedes* Wnt5 were very large proteins due to the presence of two hefty inserts, with one insert that spanned around 500AA and a second of around 120AA (Figure 10) Although *Drosophila* and *Aedes* both presented large inserts, the appeared to be no conservation between the insert sequences. Furthermore, they AA sequences of the larger insert showed that there did not appear to be any distinct large tertiary structures but instead around 4 small alpha helices (Figure 10). However, the second insert did comprise of two larger alpha helices 11 and 12. The greatest level of conservation was seen between alpha helices: 5-14 and between the following beta pleated sheets: 1-9, and once again the conserved cysteines in this case- 23, were all located in the beta pleased sheets described above (Figure 10).


η1
α1
α2
α3
TT
TTT
Drosophila_Wnt5_NP_001285407.1
Nematostella_Wnt5_
Mus_Wnt5a_EDL24737.1
Mus_Wnt5B_NP_001258686.1
Parasteotoda_Wnt5_NP_001310745.1
Platynereis_Wnt5_
Aedes_Wnt5_XP_021700182.1
Tribolium_Wnt5_XP_974684.1
Bicyclus_Wnt5_XP_023937318.2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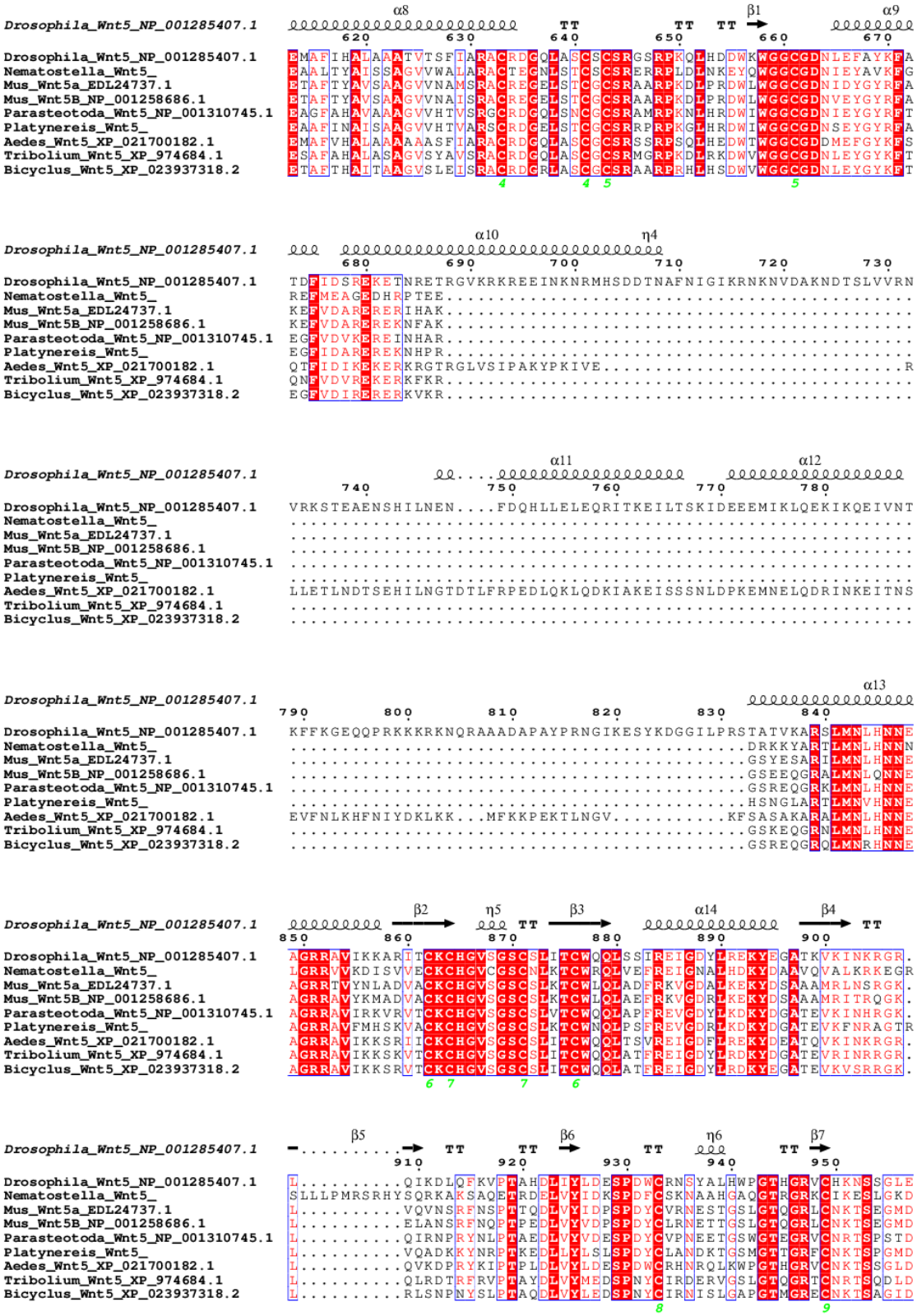
Drosophila_Wnt5_NP_001285407.1
Nematostella_Wnt5_
Mus_Wnt5a_EDL24737.1
Mus_Wnt5B_NP_001258686.1
Parasteotoda_Wnt5_NP_001310745.1
Platynereis_Wnt5_
Aedes_Wnt5_XP_021700182.1
Tribolium_Wnt5_XP_974684.1
Bicyclus_Wnt5_XP_023937318.2
η2
α4
α5
α6
η3
α7
TT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Drosophila_Wnt5_NP_001285407.1
Nematostella_Wnt5_
Mus_Wnt5a_EDL24737.1
Mus_Wnt5B_NP_001258686.1
Parasteotoda_Wnt5_NP_001310745.1
Platynereis_Wnt5_
Aedes_Wnt5_XP_021700182.1
Tribolium_Wnt5_XP_974684.1
Bicyclus_Wnt5_XP_023937318.2

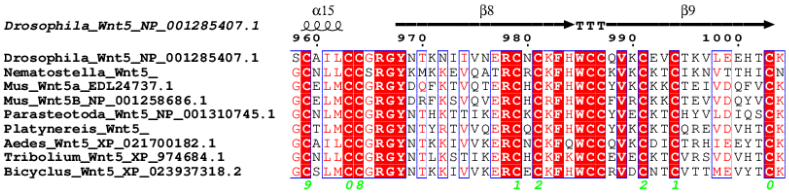
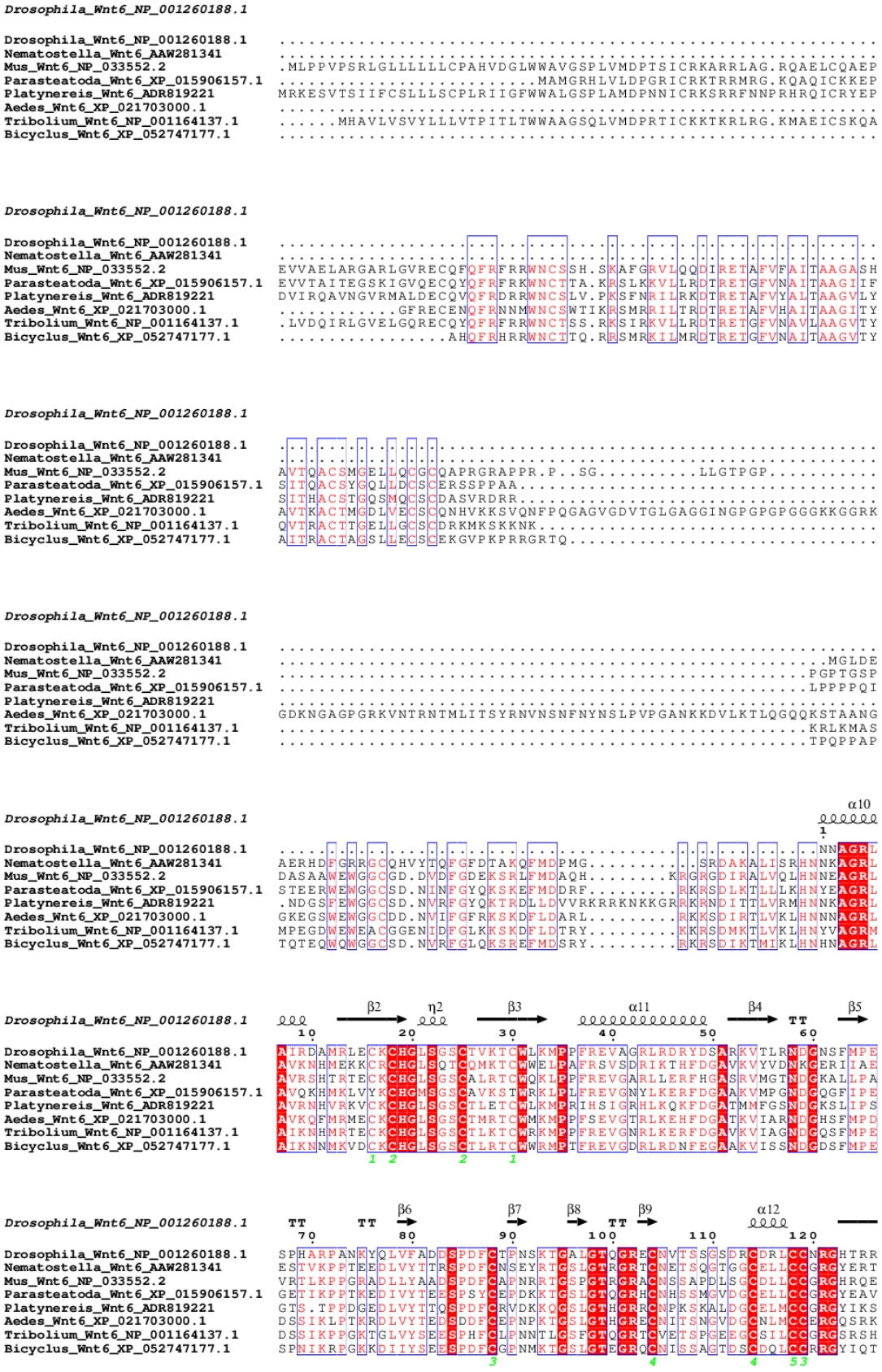


Figure 10: Wnt5 species Alignment

An ESPript output obtained from Wnt5 protein sequences extracted from NCBI data bank, aligned with CLUSTAL. The Wnt5 protein is 1004AA long. Sequences are divided into three groups according to similarity. Residues that are strictly conserved have a red background, residues that are well conserved within a group are indicated by red letters and the remaining AA are in regular black font. Residues conserved between groups are boxed. Symbols above blocks of sequences correspond to the secondary structure of the Wnt5 protein. The Wnt5 protein consists of alpha helices depicted as squiggly lines and beta pleated sheets as arrows. The numbers depicted below the alignments in green correspond to the number of disulphide bonds present.

3.2.1.5. Wnt6 protein alignment

Using Espript 3.0 multiple sequence alignment tool Wnt6 protein sequences were aligned to investigate amino acid sequences conservation. When looking at the alignment for Wnt6 the greatest conservation of AA can be seen in alpha folds: 10-12 and beta pleated sheets 2-11, with only 14 cysteines conserved among the Wnt6 orthologs. There does appear to be some conservation with groups present in the alignment, however, these AA sequences do not correspond to any tertiary structures, and there are no conserved cysteines present within these areas (Figure 11). Finally, there appears to one large insert that spans around 100AA that appeared to only be present in *Aedes* (Figure 11).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Mus_Wnt6_NP_033552.2
Parasteatoda_Wnt6_XP_015906157.1
Platynereis_Wnt6_ADR819221
Aedes_Wnt6_XP_021703000.1
Tribolium_Wnt6_NP_001164137.1
Bicyclus_Wnt6_XP_052747177.1
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β2
η2
β3
α11
β4
TT
β5
β6
β7
β8
β9
α12

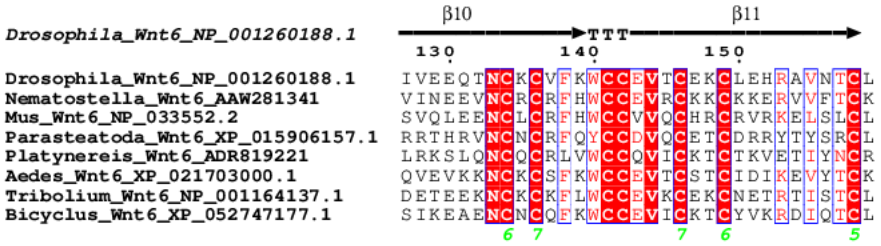


Figure 11: Wnt6 species Alignment

An ESPript output obtained from Wnt6 protein sequences extracted from NCBI data bank, aligned with CLUSTAL. The Wnt6 protein is 159AA long. Sequences are divided into three groups according to similarity. Residues that are strictly conserved have a red background, residues that are well conserved within a group are indicated by red letters and the remaining AA are in regular black font. Residues conserved between groups are boxed. Symbols above blocks of sequences correspond to the secondary structure of the Wnt6 protein. The Wnt6 protein consists of alpha helices depicted as squiggly lines and beta pleated sheets as arrows. The numbers depicted below the alignments in green correspond to the number of disulphide bonds present.

3.2.1.6. Wnt8 protein alignment

Using Espript 3.0 multiple sequence alignment tool Wnt8 protein sequences were aligned to investigate amino acid sequences conservation. The Wnt8 alignment showed significant conservation, with some form of conservation present in all the main tertiary protein structures (Figure 12). When looking at conserved tertiary structures, alpha helices 2-8 and beta pleated sheets 1-7 showed both direct amino acid and group amino acid conservation. There were 32AA that were indicated to be identical and conserved within all orthologs, of which 20AA are cysteine residues (Figure 12). There appears to be poor conservation at the beginning of the alignment, as well as, approximately 20AA that were present in *Mus* Wnt8A and Wnt8B, that were not present in the other aligned orthologs (Figure 12). Furthermore, there was an area of approximately 30AA that showed no conservation.

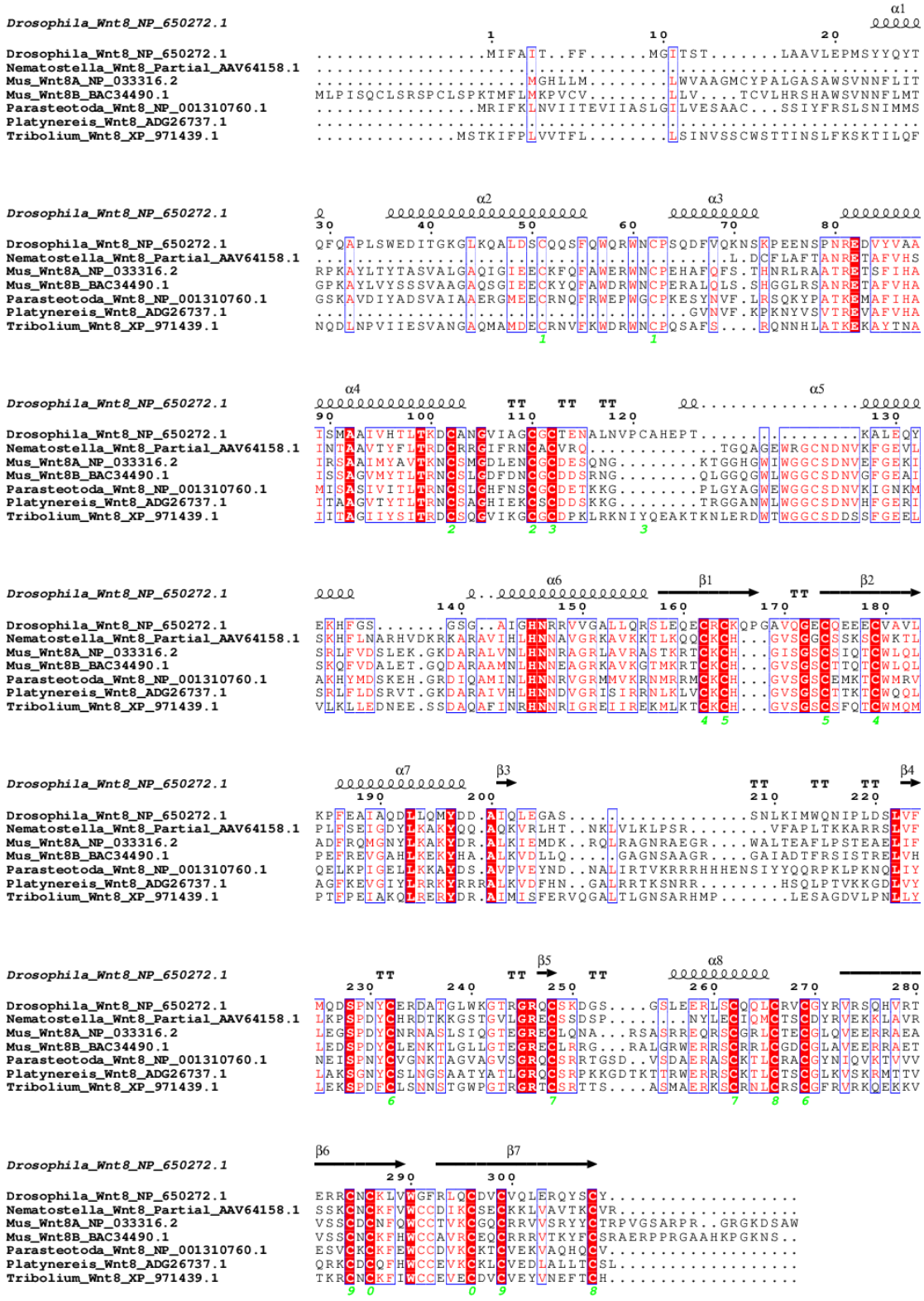
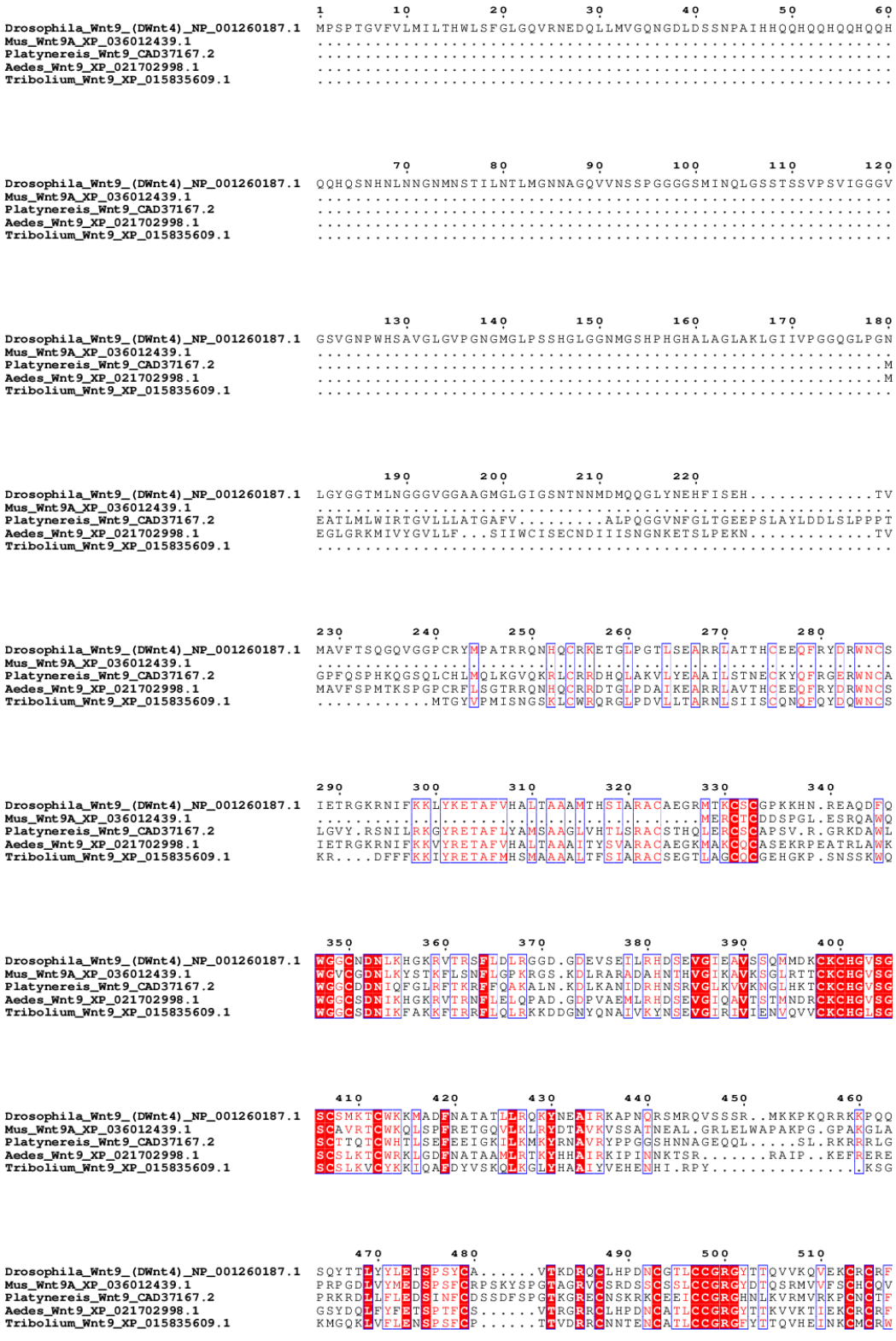

Drosophila_Wnt8_NP_650272.1
Drosophila_Wnt8_NP_650272.1
Nematostella_Wnt8_Partial_AAV64158.1
Mus_Wnt8A_NP_033316.2
Mus_Wnt8B_BAC34490.1
Parasteotoda_Wnt8_NP_001310760.1
Platynereis_Wnt8_ADG26737.1
Tribolium_Wnt8_XP_971439.1

Figure 12: Wnt8 species Alignment

The Wnt8 protein is 327AA long. Sequences are divided into three groups according to similarity. Residues that are strictly conserved have a red background, residues that are well conserved within a group are indicated by red letters and the remaining AA are in regular black font. Residues conserved between groups are boxed. Symbols above blocks of sequences correspond to the secondary structure of the Wnt8 protein. The Wnt8 protein consists of alpha helices depicted as squiggly lines and beta pleated sheets as arrows. The numbers depicted below the alignments in green correspond to the number of disulphide bonds present.

3.2.1.7. Wnt9 (DWnt4) protein alignment

Using Espript 3.0 multiple sequence alignment tool Wnt9 (DWnt4) protein sequences were aligned to investigate amino acid sequences conservation. The Wnt9 protein was around 500AA long, and 50% of the sequences (latter half) showed conservation, with 39 identical AA conserved, of which 13 AA were cysteine residues (Figure 13). Within the alignment, there appeared to be a large insertion present within *Drosophila* of around 220AA, no other orthologs has this insert present (Figure 13).


Drosophila_Wnt9_(DWnt4)_NP_001260187.1
Mus_Wnt9A_XP_036012439.1
Platynereis_Wnt9_CAD37167.2
Aedes_Wnt9_XP_021702998.1
Tribolium_Wnt9_XP_015835609.1

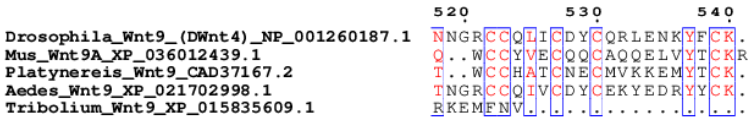
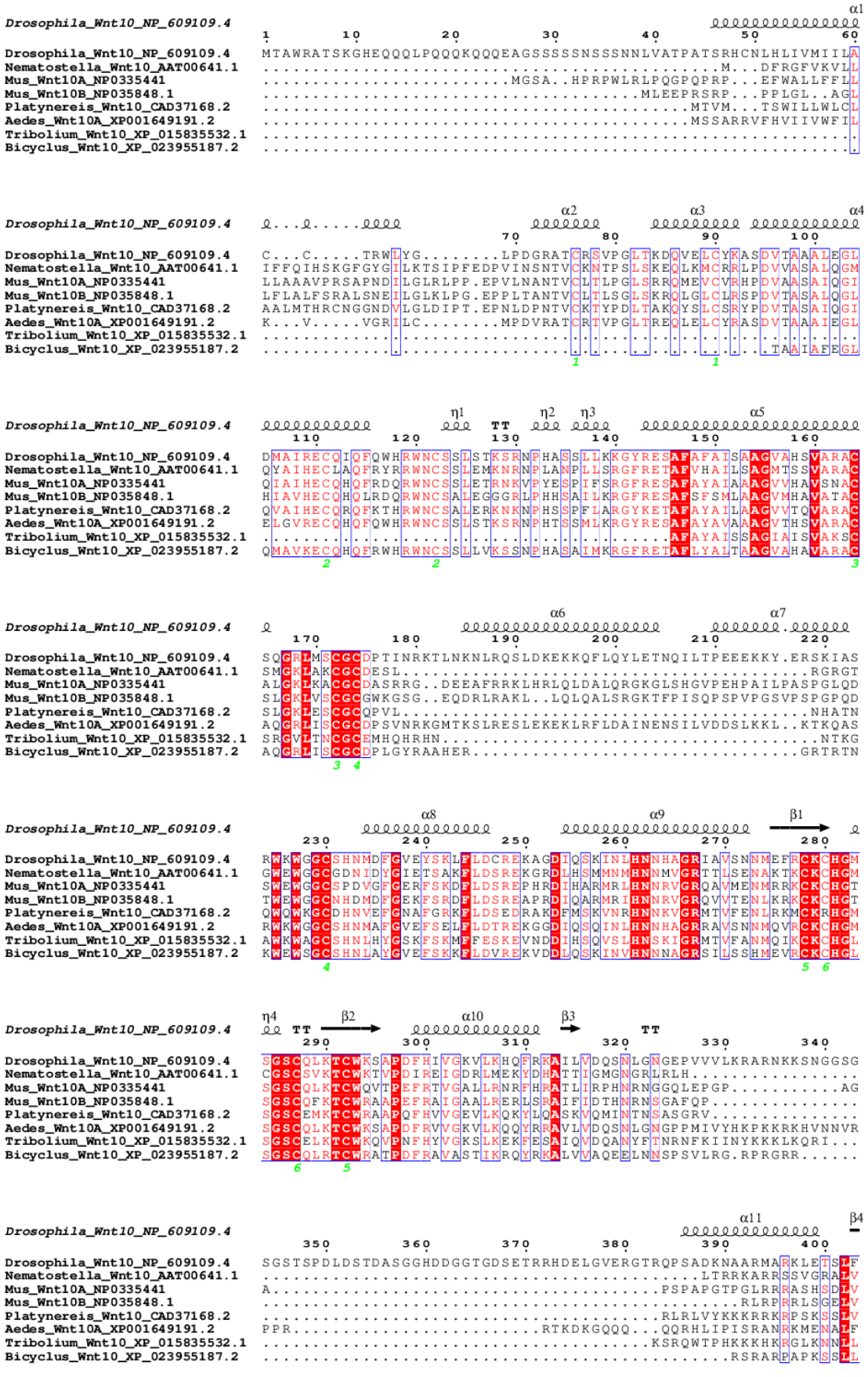


Figure 13: Wnt9 species Alignment

The Wnt9 protein is 541AA long. Sequences are divided into three groups according to similarity. Residues that are strictly conserved have a red background, residues that are well conserved within a group are indicated by red letters and the remaining AA are in regular black font. Residues conserved between groups are boxed. Symbols above blocks of sequences correspond to the secondary structure of the Wnt9 protein. The Wnt9 protein consists of alpha helices depicted as squiggly lines and beta pleated sheets as arrows. The numbers depicted below the alignments in green correspond to the number of disulphide bonds present.

3.2.1.8. Wnt10 protein alignment

Finally, using Espript 3.0 multiple sequence alignment tool Wnt10 protein sequences were aligned to investigate amino acid sequences conservation. Within the Wnt10 protein alignment, conservation appears to be present within the following tertiary structures: alpha helices 2-5, and 8-12 (not including alpha helix 11) and within all 6 beta pleated sheets (Figure 14). Within the alignment 54AA are identical and conserved within all orthologs of which 23AA are cysteine residues (Figure 14). There were three regions within the alignment that showed little or no conservation. The first being the start of the alignment itself, the second was around alpha helix 6 and 7, this region spanned around 50AA and none of the orthologs showed any conservation within these regions. The last region spanned from AA 330- 390, with *Drosophila* having a large insert present that is absent from the other aligned orthologs (Figure 14).


Drosophila_Wnt10_NP_609109.4
Drosophila_Wnt10_NP_609109.4
Nematostella_Wnt10_AAT00641.1
Mus_Wnt10A_NP0335441
Mus_Wnt10B_NP035848.1
Platynereis_Wnt10_CAD37168.2
Aedes_Wnt10A_XP001649191.2
Tribolium_Wnt10_XP_015835532.1
Bicyclus_Wnt10_XP_023955187.2

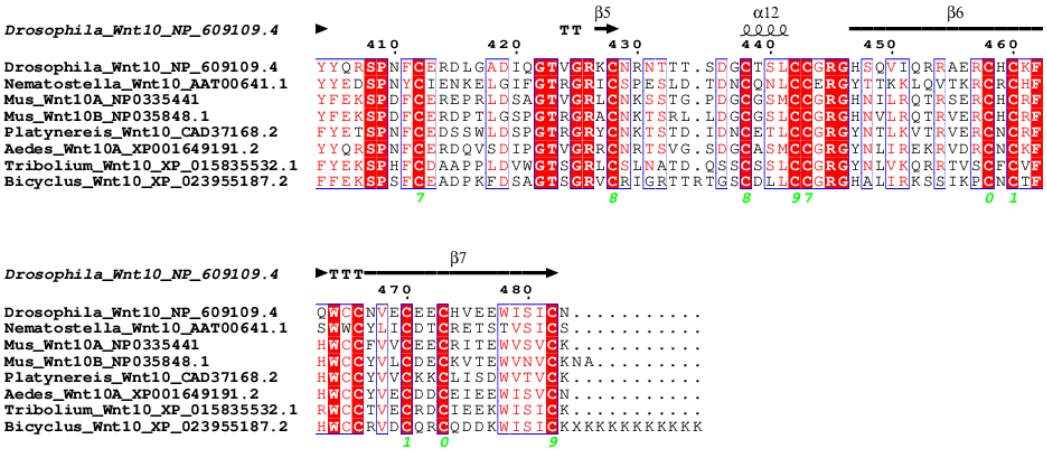


Figure 14: Wnt10 species Alignment

The Wnt10 protein is 495AA long. Sequences are divided into three groups according to similarity. Residues that are strictly conserved have a red background, residues that are well conserved within a group are indicated by red letters and the remaining AA are in regular black font. Residues conserved between groups are boxed. Symbols above blocks of sequences correspond to the secondary structure of the Wnt10 protein. The Wnt10 protein consists of alpha helices depicted as squiggly lines and beta pleated sheets as arrows. The numbers depicted below the alignments in green correspond to the number of disulphide bonds present.

3.2.2. *Xenopus* Wnt8 a template basis for Wnt Ligand Protein modelling

The structural analysis of XWnt8 in complex with mouse Fz8 indicates this ligand has an unusual two-domain structure (Figure 15) (Janda *et al.,*2013). The protein has been described as resembling a hand structure which comprises of a thumb and index finger required to grasp the Fzd8-CRD at two distinct binding sites (Janda *et al.,*2013). This predicted structure of XWnt8 in complex with Fzd8 can be used as a template to model other Wnt ligands using Swissmod (Figure 15). This program works by identifying a structure template, aligning the structure template to the target sequence, building a model, and evaluating the quality of the model (See Methods section 1). I therefore used this approach to model the structure of the seven Wnts represented in *D. melanogaster* as well as their orthologs in the six other species chosen to explore if differences in their amino acid sequences could be predicted to result in structural differences. The Wnt models produced by Swiss-mod were then compared with those generated using Alphafold protein prediction software produced by Google Deepmind (Jumper *et al.,* 2021). Swissmod is a structural bioinformatics tool used to build homology models of proteins, this is often competed using a template structure (Waterhouse *et al.,* 2018). Alphafold software predicts protein 3D structure from amino acid sequences, achieving accuracy competitive with atomic level accuracy, and differs from Swissmod by using artificial intelligence (AI) to predict 3D structure rather than dependency on just a template (Jumper *et al.,* 2021).

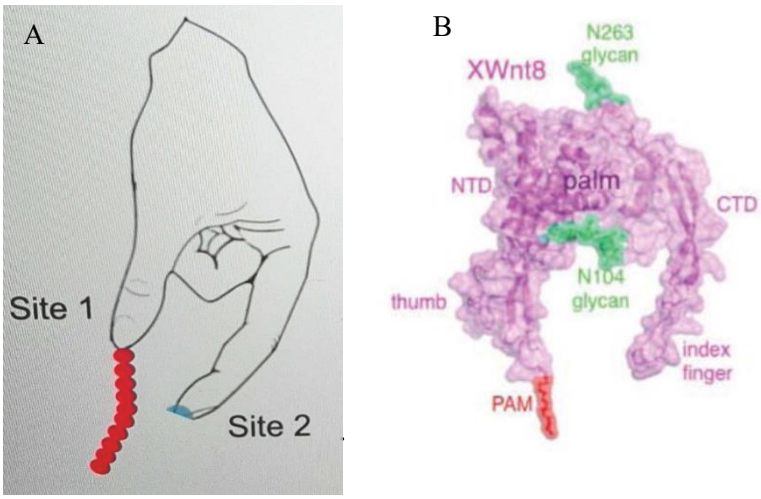


Figure 15: Structure of XWnt8 in complex with Fz8-CRD:

(A) Metaphorical representation of Wnt proteins depicted through a hand structure showing the thumb and index finger. (B) Surface representation of XWnt8 after removal of the Fz8-CRD from the complex structure. The extended palmitoleic acid (PAM) group is shown in red extending from the Wnt thumb, an exception to this is *Drosophila* Wnt8, which cannot have the PAM sequence added (adapted from Janda et al., 2013).

3.2.3. *Drosophila* Wnt Protein modelling using Swiss-mod and Alphafold

The key take-away from modelling the seven Wnt ligands from *Drosophila* and the six other species using Swiss-mod was that as expected the core protein has the same hand-like structure comprising the thumb and index finger. However, consistent with the amino acid alignments above, some *Drosophila* Wnt ligands appear to have additional domains that vary in location and contribute to structural differences. The sophistication of Google Deepmind allowed for a more detailed interpretation of the proteins, and how these novel regions may fold.

3.2.3.1. Wg

The Swiss-mod structure indicates that the core of the Wg/Wnt1 protein is conserved. When modelling Wg from, *Aedes*, *Bicyclus, Tribolium*, *Parasteatoda*, *Platynereis*, *Mus and Nematostella* using Alphafold, *Aedes* & *Bicyclus* are comparable to the models produced by Swiss-mod with small inserts at the N-terminus reflected in both the comparative models (Figure 16). However, when modelling *Tribolium* there is a much larger domain at the N-terminus. Interestingly the insert is depicted as an alpha helix with Alphafold. In the case of *Drosophila* Wg, the additional region of 85 amino acids forms an extra domain located at the top of the palm domain (Figure 16). It is important to note that both modelling approaches highlight low confidence in the regions that are specific to *Drosophila* outside of the core structure of the protein (Jumper *et al.,* (2021).

3.2.3.2. Wnt7 (DWnt2)

Swiss models for Wnt7(DWnt2) for the seven orthologues show the presence of a strikingly large region present within *Drosophila* and *Aedes* but absent from the other orthologue models (Figure 17). The insert found in *Drosophila Wnt*7(DWnt2) is present at the top of the protein structure adjacent to the index finger, whereas in the case of *Aedes* the region is located close to the thumb of the protein near the Fz binding site (Figure 17). When comparing this to models produced by Alphafold (Deepmind) only *Nematostella.*Wnt7 is predicted without any additional domains (Figure 17). However, *Drosophila.*Wnt7(DWnt2) shows striking differences between the two model types. There appears to be several new regions all around the periphery of the protein indicated in the Alphafold model. The model also depicts the additional regions as more structured alpha helices, rather than a globular tangled region as in the Swissmod model. The remaining orthologues: *Aedes*, *Bicyclus, Tribolium, Parasteatoda,*

Platynereis and Mus appear to have additional protein regions depicted as more structured alpha helices that do not correspond to the Swissmod model counterparts (Figure 17.). For DWnt2, Alphafold provides more detail of the protein regions outside of the core structure, with a further 4 alpha helices depicted (Figure 17.) compared to the other orthologues for DWnt2. The Swiss-mod model of Wnt7(DWnt2) predicts a large globular region of around 76 AA corresponding to the additional 85 amino acids in *Drosophila*, whereas Alphafold predicts the presence of an alpha helix with high confidence.

3.2.3.3. Wnt5 (DWnt3)

When looking across the orthologs for Wnt5 (DWnt3) all orthologs resemble the XWnt8 structure. The only species in which Swiss-mod and Alphafold both predict very similar structures is *Nematostella*, *Parasteotoda, Platynereis*, *Mus* and *Drosophila* all appear to have an alpha helix present at the top of the N-terminus (Figure 18). Both modelling methods highlight Wnt5 (DWnt3) has an additional domain close to the palm region of approximately 150 amino acids that is not found in the orthologs investigated (Figure 18). Swiss-mod models for Wnt5 (DWnt3) for the seven orthologues show the presence of a large region present within *Aedes*, but absent from the other orthologue models including *Drosophila* (Figure 18). When comparing this to the Alphafold models for Wnt5(DWnt3) *Aedes* and *Bicyclus* appear to be strikingly different to the Swissmod models with very large protein regions surrounding the core protein structure almost encapsulating the protein, as Alphafold uses intelligent AI the structure has not been determined using XWnt8 as a template (Figure 18).

3.2.3.4. Wnt6

When modelling Wnt6 the only orthologs that share high similarity in structure predictions from both models are *Parasteatoda* and *Nematostella.* In the case of *Drosophila* Wnt6, an additional domain can be found near the thumb of the protein (Figure 19). When modelling *Drosophila* Wnt6 using Alphafold the model predicted also shows there to be a novel region as indicated by the Swiss-mod. The novel region is shown to be located near the PAM sequence in both models (Figure 19). The Alphafold model of DWnt6 shows the novel region to be organised alpha helices, rather than a globular domain as predicted by the Swiss-mod model. Swissmod models for Wnt6 orthologs predict a large region in *Drosophila* and *Aedes* this region is not absent from the other orthologs models but it is important to note it is greatly reduced in size (Figure 19). However, this region is not predicted for *Parasteatoda* or *Nematostella*. When comparing models, Alphafold also appears to indicate the presence of a novel region in *Drosophila* but the region is more organised and represented as an alpha helix. *Aedes, Bicyclus, Tribolium, Platynereis, and Mus* all appear to show have a more unstructured protein domain at the N-terminus of the core structure (Figure 19).

3.2.3.5. Wnt 8 (DWnt8)

Alphafold modelling for *Drosophila* Wnt8 indicates the presence of a novel region located at the N-terminus of the protein and this is absent from the model predicted by Swiss-mod (Figure 20). When looking at the Swiss models for the Wnt8 for the remaining 6 orthologues there is similarity across all the models, with only *Tribolium* indicting a possible small novel region at the N-terminus of the protein (Figure 20). When comparing Swissmod to the Alphafold predictions, the models look highly similar with only *Platynereis* also showing a novel region extending out from the core region protein (Figure 20).

3.2.3.6. Wnt9 (DWnt 4)

For Wnt9 (DWnt4) Alphafold modelling, a novel domain was predicted at the top of the ligand, distal to the binding position of the ligand to the Fz receptor (Figure 21). This domain appears to loop around the protein, but appears to be globular and disorganised. However, the Swiss-mod structure does not indicate additional domains (Figure 21). When looking at the Swissmod models for *Drosophila*, *Aedes, Tribolium*, Platynereis and *Mus* the models appear to be similar in structure, with a lack of additional domains (Figure 21). When looking at the corresponding Alphafold models, the *Tribolium,* Swiss-mod and Alphafold models appear to be indistinguishable (Figure 21). When looking at *Aedes,* the Alphafold model has an alpha helix protruding from the N-terminus of the protein. The Alphafold models for *Platynereis* and *Mus* also appear to have a protein domain present at the N-terminus of the structure, but this domain is absent from the Swiss-mod structures (Figure 21). Wnt10 (DWnt10)

The Swissmod model for DWnt10 highlights two distinct areas with novel domains, one that is present at the N-terminus of the protein and one by the Fz binding site (Figure 22). Swiss-

mod models of the Wnt10 orthologues fro*m Nematostella, Platynereis* and *Bicyclus* appear to lack any novel protein regions compared to *Drosophila* Wnt10. *Aedes,* and *Mus* have an additional region close the Fz binding region, whereas *Tribolium* has a small loop domain to the left of the core protein (Figure 22).

Comparing the Swiss-mod models to the Alphafold predictions showed there are considerable differences that can be noted for all orthologues. *Drosophila* Wnt10 has 3 additional alpha helix domains surrounding the core of the protein (Figure 22). DWnt10 which has two regions that appear to unique to *Drosophila* Wnt10 however, the Alphafold model shows several alpha helices interconnected with disordered regions with high confidence when compared to the low-level confidence depicted in the Swiss-mod model. *Aedes* Wnt10 has an additional alpha helix protruding from the bottom of the protein as well as an additional loop at the top of the protein once again at the N-terminus side of the protein. *Bicyclus* Wnt10 has an additional protein region at the top of the core protein (Figure 22). The Alphafold model for *Tribolium* Wnt10 appears to show an organised region that stems from the top of the protein and loops out from the core structure. *Platynereis* Wnt10 also has an additional region located near the index finger of the protein, near the Fz binding site, and this structure strongly resembles the *Mus* Wnt10 structure. *Nematostella* Wnt10 is distinctive as the additional region for this protein appears to be linking the index finger and thumb structure together, this could be an artefact of the modelling (Figure 22).

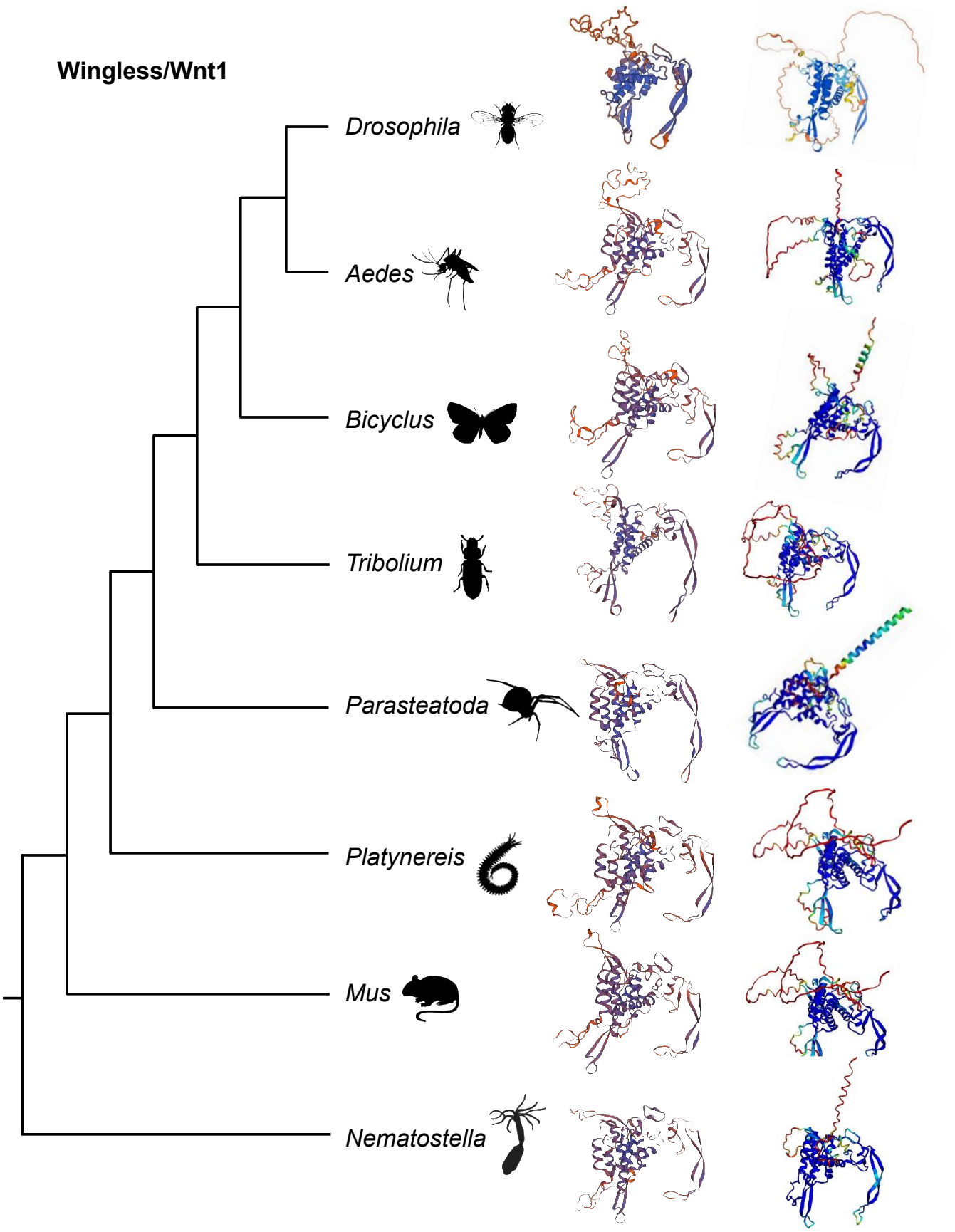
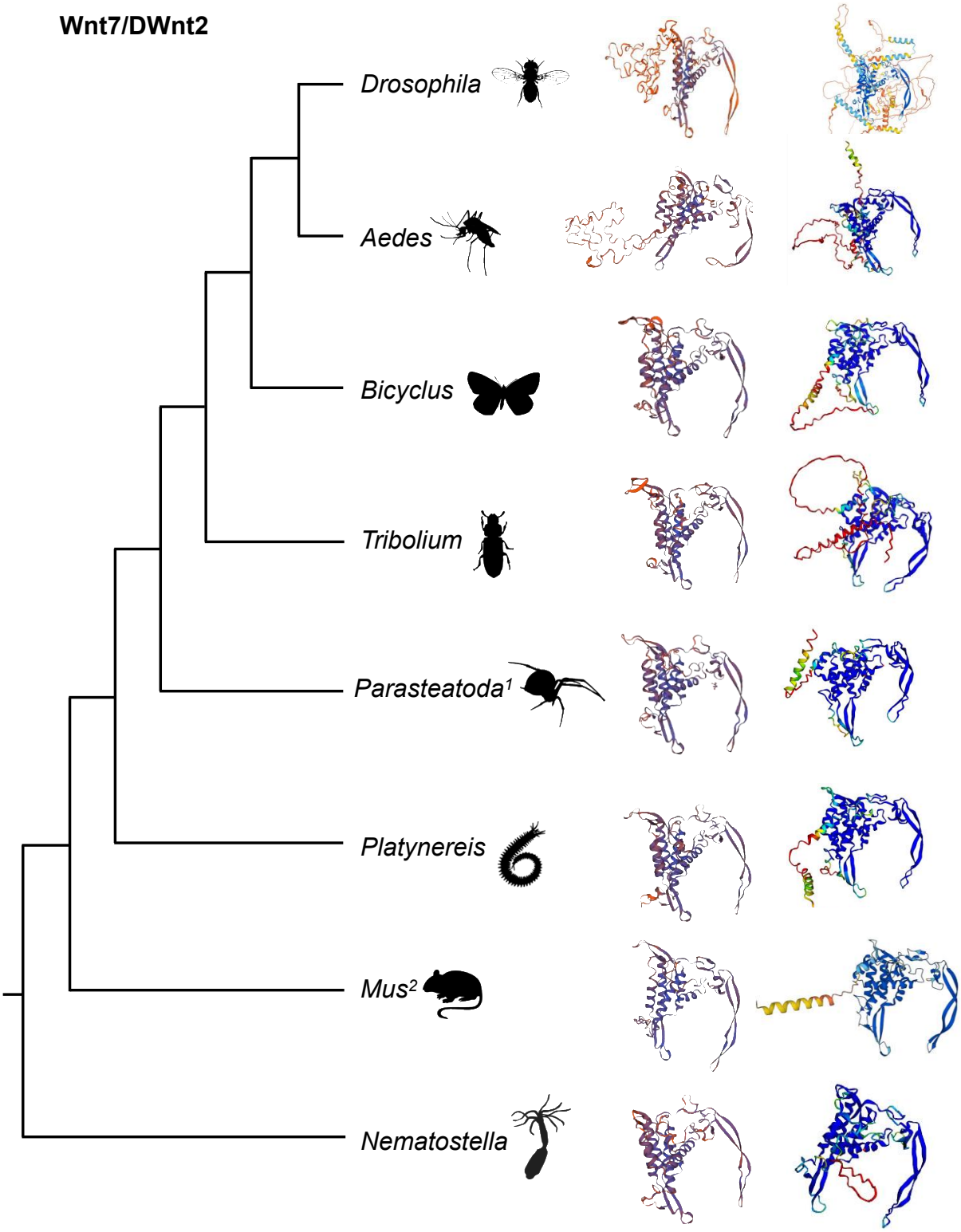

Wingless/Wnt1
Drosophila
Aedes
Bicyclus
Tribolium
Parasteatoda
Platynereis
Mus
Nematostella

Figure 16: Wingless/Wnt1 protein models.

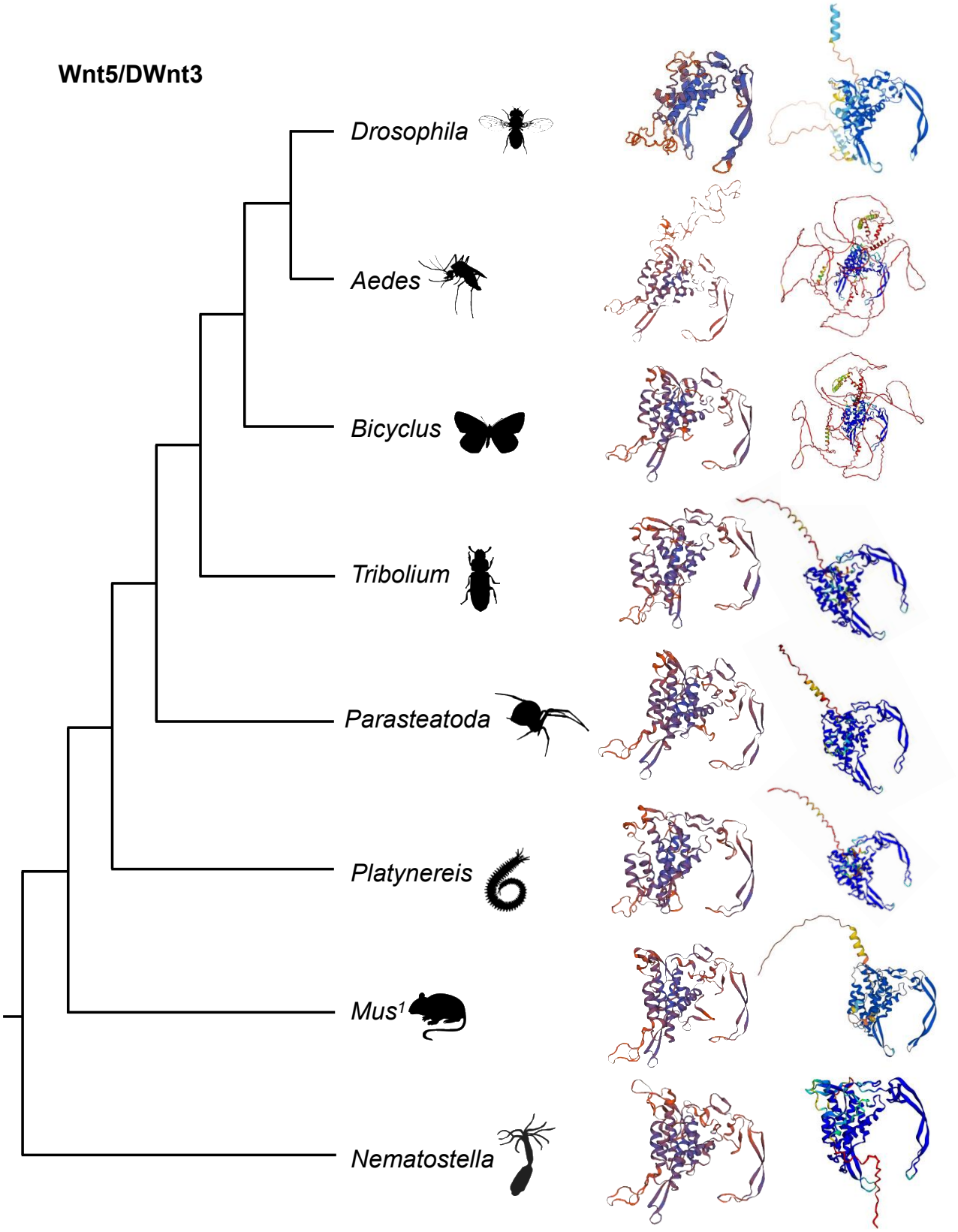
Models created by Swissmod to the left vs those produced by Alpha fold to the right. Amino acids presented in dark blue show good superposition whereas those presented in orange represent poorer superimposition. Both models highlight potential novel protein domains that appear to either be located at the top of the palm domain such as in the case of DWg or located at the thumb of the protein which is near where the Wnt ligand will bind to its corresponding Fd receptor. Both the Swissmod and Alphafold models highlight a unique region that is present in *Drosophila*, and to a lesser extent in *Aedes* but absent from the other orthologs. It is important to note that compared to models produced by Swiss mod the protein structures produced by Alphafold shed some clarity on previously more unknown regions of the protein highlighting, certain regions are in fact more structured than previously thought. The novel region in *Drosophila* Wingless is shown to be more unorganised and spread over the core of the protein than the Swissmod model of *Drosophila* Wg. The models produced by Swiss-mod show regions that are predicted with higher confidence as blue, and regions with more uncertainty as red. When looking at Alpha fold predicted models, regions with greater predicted confidence are depicted in blue, and regions of more uncertainty are presented in yellow to regions in red as most uncertain.



1 representing only Wnt7.1 of *Parasteatoda*
2 representing only Wnt7A of *Mus*

Figure 17: Wnt7/DWnt2 protein models.

Models created by Swissmod to the left vs those produced by Alpha fold to the right. Both the Swissmod and Alphafold models highlight a unique region that is present in *Drosophila* and to a lesser extent in *Aedes* but absent from the other orthologs, this region is located at the N-terminus of the protein for *Drosophila*, but close to the Fz binding site for *Aedes*. It is important to note that compared to models produced by Swissmod the protein structures produced by Alphafold shed some clarity on previously more unknown regions of the protein highlighting, certain regions are in fact more structured than previously thought. The novel region in *Drosophila* Wingless is shown to be more organised and spread over the core of the protein, there appears to be several alpha helices distributed around the core the protein vs the than the Swissmod model of *Drosophila* Wg. The models produced by Swiss-mod show regions that are predicted with higher confidence as blue, and regions with more uncertainty as red. When looking at Alpha fold predicted models, regions with greater predicted confidence are depicted in blue, and regions of more uncertainty are presented in yellow to regions in red as most uncertain.



1 representing only Wnt5A of *Mus*

Figure 18: Wnt5/ DWnt3 protein models.

Models created by Swissmod to the left vs those produced by Alphafold to the right. Only the Alphafold model highlights a unique region that is present in *Drosophila*. The novel region in *Drosophila* Wnt5/ DWnt3 is shown to be an alpha helix located at the N-terminus of *Drosophila*Wnt5/ DWnt3. The models produced by Swiss-mod show regions that are predicted with higher confidence as blue, and regions with more uncertainty as red. The *Aedes* Wnt5/DWnt3 model highlights a unique region that is similar to that found in DWg. Both the *Aedes* and *Bicyclus* Alphafold models for Wnt5/DWnt3 models show very unorganised globular regions

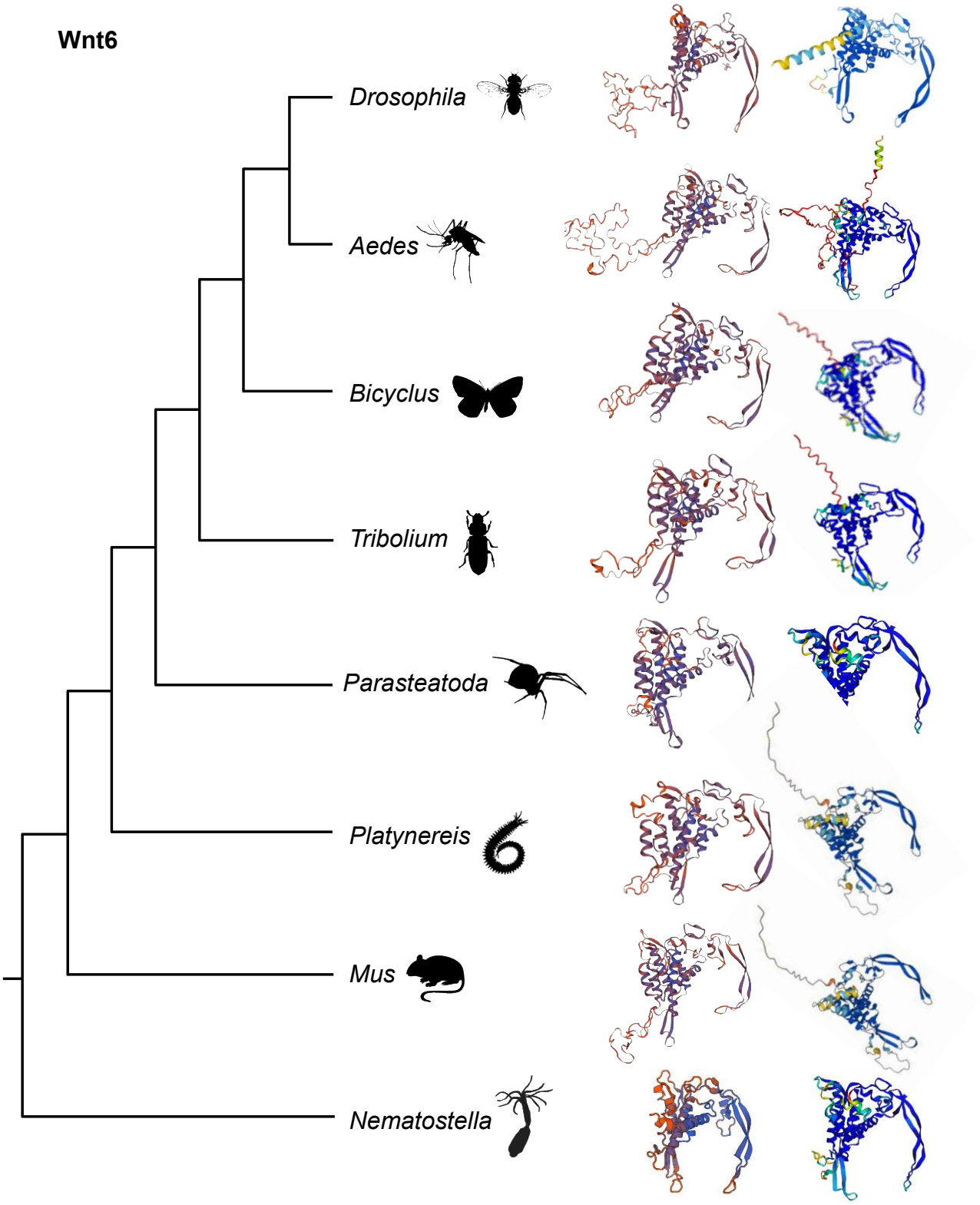
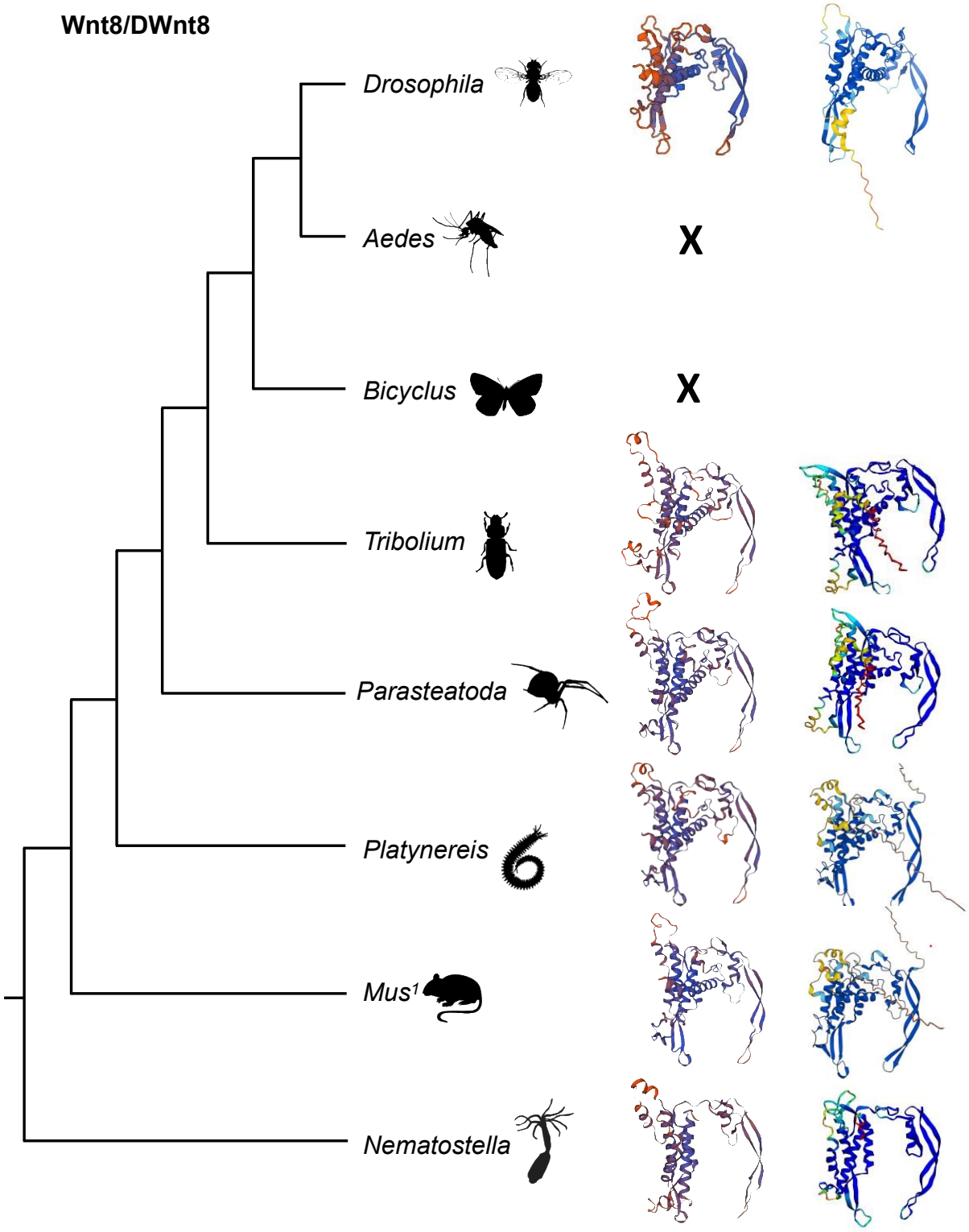

Wnt6
Drosophila
Aedes
Bicyclus
Tribolium
Parasteatoda
Platynereis
Mus
Nematostella

Figure 19: Wnt6 protein models.

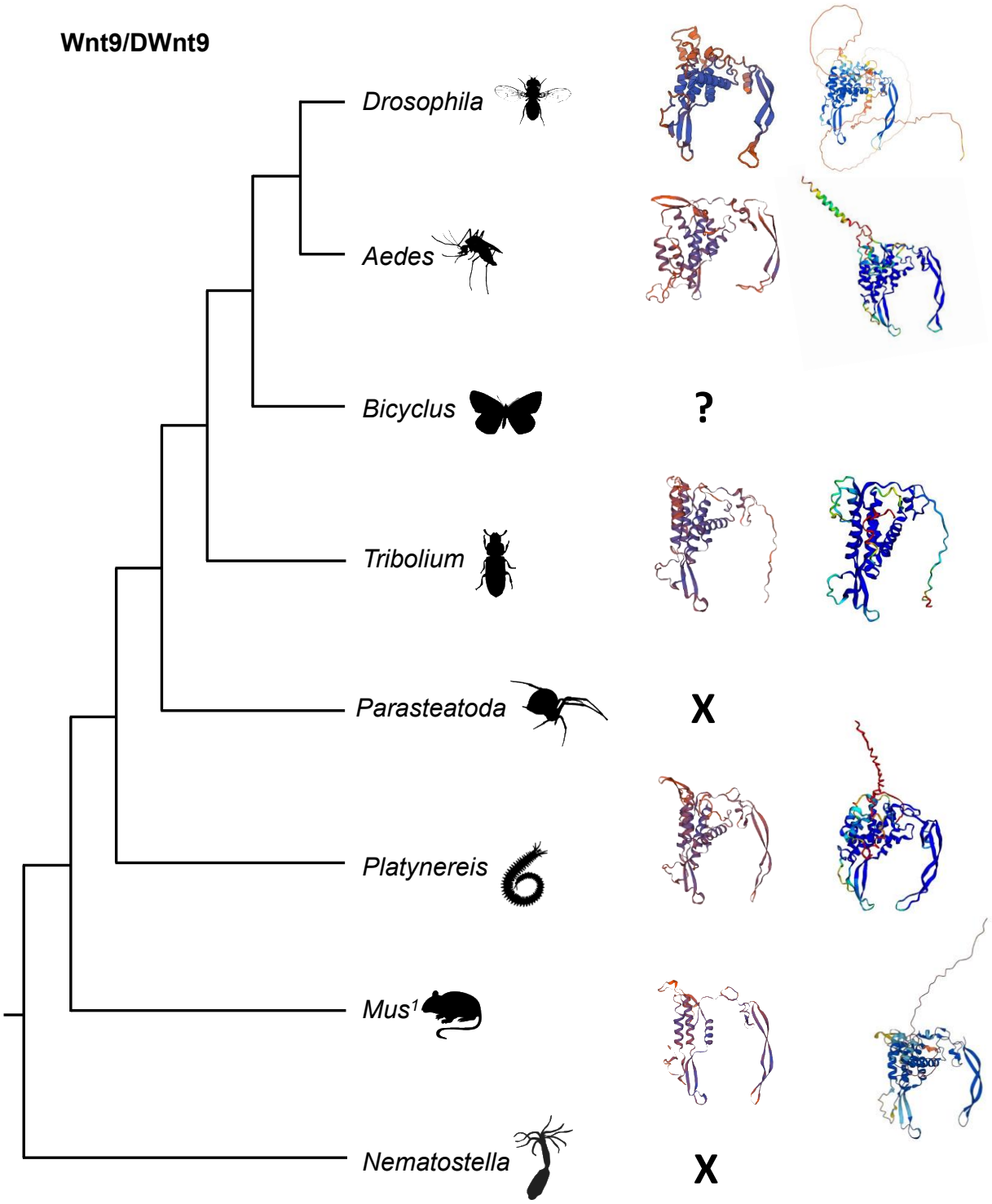
Models created by Swissmod to the left vs those produced by Alphafold to the right. The models produced by Swiss-mod show regions that are predicted with higher confidence as blue, and regions with more uncertainty as red. Excluding *Parasteatoda* and *Nematostella* all Wnt6 orthologs appear to have the presence of an additional novel region located close to the Fzd binding site. This is also reflected by the Alphafold models, with the presence of an additional Alpha helix structure at the N-terminus of *Drosophila, Aedes, Bicyclus, Tribolium, Platynereis and Mus*Alphafold models, with the presence of an additional Alpha helix structure at the N-terminus of *Drosophila, Aedes, Bicyclus, Tribolium, Platynereis and Mus.*



1 representing only Wnt8A of *Mus*
x Wnt gene/protein missing

Figure 20: Wnt8/DWnt8 protein models.

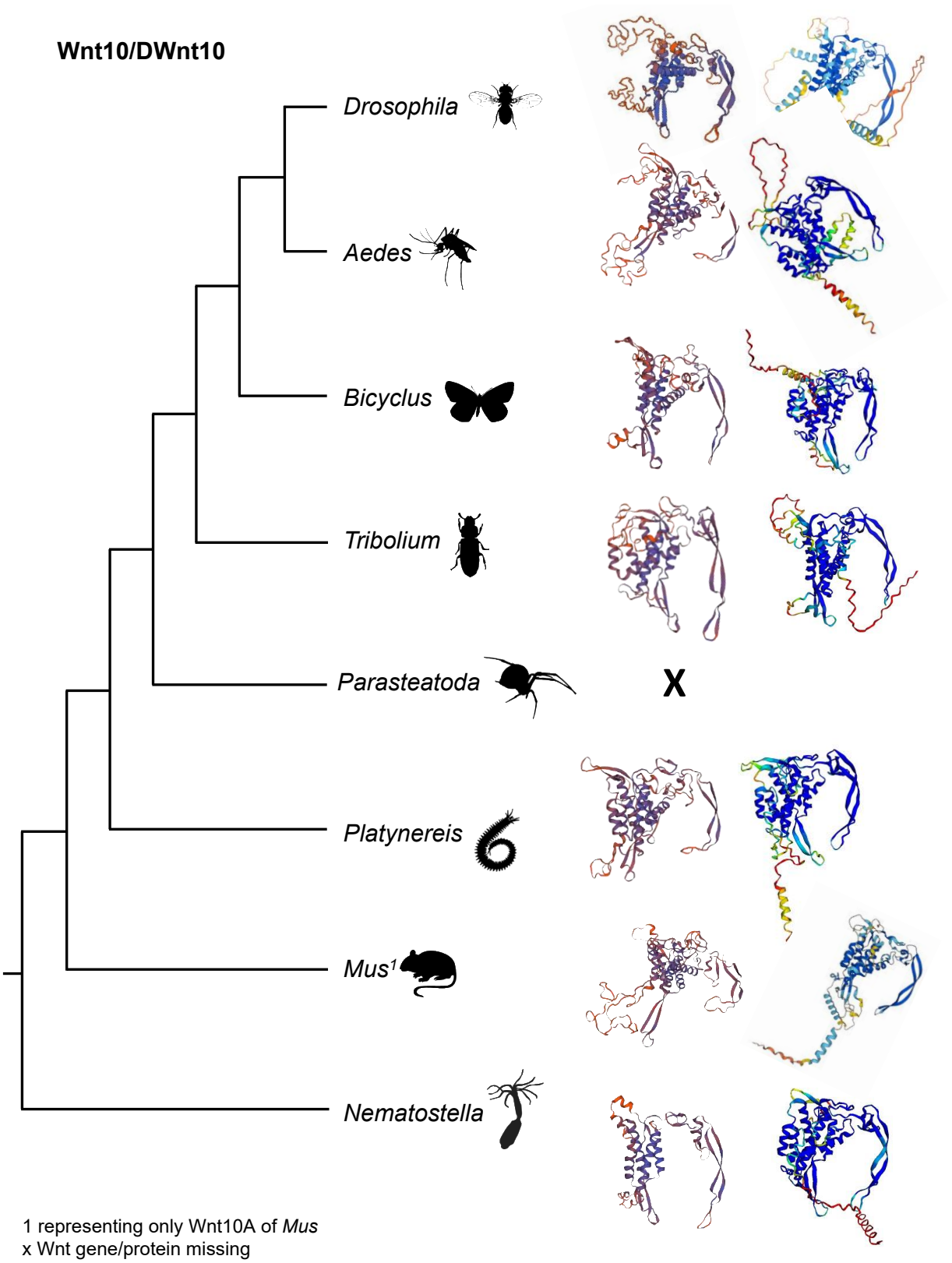
Models created by Swissmod to the left vs those produced by Alphafold to the right. The models produced by Swiss-mod show regions that are predicted with higher confidence as blue, and regions with more uncertainty as red. *Aedes* and *Bicyclus* do not have Wnt8 proteins. Swissmod models for *Tribolium*, *Parasteatoda*, *Platynereis*, *Mus* and *Nematostella* appear to show very small unorganised regions present at the N-terminus of the protein. There is a distinct lack of any additional regions present in *Drosophila* Wnt8 as shown by the Swissmod model. The *Drosophila*Wnt8 Alphafold model indicates the presence of an alpha helix at distal part of the protein close to the Fzd binding site, which is not shown in any of the other models.



1 representing only Wnt9B of *Mus*
? Sequence unavailable
x Wnt gene/protein missing

Figure 21: Wnt9/DWnt9 protein models

Models created by Swissmod to the left vs those produced by Alphafold to the right. The models produced by Swiss-mod show regions that are predicted with higher confidence as blue, and regions with more uncertainty as red. *Parasteatoda* and *Nematostella* do not contain a Wnt9 protein. The sequence for *Bicyclus* Wmt9 was unavailable at the time of this project, but *Bicyclus* is recorded in the literature to have a Wnt9 protein. For the remaining orthologs, the Swissmod models for Wnt9 appear comparable with no major differences recorded. The corresponding Alphafold model for *Drosophila* shows a large unorganised region stemming from the centre of the protein and spanning around the core structure of the protein. *Aedes*, *Platynereis* and *Mus* all appear to have an additional helix structure at the N-terminus of the protein.



1 representing only Wnt10A of *Mus*
x Wnt gene/protein missing

Figure 22: Wnt10 protein models.

Models created by Swissmod vs those produced by Alpha fold. Amino acids presented in dark blue show good superposition whereas those presented in orange represent poorer superimposition. Both models highlight potential novel protein domains that appear to either be located at the top of the palm domain such as in the case of DWg or located at the thumb of the protein which is near where the Wnt ligand will bind to its corresponding Fzd receptor. It is important to note that compared to models produced by Swiss mod the protein structures produced by Alphafold shed some clarity on previously more unknown regions of the protein highlighting, certain regions are in fact more structured than previously thought. The models produced by Swiss-mod show regions that are predicted with higher confidence as blue, and regions with more uncertainty as red. When looking at Alpha fold predicted models, regions with greater predicted confidence are depicted in blue, and regions of more uncertainty are presented in yellow to regions in red as most uncertain.

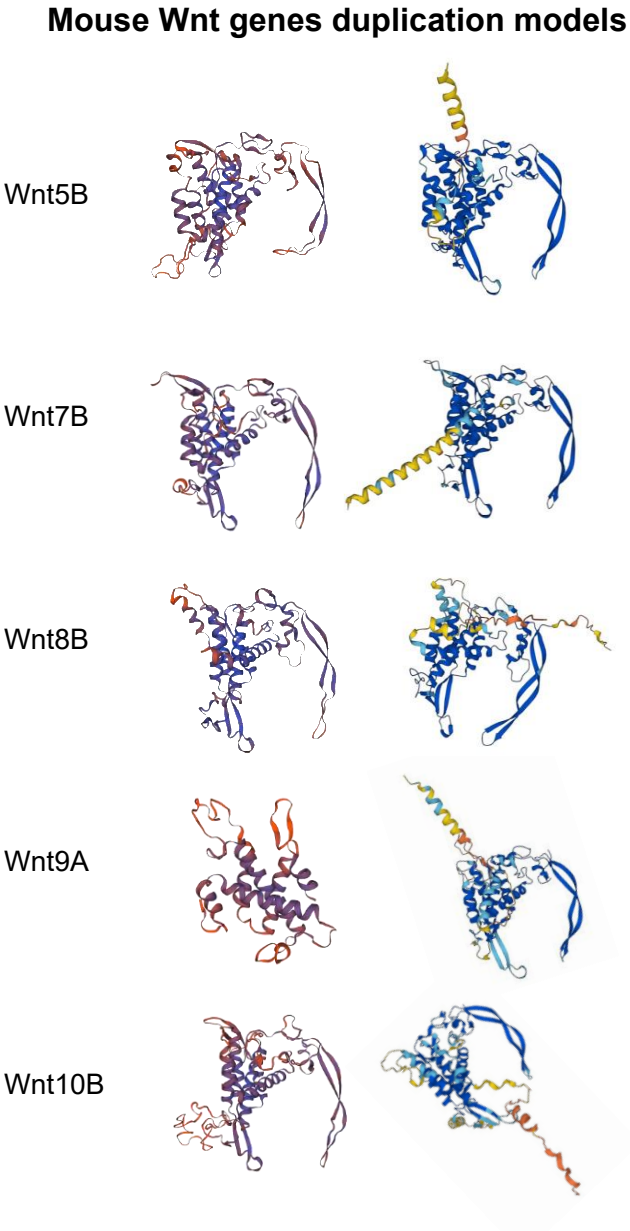


Figure 23: *Mus* gene paralog duplication protein models

Models created by Swissmod vs those produced by Alpha fold. Amino acids presented in dark blue show good superposition whereas those presented in orange represent poorer superimposition. Both models highlight potential novel protein domains that appear to either be located at the top of the palm domain such as in the case of DWg or located at the thumb of the protein which is near where the Wnt ligand will bind to its corresponding Fz receptor. It is important to note that compared to models produced by Swiss mod the protein structures produced by Alphafold shed some clarity on previously more unknown regions of the protein highlighting, certain regions are in fact more structured than previously thought. The models produced by Swiss-mod show regions that are predicted with higher confidence as blue, and regions with more uncertainty as red. When looking at Alpha fold predicted models, regions with greater predicted confidence are depicted in blue, and regions of more uncertainty are presented in yellow to regions in red as most uncertain. Paralog duplications for *M. Musculus* : Wnt 5,7, 8, 9 & 10 have been represented above for ease of viewing.

3.3.Presence of Novel protein domains found in *Drosophila*.

When modelling the *Drosophila* Wnt proteins there appears to be a novel protein domain observed in all *Drosophila* Wnts except Wnt9 (DWnt4) and Wnt8, which are not observed in the other species orthologues modelled, with the exception of some *Aedes* Wnts. Upon further inspection the novel protein regions within *Drosophila* appear to be globular, for example, in the case of *Drosophila* Wg, there is a globular region part of the protein away from the core structure (Figure 16) (Hays *et al.,* 1997).

Intrinsically (or structurally) disordered proteins (IDPs) are defined as biologically active proteins, which do not adopt a well-defined tertiary structure (Frank *et al.,* 2016). The extra protein domains detected in *Drosophila* Wnts were therefore further investigated to explore whether these regions are intrinsically disordered domains. To do this I used MobiDB, a biological database that looks at intrinsic protein disorder within a structure, to assess the *Drosophila* Wnts.

One analysis feature of MobiDB, IUPRED, is designed to predict IDP segments that are at least 30 residues long (Meszaros *et al.,* 2018). For each residue in the protein sequence a disorder probability is given; protein structures with overall disorder scores greater than 0.5 are predicted to be disordered (Meszaros *et al.,* 2018). Many disordered proteins work by binding to a structured partner, and in doing so can change from a disordered to order protein structure (Peng *et al.,* 2015). Anchor shows the probability of each residue to be part of a binding region, as if this binding region in unstable it is referred to as a disordered binding region (Meszaros *et al.,* 2018).

When using MobiDB to analyse the *Drosophila* Wg structure, amino acids 320-380 are above the threshold score of 0.5, this indicates that the tertiary structure of this region exists in an intrinsically disordered form (Figure 24). Interestingly, this region corresponds to the additional 85 amino acids found in *Drosophila* Wg with respect to other species.

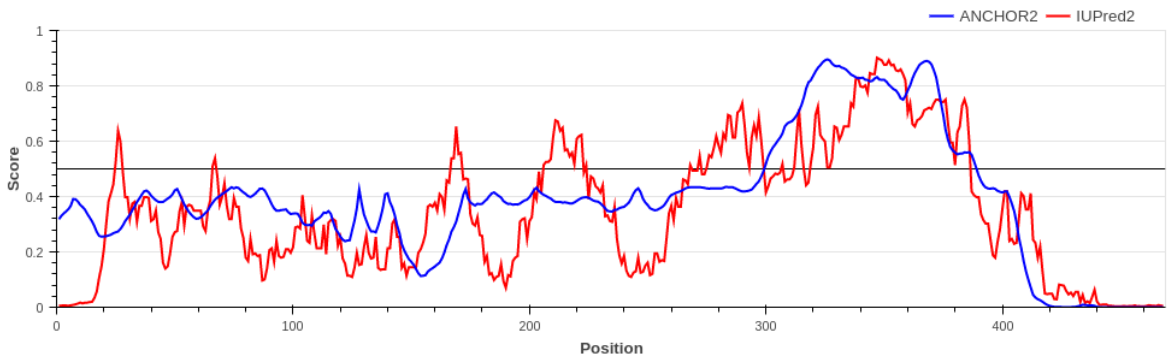


Figure 24: Disordered and conditionally ordered regions for Wg.

The IUPred2 analysis shows which regions of the protein is ordered and disordered. An IUPred2 score above 0.5 indicates position 320-380 of the protein are disordered. Anchor2 analysis shows within regions that express a high IUPred score, to what extent are they actually disordered, as this can vary depending on where in the protein, they are present and the role they are playing. X axis units = amino acids.

Analysis of DWnt6 also indicates the presence of a disordered region, between amino acids 140-180, as this region of the protein is above the threshold score of 0.5 (Figure 25). This region also corresponds to the additional amino acids found in the *Drosophila* protein with respect to other orthologues (Figure 25).

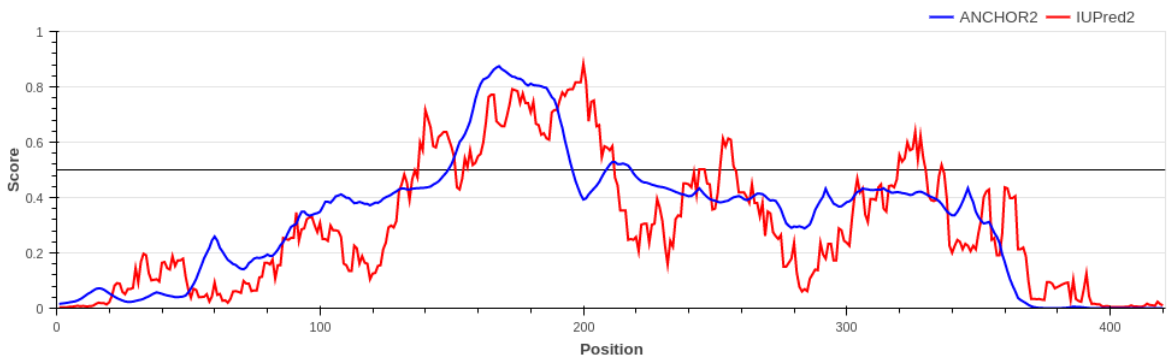


Figure 25:Disordered and conditionally ordered regions for Wnt6

The IUPred2 analysis shows which regions of the protein is ordered and disordered. An IUPred2 score above 0.5 indicates position 140- 180 are disordered. Anchor2 analysis shows within regions that express a high IUPred score, to what extent are they actually disordered, as this can vary depending on where in the protein, they are present, and the role they are playing. X axis units = amino acids.

Analysis of Wnt5 (DWnt3) indicates that a large region of the protein which stems from amino acids 80-480 are classed as disordered (Figure 26). Amino acids 320-360 have an IUPred score of 0.95, but an Anchor score of 0.1, indicating that although the region is highly likely to be disordered, (Figure 26).

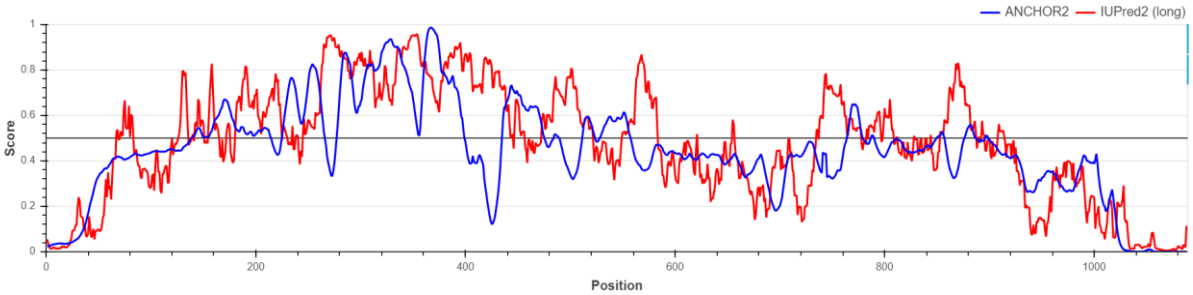


Figure 26: Disordered and conditionally ordered regions for Wnt5 (DWnt3).

The IUPred analysis shows that amino acids in position 80-480 show a high likelihood of being disordered, as they are above the threshold score of 0.5. X axis units = amino acids.

The IUPred analysis indicates that Wnt7 does not contain any major disordered regions, as they do not cross the threshold for disorder, this is consistent with the Anchor analysis (Figure 27).

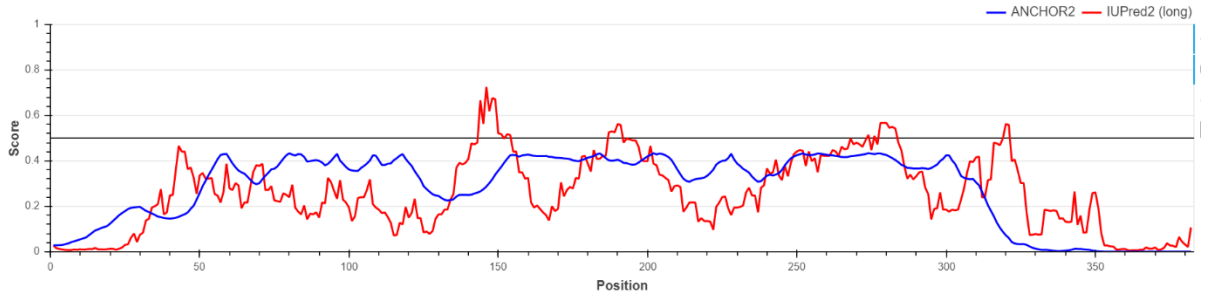


Figure 27: Disordered and conditionally ordered regions for Wnt7 (DWnt2).

The IUPred analysis shows that generally DWnt2 is a stable protein with most of the protein falling under the threshold score for disorder, and the Anchor score also falling under the threshold highlighting that the protein is generally not disordered and quite stable. X axis units = amino acids.

IUPred analysis of Wnt8 shows that generally this is not a disordered protein, with only amino acids in position 75 to 85 reaching an IUPred score of 0.8 and therefore, classed as disordered (Figure 28). When looking at the Anchor analysis it also reflects the lack of disorder in the binding regions of the protein, as the anchor analysis does not exceed the 0.5 protein threshold (Figure 28).

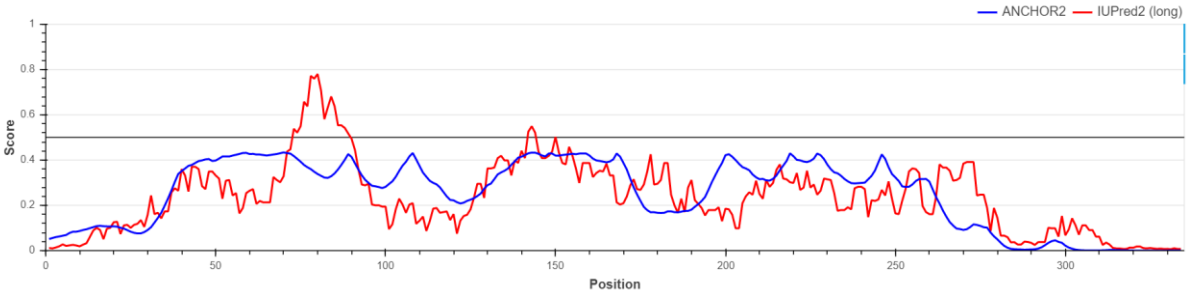


Figure 28: Disordered and conditionally ordered regions for DWnt8.

IUPred analysis shows that generally DWnt8 is not a disordered protein, with only amino acids in position 75 to 85 reaching an IUPred score of 0.8. When looking at this region in more detail, the Anchor analysis also indicates the region itself is quite stable. X axis units = amino acids.

Wnt9 (DWnt4) amino acids 25-90 represent a minor disordered region and a second region that exists from 460-500. There is an indication of a further region of disorder between 260-300 amino acids (Figure 29). With Anchor analysis the amino acids in regions 25-90 are the only part of the protein that pass above the 0.5 threshold to be labelled as disordered (Figure 29).

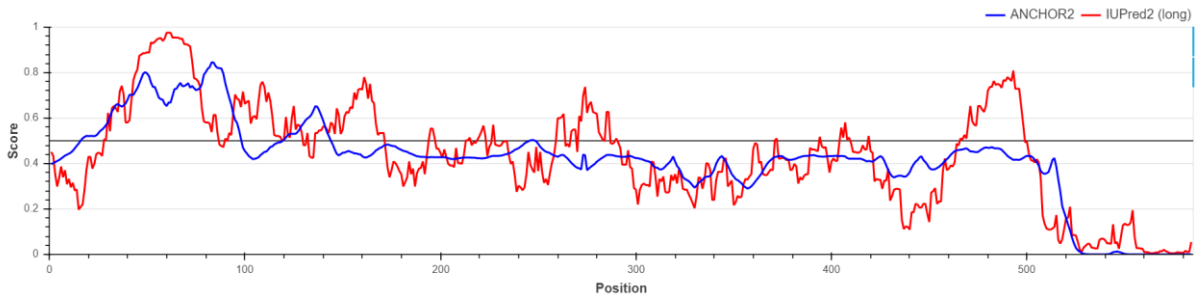


Figure 29: Disordered and conditionally ordered regions for DWnt4 (Wnt9). The

The IUPred analysis shows that there appears to be one major disordered region spanning from amino acids 25-90 and a minor disordered region spanning from amino acids 460-500. Anchor analysis indicates that the major disordered region also has a high Anchor score indicating that it is also highly unstable, however, the minor disordered region is more stable and below the 0.5 threshold. X axis units = amino acids.

The IUPred analysis for Wnt10 indicates a large disordered region between amino acids 360-420, there is also a much smaller disordered region at the very start of the protein between amino acids 10-60. The Anchor analysis indicates that the second larger disordered region also has a disordered binding region (Figure 30).

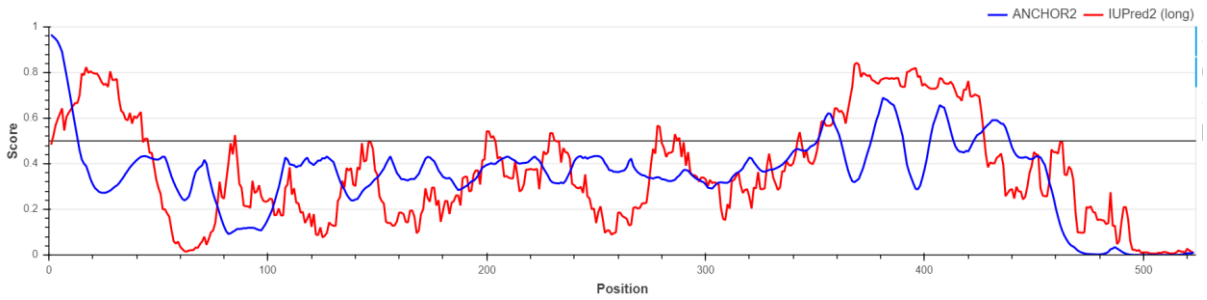


Figure 30: Disordered and conditionally ordered regions for Wnt10.

The IUPred analysis for Wnt10 shows that there is a disordered region of the protein that exists from amino acids 360-420 with a disorder score of around 0.8, with a variable anchor score ranging from 0.3 to 0.6, indicating that there is a high likelihood this part of the protein may switch from being ordered to disorder. X axis units = amino acids.

3.4.Discussion

In this chapter, the seven Wnt ligands present in *Drosophila* were aligned to the corresponding Wnt ligands from the following species *Aedes, Bicyclus, Tribolium*, *Parasteatoda*, *Platynereis*, *Mus and Nematostella where* the given Wnt ortholog was present. Proteins models for each Wnt ligand were then modelled using two different approaches - Swiss mod and Alphafold - to begin to explore Wnt protein sequence conservation and protein structure conservation and divergence. Furthermore, I examined the evolution of an insert in Wg, and explored the possibility of this region being an intrinsically disordered domain.

3.4.1. The evolution of metazoan Wnt proteins: implications for structure and function.

Disulfide bridges formed between cysteine residues are fundamental for stabilising proteins, and are thus important for governing several biological processes (Wiedemann *et al.,* 2020). Wnt proteins exhibit conserved cysteines and these are crucial for the function of these ligands (MacDonald *et al.,* 2014). Wnt ligands bind to Fzd receptors through the protruding thumb and index finger domains, which are stabilised by disulfide bonds, allowing for the Wnt thumb and index finger to grasp the two the sides of the FZD domain, and mutations to cysteines can impact Wnt signalling (MacDonald *et al.,* 2014). MacDonald *et al.,* (2014) found that mutation of any individual cysteine within the Wnt3a ligand resulted in covalent Wnt oligomers through ectopic intermolecular disulfide bond formation and as a result diminished Wnt signalling. Aliment of the amino acid sequences of the orthologs for each of the seven Wnt ligands highlighted the conservation of key cysteines, which varied from 10-22 cysteine residues in total depending on the particular Wnt ligand. As previously reported by Dierick and Bejsovec (1998), alanine 136 and cysteine 242 are absolutely invariant in Wnts across animals, as seen

in the alignments in this chapter. Therefore, the alignments completed in this chapter add to our understanding of the functional importance of these conserved disulfide bonds, with reference to Wnt-fzd interactions across a diverse selection of animals. Ultimately, adding to our understanding on the contribution of these cysteines to the overall stability and dynamics of Wnt ligands.

When examining the alignment of Wnt7(DWnt2) there appeared to be around 120 AA residues present in *Tribolium* absent from the other orthologs. Although there were amino acids present within this region within *Platynereis* and *Parasteatoda* there was no amino acid conservation among the three orthologs, indicating this region was not conserved among the orthologs surveyed. Alphafold models of Wnt7(DWnt2) highlighted that within *Drosophila*, *Tribolium* and *Aedes* there did appear to be a globular unformed tertiary structure that looped out of from the core of the protein from the N-terminus, but this appeared to be absent from the remaining orthologs investigated. Interestingly, when looking at Wnt5, the appearance of large inserts appeared to also be the case here and once again these inserts were present in *Drosophila* and *Aedes*.

The alignment of Wnt6, the ancient paralog of Wg, also indicate differences in the amino acids across the orthologs surveyed. With the presence of a unique domain of hydrophobic amino acids present in *Drosophila* Wnt6 and *Aedes* Wnt6, that are located close to the Fz binding site. The particular location of the unique domain in Wnt6, is within close proximity to the Fz receptor, however, the region adopts a flexible unordered structure. As described by Babu, (2016) such unordered domains are able to adopt different conformations, therefore, there is the possibility that the Wnt6 disordered region is able to elicit different responses depending

on the interacting partner. This could suggest that the regions in *Drosophila* and *Aedes* are functional innovations in these insects, but this requires further testing. The inserts could begin to shed a light on specific roles of such looped protein domains within Wnt proteins.

As described by Janda *et al.,* (2012), Wnt proteins have two linker connected domains of N-terminal (NTD) Saposin-like domain and C-terminal (CTD) cytokine-like domains with a special hand-shaped fold and thumb and index finger structure grasping FZ receptor in two interacting sites (Dehghanbanadaki *et al*., 2023: Janda *et al.,* 2012) (Figure 15). This unique structure that defines Wnt ligands was seen for all Wnt ligands described in this chapter. However, several protein models for Wnts of different animals surveyed in this chapter also indicated large protein regions that were not depicted as alpha helices or beta pleated sheets. For example, DWg has an insert of 85 amino acids, and this insert is present within Drosophilids (280AA- 365AA), however, the insert does not appear to be conserved among the Drosophilids. Furthermore, the insert in not present outside of the insects, for example it is completely absent from *Nematostella*. This particular region has been described previously by Hays *et al.,* (1997). The 85 amino acids do not contain any conserved cysteines, and the insert was predominantly hydrophobic (Hays *et al.,* 1997). This could suggest the region is not part of the core structure of the protein, as shown by the Swissmod and Alphafold molecules, the region loops out from the folded structure as hypothesised by Hays *et al.,* (1997). However, when looking at other Drosophilids the insert appears to be present throughout, furthermore, *Musca domestica* which belongs to the *Mus*cidae family also has the insert present, as did *Aedes*. Interestingly, although there are minimal mentions of the 85AA insert present in *Drosophila* described in the literature, there appears to be a great lack of understanding as to

the role and function of this region, and no mention of this region outside of *Drosophila* such as within the insects (Bejsovec, 2013).

3.4.2. *Drosophila* Wnt proteins contain novel intrinsically disordered regions & Wnt Orthologs lack additional novel regions

Within this chapter protein alignments and protein models were created for the seven *Drosophila* Wnt ligands, and the orthologs of each Wnt ligand from the following species: *Aedes, Bicyclus, Tribolium, Parasteatoda, Platynereis, Mus and Nematostella*. The alignments and protein models indicated that within certain species including *Drosophila*, *Aedes* and *Tribolium* certain large protein inserts appeared to be present. The Anchor analysis tool was used to determine whether the large insert regions were ordered in structure, and whether upon binding to other proteins do these regions switch to an ordered structure. The insertion present in *Drosophila* Wg, (amino acids 278-368), were depicted above the 0.5 threshold for order, and thus were classed as disordered. Furthermore, as these amino acids were also above the 0.5 threshold for IUPRED analysis, it suggests that upon binding of other proteins the region does not resume to become ordered but in fact remains disordered. Disordered proteins contain important functional elements involved in protein–protein interactions, and disordered regions play a critical role in various biological processes, for example signalling (Dosztányi *et al,.* 2009). These protein structures differ from protein interaction sites of globular proteins due to their distinct structural properties (Mészáros *et al.,* 2009). Such regions exist as a highly flexible structural regions when isolated but can and adopt a well-defined tertiary structure when binding to specific molecules (Dosztányi *et al,.* 2009). The intrinsically disordered region present in DWg is unlikely to interact with Fz binding. However, as mentioned above, the disordered region for Wnt6 is located near the Fz binding site. Anchor analysis for DWnt6 shows the insert looks to be ordered but with borderline disorder. Hibino and Hoshino, (2020)

describe a mechanism whereby intrinsically disordered proteins, and disordered regions within proteins, are able to change conformations so that they can become compatible with required interacting partners. This mechanism could allow Wnt6, to interact with Fz receptors, in a different way to other Wnts and compared to their orthologues in other species. When analysing the six other Wnt family members from *Drosophila*, alignments and modelling indicate a great level of variability in the position of the disordered regions, the size and the number of regions. *Drosophila* Wnt10 is particularly interesting as it possessed two disordered regions.

I investigated if the occurrence of the disordered region in *Drosophila* was specific or if the selected Wnt orthologs also possess some form of disordered region. When surveying the predicted models of the orthologs across the metazoan tree, there appeared to be no consistent pattern for the presence of additional regions predicted. Six of the seven orthologs investigated showed no indication of an additional predicted regions similar to that in *Drosophila*. The most closely related species *Aedes* appeared to be the only ortholog that could arguably possess a domain like that in characteristics to the disordered region present in *Drosophila melanogaster*. Such regions seem to be present in all the Wnts surveyed in *Aedes*. It is intriguing that only *Drosophila*, *Aedes and Tribolium* (to a much lesser extent) possess these regions.

The presence of these disordered regions within insects and specifically within *Drosophila* thus raises the question- what role are these disordered proteins playing? Findings from Andersen, (2011) suggest that highly elastic cuticular proteins such as resilin, are often intrinsically disordered proteins. Therefore, could the disordered regions highlighted in this work, have a role within cuticle functionality.

Finally, as intrinsically disordered proteins exist as fluctuating structures they may offer advantages in certain cellular responses, such as binding of ligands or membrane surfaces. As disordered regions are relatively accessible, they may contain multiple binding motifs, and can be sites for post-translational modifications- an important mediator for the control of cell signalling pathways (Wright and Dyson, 2015). Furthermore, disordered proteins often also have the potential to bind multiple partners (Wright and Dyson, 2015). The findings from this chapter could be suggestive of specific roles played by disordered proteins in insects.

This chapter aimed to align, model and investigate *Drosophila* Wnt paralogs and Wg orthologs across selected animals. Seven animals were selected and investigated in this work as they almost all possessed a near complement of the seven Wnt genes present in *Drosophila*. Although not in the scope of this work, future work would look to investigate a wider range of species through further alignments and protein modelling, particularly within the insect class, and the order Diptera, to begin to tease apart differences that exist with Wnt ligands between closely related species. Further investigations into the role of disordered protein regions are imperative, and this could be achieved by surveying a greater array of insects to investigate the presence of disordered domains, coupled with ANCHOR and IUPRED analysis to determine the level of disorder. Furthermore, investigations and experimentation into change in conformation upon binding would provide insights into how proteins domains switch between order and disorder. Finally, this chapter only aligned, modelled and accessed disorder in Wnt ligands. A similar approach could be used to investigate Fz receptors, and other known signalling molecules that interact with Wnt ligand. To further uncouple Wnt complexity it is also important to study Wnt ligand function within *D. melanogaster* and among orthologs. For

this reason, the subsequent chapters will begin to explore Wnt paralog and Wg orthologs function in vivo.

4. Testing the functional equivalence of Wnt ligands

Analysis of the genomic organisation of *Wnt* genes in metazoans has shown the existence of an ancestral cluster the *Wnt9–wg–Wnt6–Wnt10* cluster is found in insects, albeit with lineage specific rearrangements (Murat *et al.,* 2010). *Wg* and *Wnt6* are often expressed in the same developing tissues, for example expression of *Wnt6* is observed in *Drosophila* wing antennal imaginal discs in identical patterns to *wg* (Janson *et al.,* 2001). This may reflect some level overlapping function between these two genes. However, identical expression patterns results could also be interpreted as evidence that the functions of Wnts are redundant in some contexts, although complete redundancy would seem unlikely since all metazoan species have retained a large number of different Wnt proteins throughout evolution, as discussed in the introduction (Bazan *et al.,* 2012). It appears more likely that Wnt ligands act combinatorially in Wnt signalling landscapes in different tissues (Llimargas and Lawrence 2001; Amerongen and Nusse 2009). With this in mind it raises the question: Is Wg functionally equivalent to Wnt6 in *Drosophila*? This can be tested by investigating the rescue capacity of Wnt6 in the place of endogenous Wg. In the absence of any rescue capacity, we can begin to deduce protein domains that may have similar functionality, and conversely regions that are ligand and function specific thus helping to describe the basis of Wnt ligand specificity.

4.1. Accelerated homologous recombination and genome modification

Gene targeting via homologous recombination enables the deletion of genomic sequences and concurrent introduction of exogenous DNA, however this can often be laborious (Baena-Lopez *et al.,* (2013). Therefore, Baena-Lopez *et al.,* (2013), designed a targeting vector that replaces genomic sequences with a multifunctional fragment that includes an easily selectable marker, as well as an attP site, which acts as a landing platform for reintegration vectors (Figure 31).

This system was used to engineer the *wg* locus to allow for the desired modified Wnt gene of choice to be incorporated (Alexandre *et al.,* 2014) (Figure 31). As well as allowing the removal of Wg globally, this technique can be used to remove Wg in a specific tissue using specific GAL4 drivers. This technique can also be used to test other proteins at endogenous levels, such as in the case of Alexandre *et al.,* (2014) that tested a membrane tethered version of Wg. Using this technique, the modified *wg* locus, was used to test rescue capacity of paralogs and orthologs of Wg in the absence of endogenous Wg (Figure 31).

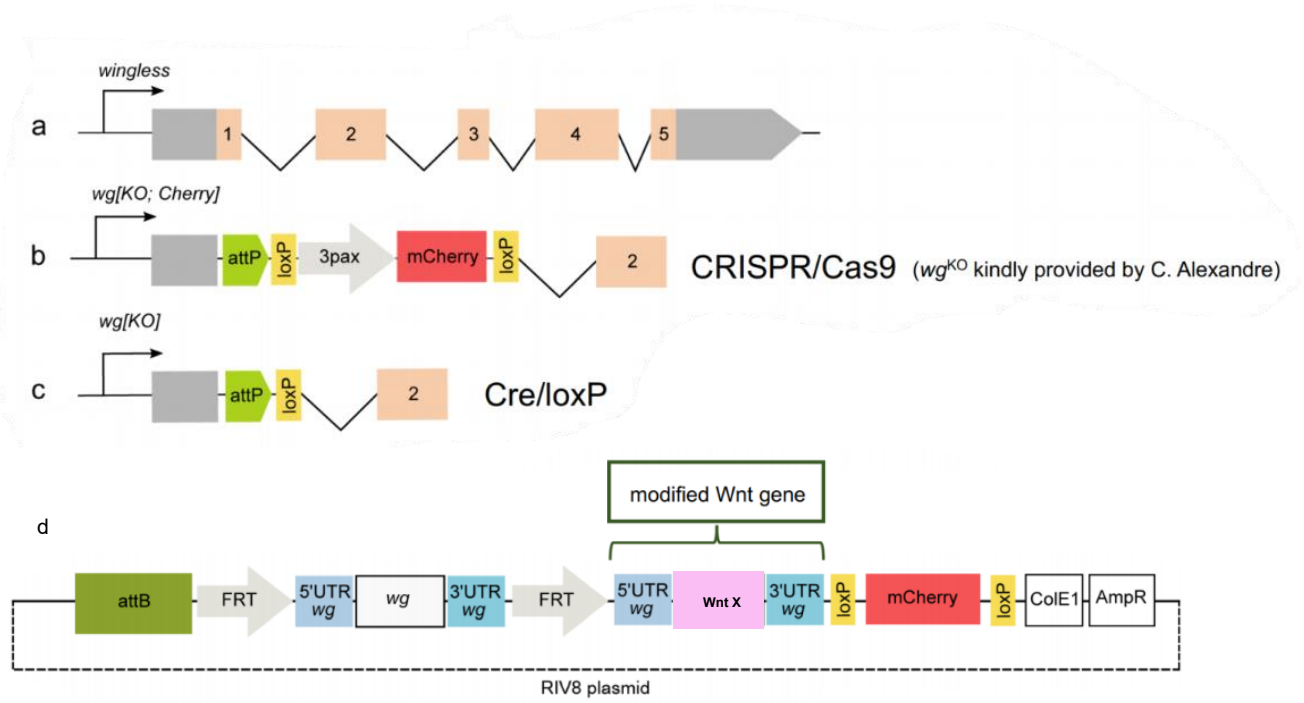


Figure 31:The targeting vector used to test Wg paralog and Wg ortholog rescue

a) The structure of the wg locus before targeting. b) Structure of the wg locus after targeting with the presence of the mCherry marker. c) The wgKO founder line that can be used for phic31 integration. d) The targeting vector, pTVCherry, showing its key features, including mCherry. The first multiple cloning site, flanked by FRT sites, allows for wg to removed, leaving behind the modified Wnt gene.

4.2.Results

4.2.1. Riv8-WgKO cannot rescue Wg function

Firstly, as a control it was important to establish that the flipping system, that was being used to remove the only source of *wg* worked efficiently. The Riv8-Wg plasmid (Figure 32) was therefore injected into the WgKO stock and four transgenic lines were established. These lines were then crossed to the WgKO line and the KB19-germline flippase which induces the recombination, to recombine the FRT sites to flipping out *wg* and leave only a QF marker (Figure 32). This experiment resulted in no surviving flies homozygous for the flip-out from any of the four lines tested as predicted, as all flies still has a balancer as evidenced by the CyO balancer. This experiment verified that the FRT flipping and the crossing scheme works effectively.

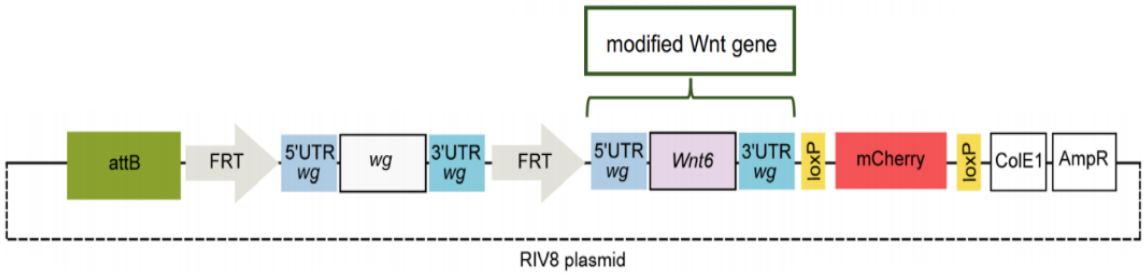


Figure 32: Riv8- WgKO targeting vector

The targeting vector, allowing for the removal of wg leaving behind just a QF marker, therefore, no modified Wnt gene.

4.2.2. NRT-Wg can rescue Wg function

I then carried out a positive control to show that the flipping out *wg* could be rescued by a second sequence introduced at the other MCS (Figure 33). For this I used a modified *wg* encoding the membrane tethered form of Wg previously described by Alexandre *et al.,* (2014). As shown previously, membrane tethered-Wg (NRT-Wg) can successfully replace the endogenous *wg* coding sequence, (Alexandre *et al.,* 2014). However, it is important to note that, Alexandre *et al.,* (2014) directly introduced *nrt-wg* into the WgKO rather than flip out *wg* cDNA from the first MCS to leave *nrt-wg* introduced at the second MCS. Hence, I repeated the experiment to test if flipping out the *wg* cDNA using the FRT system to then leave NRT-Wg as the sole source of Wg protein could rescue (Figure 33). I injected plasmid Riv8-Wg Nrt-Wg (Figure 33) into the WgKO line and generated 15 transgenic lines. I then crossed these WgKO and the KB19-germline flippase which induces the recombination to flip out the *wg* gene from the first MCS (crossing scheme in appendix).

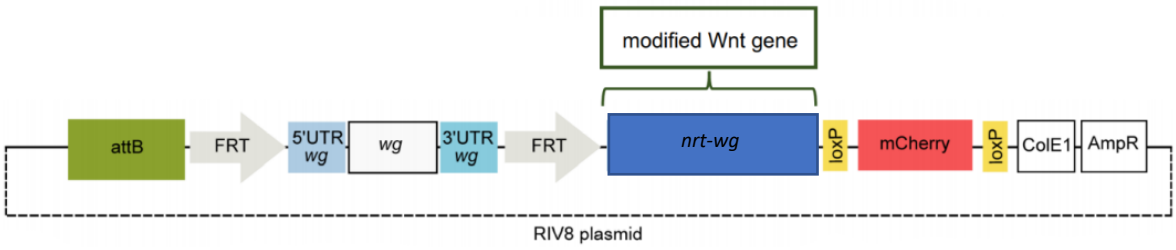


Figure 33: Riv8-Wg nrt-wg targeting vector

The *nrt-wg* targeting vector, allowing for the removal of *wg* and leaving *nrt-wg* as the sole source of *wg*.

Flipping out *wg*, to leave only the *nrt-wg* resulted in homozygous viable adult flies as scored by loss of the balancer CyO. However, the NRT-Wg flies seemed generally very unwell, siblings were unable to mate and therefore a stock of the flies could not be maintained.

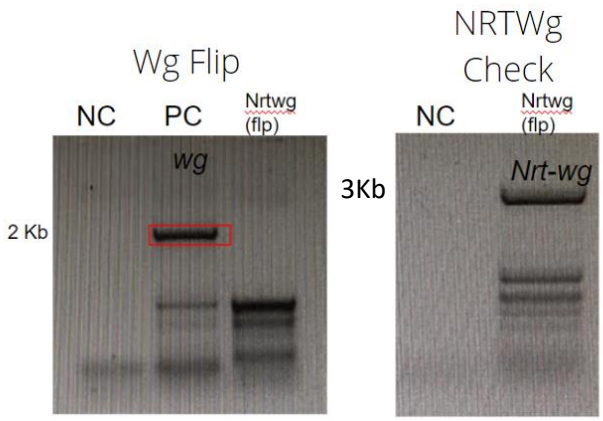


Figure 34: nrt-wg gel result

Gel result showing the successful removal of Wg, and the presence of NRT-Wg. The negative control, is the presence off all other materials, minus the DNA. Primers to conduct verification included a plasmid specific primer and a reverse plasmid specific primer that covered wg, with a PCR product of 2Kb, this PCR product would be missing if a successful flip occurred. To then test for *nrt-wg*, plasmid specific primers were designed to cover the length of nrt-wg approximately 3Kb.

4.2.3. Wnt6 cannot functionally replace of Wg

To investigate if Wnt6 can functionally replace Wg, I applied the same approach when testing NRT-Wg. Wnt6 cDNA was cloned into the second multiple cloning site of the RIV8-Wg plasmid (Figure 35) and injected into the WgKO line. Three transgenic lines were established, which were crossed to KB19-flp to flip out *wg* leaving only *Wnt6*. Removal of *wg* leaving *Wnt6* under the control of wg regulatory sequences and thus presumably expressed at the same physiological level, did not produce any viable homozygous flies because all flies from this cross retained the balancer chromosome CyO (Figure 35). This was repeated a minimum of three times in three separate crosses, with approximately 100 progenies generated. I used PCR

to verify that the flipping was successful, as can be seen in Figure 23B. This experiment determined that Wnt6 cannot functionally replace Wg to generate viable flies.

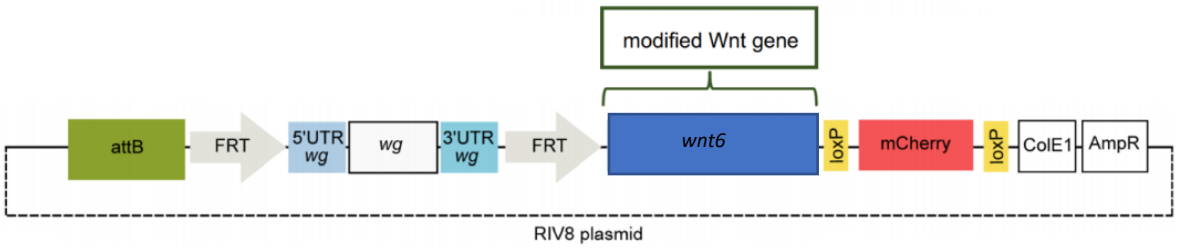


Figure 35: Riv8-Wg Wnt6.

Riv8-*wg wnt6* targeting vector, allowing for the removal of *wg* and leaving *wnt6* as the sole wnt ligand source.

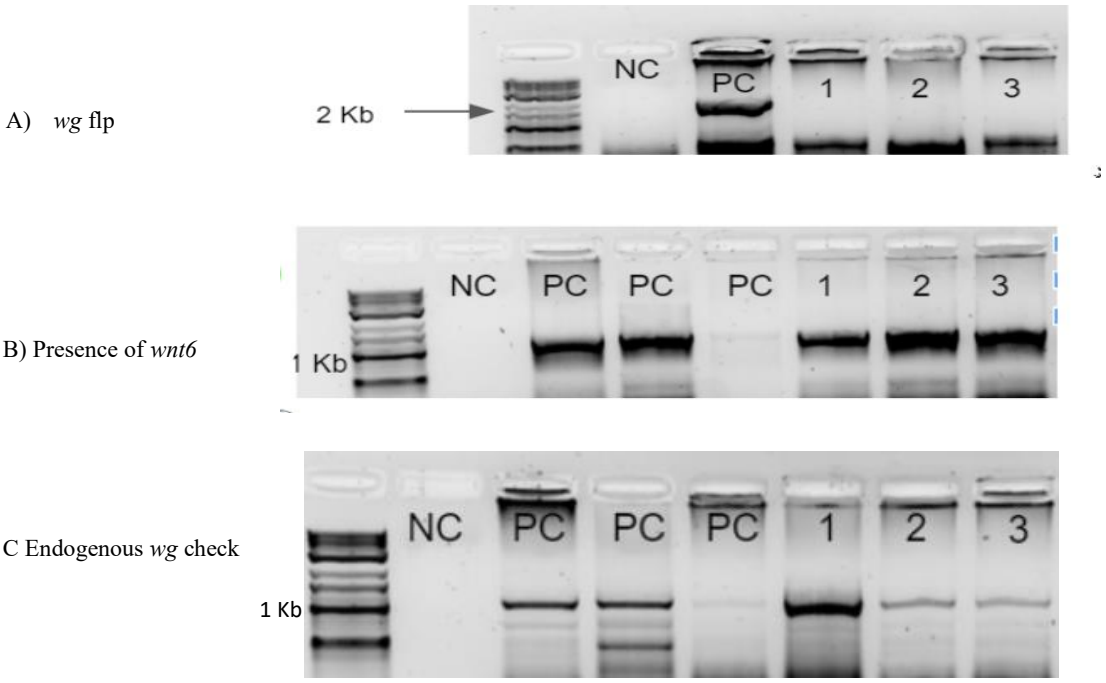


Figure 36: Gel images testing Wnt6KO

A) The gel image indicates that Wg was successfully flipped out from the first multiple cloning site of the inserted the RIV8-Wg plasmid. Primers to test the absence of Wg were designed to be plasmid specific flanking the first multiple cloning site. The PC control indicates a band at 2 kb, which is absent from the three other flies tested from three separate transgenic lines. B) The gel image confirms the presence of Wnt6 with a band at 1 kb, indicating wnt6 as the sole wnt ligand acting in place of wg. C) As Wnt6 could not replace Wg, a stock of Wnt6 (Dwg flp)/CyO flies was established to prove that this stock of flies is viable due to the presence of endogenous wg, this was indicated by a PCR product of 1 Kb

4.2.4. Synthetic Chimeras Incorporate Disordered Regions

From the result of the experiment above, I reasoned that specific domains of Wg and Wnt6 may be functionally distinct and so a series of chimeric genes were designed that express Wg containing domains of Wnt6 (Figure 37). In Chapter 3 it was observed that Wg and Wnt6 contain different disordered domains. Therefore, to expand upon this finding four different chimeras were designed by Holzem, (unpublished) in which the Wg and Wnt6 disordered regions were featured (Figure 37 and 38). The chimeras were designed to feature the disordered regions:

- Chimera A-the 5' region of *wg* and the 3' region of *Wnt6* including the disordered region of *Wnt6*
- Chimera B- the 5' prime region of Wnt6 and the 3' region of wg including the disordered region of wg
- Chimera C- the 5' region of Wnt6 and the 3' region of wg including the disordered regions of both genes
- Chimera D- the 5' and 3' region of Wnt6, excluding the disordered regions.

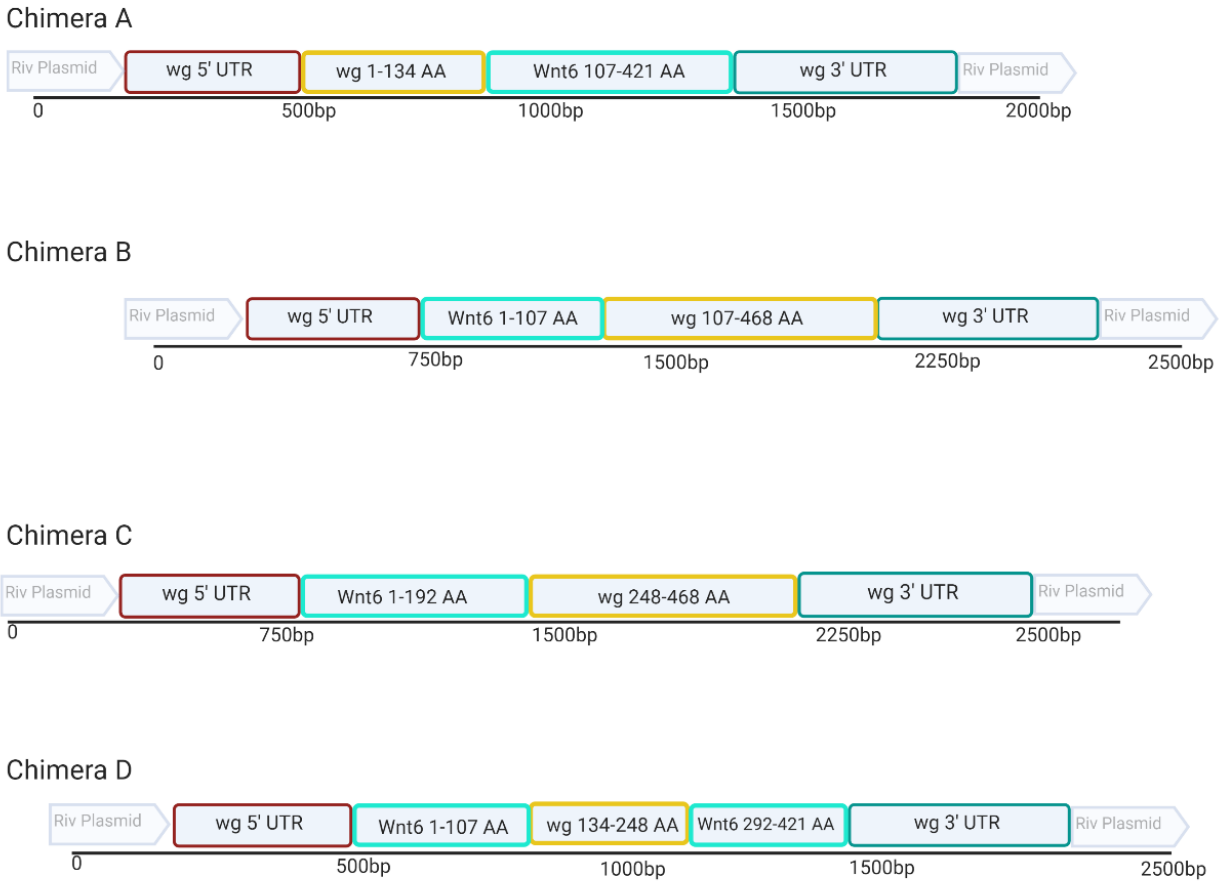


Figure 37:Chimeric wg/Wnt6 genes.

Sequences depicting the four synthetic chimeric gene sequences. All four synthetic Wnts include the 5' and 3' UTR of *wg*. Chimera A incorporated the region encoding amino acids 1-134 amino acids from Wg, and encoding 107-421 amino acids from Wnt6, including the disordered region of Wnt6. Chimera B incorporated the region encoding amino acids 1-107 from Wnt6 and encoding 107-468 from Wg, including the disordered region of Wg. Chimera C incorporated the region encoding amino acids 1-192 from Wnt6 and encoding 248-468 from Wg, including the disordered regions of both.

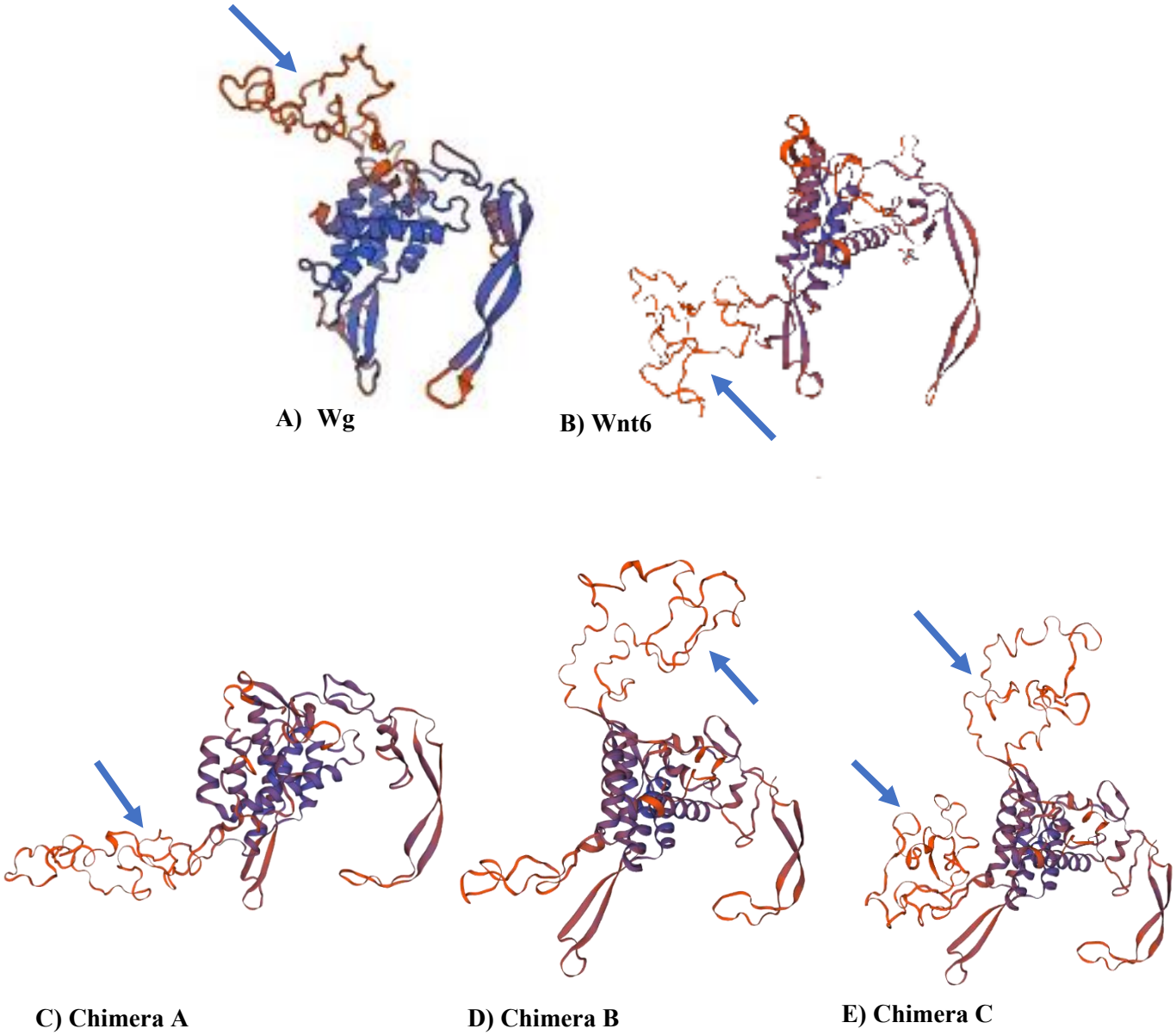


Figure 38: Protein models of Wg, Wnt6 and Chimeras A, B and C.

A & B depict protein models for Wg and Wnt6, the models indicate the disordered regions present in each of the models. Protein models of Chimeras A-C modelled using Swiss-mod. C) Chimera A has the disordered region from Wnt6 located near to the FZ binding region. D) Chimera B has the disordered region of Wg located near the N- terminus of the protein. E) Chimera C has both disordered regions. The arrows indicate the disordered regions present in each of the protein models. The chimeras used in this chapter was designed by (Holzem, unpublished).

4.2.5. Wg/Wnt6 Chimeras cannot rescue in the place of Wg

The chimeric proteins showed that different protein regions that can be found in either *wg* and or *wnt6* were incorporated when designing the chimeras. For Chimeras A-C a total of three transgenic lines were generated. They were then crossed to KB19-FLP to flip out wg leaving the specific chimera as the only source of wnt ligand in the place of wg, and the progeny checked for the presence of straight wings indicating the loss of the CyO balancer. Although the flipping out of wg was successful (Figure 39), it was not possible to generate homozygotes for any of the three chimeras. This suggests that like Wnt6, Chimera A, B and C cannot rescue Wg.

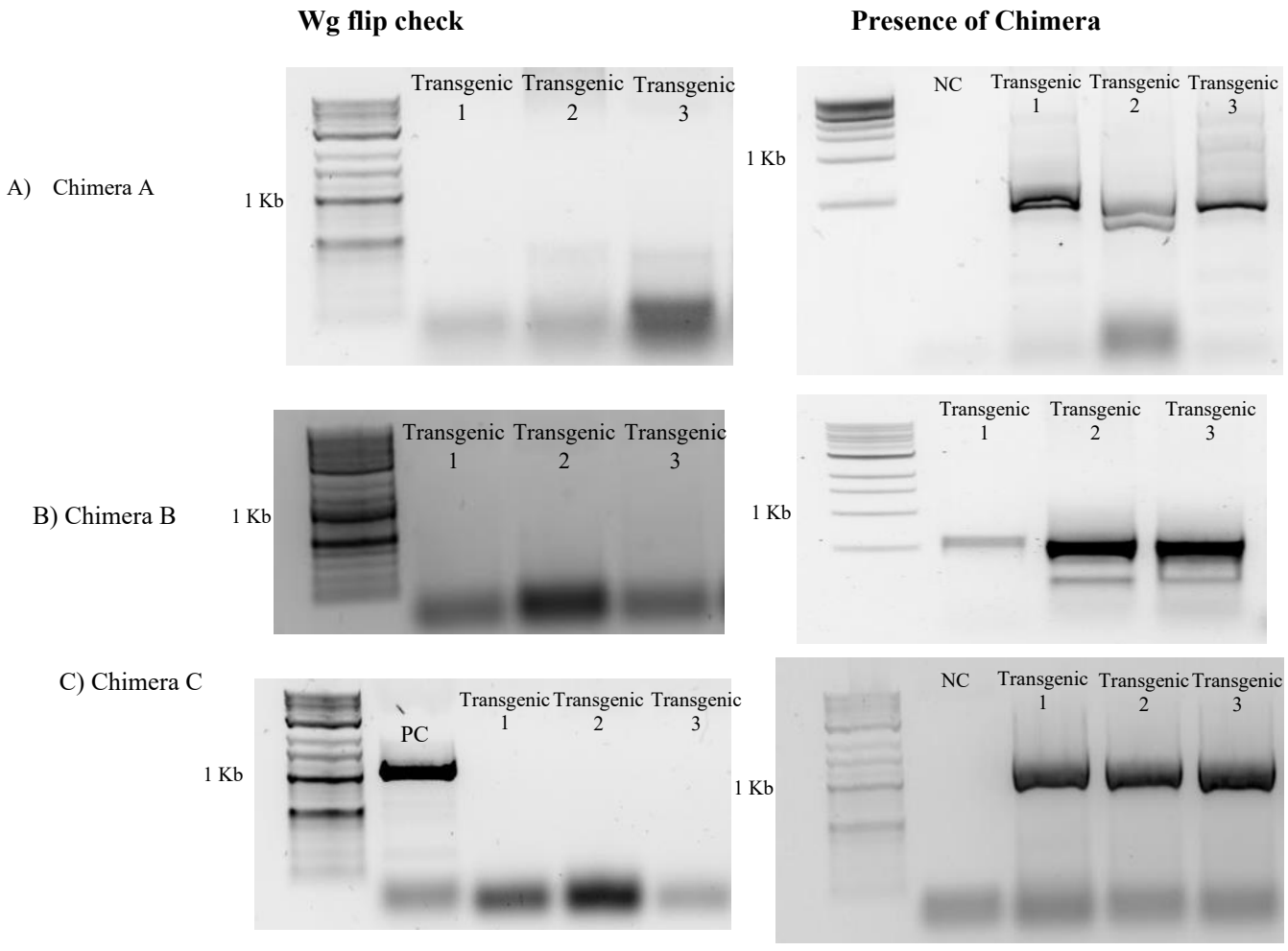


Figure 39:Genetic conformation of flip of wg and presence of chimeric in the absence of wg.

A) PCR verification for Chimera A indicating three transgenic flies with conformation of the flipping of *wg* as there is no PCR product, and the same three flies with the presence of Chimera A PCR product of 500 bp to confirm the only Wnt source is Chimera A. B) PCR verification for Chimera B indicating three transgenic flies with the conformation of the flipping of wg as there is no PCR product, and the same three flies with the presence of Chimera B PCR product of 500 bp to confirm the only Wnt source is Chimera B. C) PCR verification for Chimera C indicating three transgenic flies with the conformation of the flipping of wg as there is no PCR product, as there is conformation via the positive control a non- flipped chimera C fly. The same three transgenic flies have also been tested for the conformation of Chimera C as there is a PCR product of 1 Kb.

Wnt6 minus the disordered region (Wnt6-dr) is unable to rescue Wg function

To investigate the role of the Wnt6 insert, the 63 amino acid encoding region was removed using restriction endonucleases (synthesised externally via Genewiz) to generate *Wnt6-dr* in the second MCS of Riv-8Wg (Figure 40). This construct was injected into the WgKO strain as described for Wnt6 above and four lines were established. Flipping was then carried out by crossing to the KB19 germline flippase line. Flipping out of *wg* was successful as shown in (Figure 40). However no homozygous flies were recovered. Thus, attempting to rescue Wg function with *Wnt6-dr*, did not work, suggesting that the disordered region does not inhibit Wnt6 rescue of Wg.

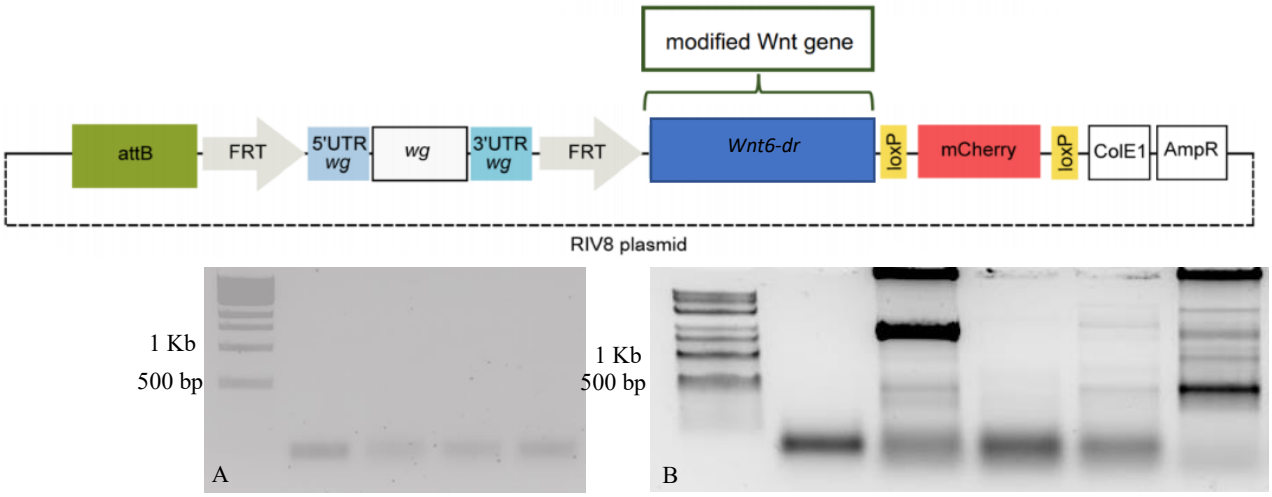


Figure 40:Riv8-Wg Wnt6- dr.

The Riv8-wg Wnt6-dr targeting vector, allowing for the removal of wg and leaving wnt6-dr as the sole wnt ligand source. A) Gel image A verifies the successful flipping of wg as there is a lack of any PCR products expected at 1 Kb. B) Gel image indicating the presence of Wnt6 as indicated by the 1 Kb PCR product.

4.3.Discussion

4.3.1. The ancient paralog Wnt6 cannot rescue Wg function globally

I showed that Wnt6 is unable to functionally replace Wg globally. This suggests that there are certain protein domains unique to Wg function, which are absent or have diverged in Wnt6. *wg* is essential for many aspects of *Drosophila* development, including segmentation and development and differentiation of structures from the imaginal discs (Sharma, 1973; Swarup and Verheyen 2012). Compared to the broad spectrum of functions in which *wg* is involved, Wnt6 appears to play fewer roles during development (Murat *et al.,* 2010) However, similar to *wg, Wnt6* is expressed in the imaginal discs, and Wnt6 expression is seen in the developing foregut and midgut (Janson *et al*. 2001). Therefore, both their individual roles in some tissues and their combinatorial activity in other suggests that Wg and Wnt6 are functionally distinct at the molecular level and consistent with the results of my rescue experiments in this chapter.

Furthermore, the Wg/Wnt6 chimeras and their inability to rescue suggests that specificity may be encoded in more than one domain or that domains have co-evolved. It is plausible that the chimeras do not fold correctly. However, modelling of the chimeras indicates structures that are very similar to those predicted when modelling Wg and Wnt6. More sophisticated structure analysis of the chimeras and how they are interacting with key players in the Wnt pathway is required to then design a new series of chimeras that incorporate conserved and non-conserved protein regions. These would help begin to tease apart structural differences that exist among Wnt ligands that could underlie functional differences. Chimeras have successfully been used previously to explore Wnt ligand functionality. Julius *et al.,* (1999) tested eleven chimeric genes of the *Wnt1* and *Wnt5a* sequences. They deduced that the amino terminus was necessary for cytosolic accumulation of beta-catenin due to the fact a chimera that contained as few as

110 residues from the Wnt5a amino terminus was not functional (Julius *et al.,* 1999). Another study tested membrane-tethered Wg-CD4 and Wg-CD8 chimeras. These chimeric proteins were able to elicit partial morphological transformation in C57MG cells either by direct expression or by co-culture with donor cells producing the chimeras, indicating the potential for Wnt1 to be altered at the carboxyl terminus but still able to function when membrane tethered as also demonstrated later by Nrt-Wg (Parkin *et al.,* 1993). Another study that looked at Wnt action focused on the role played the Fz receptor. Bhat *et al.,* (2010) showed that a human Wnt3–Fzd1 chimera is more efficient that Wnt3 along in activating canonical Wnt signaling Furthermore, the authors discovered an eight-fold decrease in activity when deleting the cytoplasmic portion in the Wnt3-Fzd1 chimera, indicating specific parts of the protein play a critical role in Wnt ligand function.

One of the major unanswered questions in Wnt biology is the specificity of interaction between different ligands and Fzd receptors, as well as the various downstream pathways that these ligand-receptor pairs stimulate. The Wnt chimera approach and the Wnt–Fzd chimera approach may therefore provide a method to study this selectivity issue between Fz receptors and Wnt ligands. Furthermore, to study the subsequent downstream pathways that these receptor–ligand pairs activate in cells, and by doing so will shed light on specific functional domains that exist per Wnt to provide specificity, and uniquely shape the specific Wnt ligand. The removal of the Wnt6 disordered region indicates that this domain does not inhibit Wnt6 ability to rescue Wg. Further testing is required to understand the functional role of this domain. Further testing of these disordered regions will begin to shed a light on the regions that are imperative to Wnt6 function.

5. Testing the functional equivalence of Wg Orthologs

Are Wg orthologs functionally equivalent to Wg in *Drosophila*?

Similarities and differences in *wg* function from different animals can help reveal the key amino acids for the function of this ligand in different lineages. Functional rescue experiments have been carried out for orthologs from other protein families from partial/tissue-specific rescues, for example *Pax6* overexpression to generate ectopic eyes in *Drosophila*, to attempted full rescue such as in the case of *engrailed* (*en*) genes (Manuel *et al.,* 2008; Hanks *et al.,* 1998; Halder *et al.,* 1995). A study conducted by Halder *et al.,* (1995) showed conservation of Pax6 function, by placing an eye-enhancer region of *Drosophila Pax6* upstream of the mouse *Pax6* gene transgenic mice can accurately reproduce endogenous Pax6 expression. However, there are caveats to using overexpression, as overexpression implies expression beyond the norm, without knowledge of the normal expression this can possibly result in mutant phenotypes, and it is argued that rescue experiments are a more sophisticated method to test rescue capability, such as in the case of the *engrailed* (*en*) genes (Prelich, 2012). Hanks *et al.,* (1998) used a gene targeting knock-in strategy in mouse embryonic stem (ES) cells to replace the coding sequences of mouse *En1* with those of *Drosophila en*, and were able to achieve a near complete rescue of the *en* mutant brain defect. This experiment suggested *En1* has the capacity to substitute for *Drosophila en*. I have applied the same logic here and have sought to fully test *wg* functionality with rescue experiments using a variety of *wg* orthologs. for the first time.

Specifically, in this chapter I tested the ability of Wg orthologs to rescue Wg function in *Drosophila*. I focussed on seven Wg/Wnt1 orthologs from the following species: *Nematostella vectensis* (Cnidaria), *Mus Musculus* (Vertebrata), *Platynereis dumerilii* , (Annelida),

Parasteatoda tepidariorum , *Tribolium castaneum* , *Bicyclus anynana* and *Aedes aegypti* (spider/ beetle/butterfly/mosquito, Arthropoda) to test species that have retained the same Wnt ligands that are found in *Drosophila*. As shown in Chapter 1, all these Wg orthologues except for that of *Aedes* lack the disordered region found in *Drosophila*. This therefore also allowed me to test whether Wg orthologs that lack this insert, can rescue Wg function in *Drosophila* to shed further light on the role of this domain in *Drosophila*.

5.1. Accelerated homologous recombination and genome modification in *Drosophila*

I utilised the modified *wg* locus described in previous chapters to test the functionality of seven orthologs of Wg in the absence of *Drosophila* Wg (Alexandre *et al.,* 2014). This system was used because it allows the desired Wg ortholog of choice to be incorporated directly into the *Drosophila wg* locus and therefore to test the protein sequences directly by expressing them the same as the endogenous gene in space and time (Alexandre *et al.,* 2014) (Figure 41).

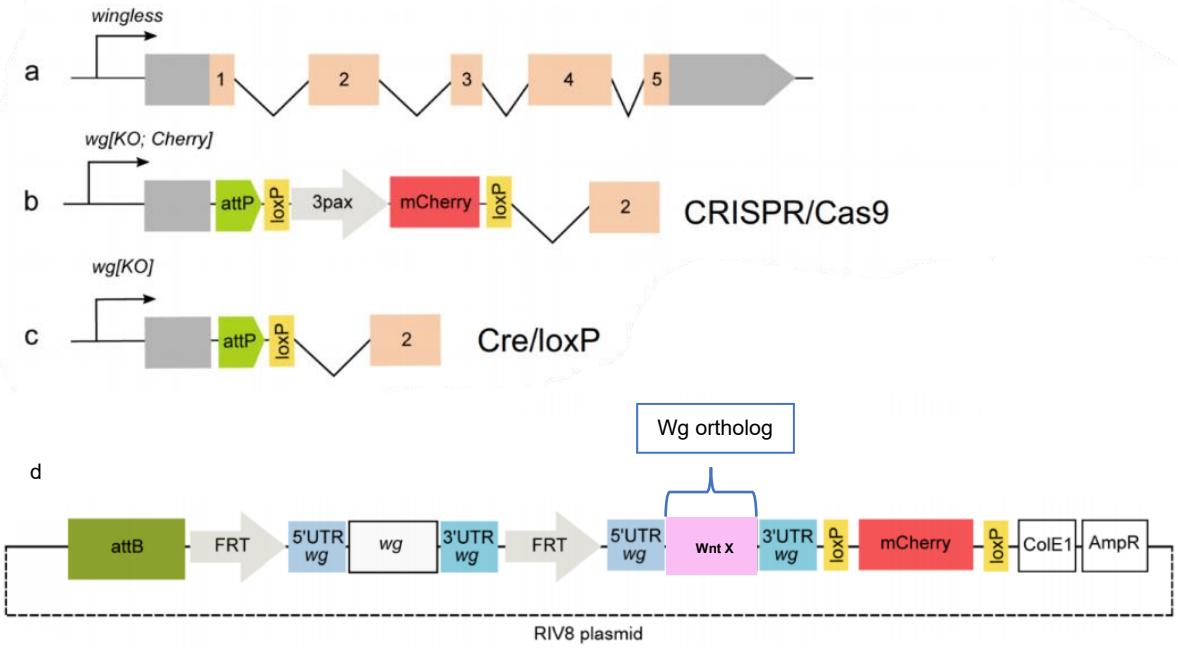


Figure 41:The targeting vector used to test Wg ortholog rescue

a) The structure of the *wg* locus before targeting. b) Structure of the *wg* locus after targeting with the presence of the mCherry marker. c) The *wg*KO founder line that can be used for phic31 integration via the attP site after removal of other sequences using Cre/loxP. d) The targeting vector, pTVCherry, showing its key features, including mCherry. The first multiple cloning site is flanked by FRT sites, allows for *wg* to removed, leaving behind the *wg* ortholog of choice inserted into the second multiple cloning site.

5.2.Results

5.2.1. Wg ortholog alignments

In chapter 1 the *Drosophila* (D-Wg) protein sequence was compared to the Wg/Wnt1 orthologues of *Nematostella*-Wg, *Mus*-Wg, *Platynereis*-Wg, *Parasteatoda*-wg, *Tribolium*-wg, *Aedes*-Wg & *Bicyclus*-Wg by aligning the full-length protein sequences. As expected, given the relationships of these eight species, Wg from the dipteran *Aedes* has the greatest level of sequence similarity to *Drosophila* with a 69.3% identity match (conserved amino acids) and ~77% sequence similarity (conserved and similar amino acids), with *Nematostella* only sharing 47.29% sequence similarity. The most striking difference between the species is a domain of up to around 85 amino acids found in *Drosophila* that is not present in the other orthologs as previously noted by Hays *et al.,* (1997) and in chapter 1. While the *Aedes* and *Tribolium* Wg proteins also have a few additional amino acids at this position, they do not align very well with the *Drosophila* Wg sequence.

5.2.2. Cloning and Injection of Wg Orthologs

To investigate if *Nematostella*-Wg, *Mus*-wg, *Platynereis*-Wg, *Tribolium*-Wg and *Bicyclus*-Wg, *Parasteatoda*-Wg and *Aedes*-Wg can functionally replace *Drosophila* Wg, I applied the same approach as described in Chapter 4. *Nematostella*-Wg, *Mus*-Wg, *Platynereis*-Wg, *Parasteatoda*-wg, *Tribolium*-wg, *Aedes*-Wg & *Bicyclus*-Wg coding sequences were cloned into the second multiple cloning site of the RIV8-Wg plasmid (Fig 30.). Synthesis and cloning for *Nematostella*-Wg, *Mus*-Wg, *Platynereis*-Wg, *Parasteatoda*-wg, *Tribolium*-wg, *Aedes*-Wg & *Bicyclus*-Wg was carried out by by Genewiz and injections into the *wg*KO founder line were performed by the Cambridge Fly injection facility. I cloned *Parasteatoda-wg* into Riv8Wg

plasmid and injected this construct into the *wg*KO founder line, and established three lines for *Parasteatoda*-Wg. For the remaining orthologs three lines were provided for *Nematostella*-Wg, *Mus*-Wg, *Platynereis*-Wg, *Tribolium*-wg, and *Platynereis*-Wg, and two for *Bicyclus*-Wg and *Aedes*-Wg by the Cambridge fly injection facility.

5.3.Flipping and functional rescue assays

5.3.1. The Wg orthologs from *Nematostella vectensis*, *Mus Musculus*, *Platynereis dumerilii*, *Tribolium castaneum*, *Bicyclus anynana* and *Aedes aegypti* cannot functionally replace *Drosophila* wg.

To investigate if *Nematostella*-Wg, *Mus*-Wg, *Platynereis*-Wg, *Tribolium*-Wg, and *Platynereis*-Wg, and two for *Bicyclus*-Wg and *Aedes*-Wg can functionally replace *Drosophila* Dm-Wg, I applied the same approach as described in Chapter 4. The *wg*KO lines with the inserted RIV8-*wg* carrying *Nematostella*-Wg, *Mus*-Wg, *Platynereis*-Wg, *Tribolium*-Wg, *Bicyclus*-Wg, *Aedes*-Wg were crossed to KB19-flp to flip out *wg* (from MCS 1) leaving only the desired ortholog (Fig. 30). Removal of *wg* resulting only in the expression of *Nematostella*-Wg, *Mus*-Wg, *Platynereis*-Wg, *Tribolium*-Wg, *Bicyclus*-Wg, *Aedes*-Wg under the control of *wg* regulatory sequences and thus presumably expressed at the same physiological level as *Drosophila* Wg, did not produce any viable homozygous flies because all flies from the crosses retained the balancer chromosome CyO (crossing scheme- Appendix 1). This was repeated a minimum of three times via three separate crosses. This experiment suggests that the orthologs tested cannot functionally replace Wg to generate viable flies. It is important to note that NRT-Wg did rescue using the same crossing scheme was applied (crossing scheme- Appendix 1).

5.3.2. *Parasteatoda* may be able to functionally replace *Drosophila* wg

To investigate if *Parasteatoda wg (Pt-wg)* can functionally replace Wg, I applied the same approach when testing NRT-Wg, and the other orthologs by crossing to KB19-flp to flip out *wg* leaving only *Pt-wg*. Removal of *wg* leaving *Pt-wg* did produce viable homozygous flies as straight wings were visible and thus the flies lacked the CyO balancer (Fig. 42, crossing scheme- Appendix). This was repeated a minimum of three times in three separate crosses, with approximately 100 progenies. PCR was used to verify that the flipping was successful in six progeny (Fig. 42). However, when repeating this experiment, no homozygous flies were produced.

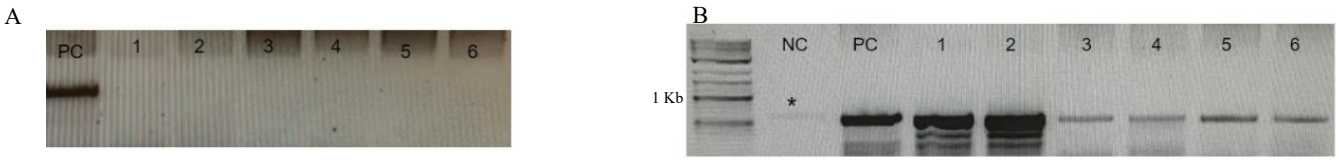


Figure 42:Genetic verification for the flip of DWg and presence of PtWg.

A) This gel indicates the lack of *Dwg* in the first multiple cloning site (MCS), to confirm the *wg* has been flipped. The gel indicates that the PC, which is a- non flipped individual still possessed *Dwg*. Primers used to test wg in the first MCS are Riv8-Wg plasmid specific. B) This gel tests for the presence of *Ptwg* indicating it is able to function in the place of *Dwg,* six progeny with straight wings from one parent sibling cross were tested, as represented by the lanes 1-6. There appears to be a band in the NC, which was most likely due to contamination. The PCR products were verified by sequencing to confirm the sequence was indeed from *Ptwg.*

5.4.Discussion

The results from this chapter indicate that rescue attempts with Wg orthologs from other species did not generate viable flies and shows that these proteins are not functionally able to replace Wg in *Drosophila*. Although *Parasteatoda*-Wg initially appeared to rescue, I was unable to repeat this result. Therefore, although Wg is found in all metazoans, my results suggest that these proteins could be species-specific in the lineages I tested, including other arthropods like a spider, beetle, butterfly and mosquito. It could be hypothesised that protein-protein interactions, for example the ways in which the orthologs tested in this work interact with Fzd proteins could be preventing rescues.

Partial rescues using other proteins have been attempted in *Drosophila*. A classic example is *Pax6/eyeless*. Halder *et al.,* (1995) expressed the mouse *Pax6* gene ectopically in *Drosophila* and were able to show the mouse homolog could induce ectopic eyes. However, this overexpression may not mean that mouse *Pax6* can functionally replace *ey* in all contexts to generate a viable fly. This work shows that there is definitely some capacity for even distantly related orthologs to have conserved functionality in some developmental contexts at least. For example, the *Drosophila* gene *en* can partially substitute for mouse *Engrailed 1* in the hindbrain, but is unable to do so in limb development (Hanks *et al.,* 1998). Weinberger *et al.,* (2017) directly replaced *Drosophila* atonal coding sequences with that of atonal homologues. They found that atonal homologues rescued sensory organ fate in atonal mutants. Lastly, Lutz *et al.,* (1995) tested rescue capacity using the chicken ortholog *gHoxb*-1 the ortholog of the *Drosophila labial* gene. They fused the entire protein-coding region of *gHoxb-1* with previously identified regulatory sequences of *lab*. In doing so they were able show that the gHoxb-1 protein can regulate lab target genes. Therefore, were able to demonstrate functional

conservation of the lab class homeo-proteins between insects and vertebrates (Lutz *et al*., 1995).

Therefore, given the results of other studies that compared the function of orthologs, it could be that expecting a full rescue of all Wg functionality in every functional context in *Drosophila* was too great an ask. To follow this series of work up, more context-specific flipping is needed. For example, testing the capacity of Wg orthologs to replace Wg function in the wings or legs specifically using drivers to induce flipping during the development of these tissues. Therefore, providing the Wg orthologs with an opportunity to rescue in a less complicated manner. As far as I am aware, using orthologs from other species to rescue *Drosophila* Wg in a specific developmental context has yet to be demonstrated but this would definitely be a profitable approach to inform the evolution of the functionality and specificity of these proteins.

6. Discussion

This work set out to investigate three elements:

1. Can modelling the structure of the seven *Drosophila* Wnt ligands and their orthologues help shed light on the evolution of Wnt protein structure?
2. Is *Drosophila* Wnt6 functionally equivalent to Wg?
3. Can Do Wg orthologues have the capacity to rescue a *Drosophila wg* null mutant.

6.1.Chapter 3

In chapter three, I explored the predicted protein structures of the representative of the seven Wnt ligand subfamilies found in *Drosophila* compared to their orthologs in seven other species: *Nematostella* (Cnidaria)*, Mus* (Vertebrata)*, Platynereis* (Annelida)*, Parasteatoda, Tribolium, Bicyclus* and *Aedes* (spider/ beetle/butterfly/mosquito, Arthropoda). This chapter aimed to investigate differences and similarities between Wnt ligand orthologs, to begin to shed light on the evolution of the *Drosophila* Wnt ligands, particularly Wg (Marino and Gladyshev, 2010).

6.1.1. Cysteines are conserved among Wnt ligands

Among the 20 common amino acid in proteins, cysteine (Cys) is one of the least abundant, but it is frequently observed in functionally important sites of proteins, for example regulatory sites of proteins (Marino and Gladyshev, 2010). Disulfide bridges are covalent bonds formed between two cysteine residues. Cysteines contain a thiol group, that allows for the formation of disulphide bonds with other cysteine residues. Disulphide bonds are essential for proper protein folding (Marino and Gladyshev, 2010; Wiedemann *et al.,* 2020). Disulfide bridges can

occur in both the secondary and tertiary structure of a protein, and are vital to support stabilisation of alpha helices and beta sheets, furthermore, they create rigid connections that help hold the structure in place and prevent it from unfolding (Wiedemann *et al.,* 2020).

It is known that Wnt proteins are typically 300-400 AA long with around 22-24 conserved cysteine residues that are essential to form disulphide bonds, to support protein folding and function of all Wnt ligands (Mikels and Nusse, 2006). The alignments produced in this work highlighted the conservation of cysteine residues among all the different orthologs surveyed, and with species closely related to *Drosophila melanogaster.* Studies conducted by Dhasmana *et al.,* (2021) showed that zebrafish Wnt3 has lipid modifications at its conserved cysteine (C80), which is essential for regulating its secretion and activity in neural cells. Ooyen *et al.,* (1985) were first to highlight that a salient feature of mouse Wg was the high content of cysteine residues present, that were often found in pairs near the COOH terminus, which I have also found in my work. Further investigations into non-conserved cysteines and other key amino acids such as serine residues, would help to explore differences that are present among Wnt ligands and if particular ligands are modified differently, contributing to our understanding of Wnt ligand dynamics.

6.1.2. The roles of intrinsically disordered regions

Intrinsic protein disorder is defined by the lack of three-dimensional structures (Wright and Dyson, 2015). Intrinsically disordered proteins (IDPs) are important components of the cellular signaling network, as they allow for the same polypeptide to undertake different interactions (Wright and Dyson, 2015). These domains are therefore thought to have an important role in protein-protein interactions due to their conformational flexibility altering their interactions

with other proteins depending on different environmental conditions (Shimizu *et al.,* 2009). The protein models produced in chapter 3 indicated the presence of globular protein domains in *Drosophila* Wnts that were absent from most other species surveyed. When investigating these domains using Anchor and IUPRED tools, these domains were predicted to be intrinsically disordered. Each of the seven *Drosophila* Wnts appeared to possess such a disordered region at different positions in the protein with the exception of Wnt5/DWnt3. The roles played by these disordered proteins is not clear, and requires further exploration.

Drosophila Wnt ligands are particularly interesting as it appears that they have evolved novel specific disordered regions over time. Mosca *et al.,* (2012) suggest that conservation of disordered protein regions provide an evolutionary advantage due to conferring the ability to alter protein interactions via binding to several different partners. Dm-Wg may have an increased capacity to interact with a number of different proteins, and therefore, this may help explain how this protein has evolved to in involved in a huge number of different roles in *Drosophila* and make it more difficult for even an ancient paralog, like Wnt6, that lacks this domain to functionally replace it. However, this domain does appear in more distantly related *Drosophila* species such as *Drosophila virilis* and the predicted Wg sequence of another higher dipteran *Musca domestica* also has an insertion, albeit shorter, like *Aedes* Wg, indicating a possible advantage of this region within Dipterans.

Disordered domain functions have been described in the literature for example Hsiao *et al.,* (2014) evaluated the interactions *Drosophila* Ultrabithorax (Ubx) forms with other proteins as this protein contains both structured and disordered regions. They found that *Ubx* disordered regions are required for binding other proteins. They hypothesised that one of the roles the

disordered regions of *Ubx* could be to coordinate multiple molecular functions to respond to cues from specific tissues (Hsiao *et al.,* 2014). The findings of Rogers *et al.,* (2021) shed further light on the potential role intrinsically disordered regions are playing during cell signalling. They tested if *Abi* mutants lacking the disordered regions could rescue adult flies that were heterozygous for *Abi* null. They found that the disordered region of the non-receptor tyrosine kinase- Abelson (ABl) is essential for Abl's to function at multiple steps in *Drosophila* morphogenesis. The findings of this chapter therefore begin to question the possible roles *Drosophila* disordered regions play in cell signalling and highlight future directions to explore to better understand Wnt function (Rogers *et al.,* 2021).

Findings in chapter 3 provide evidence that the previously described 85 amino acid domain in *Drosophila* Wg, and large inserts within the other Wnt ligands appear to be disordered in structure. In order to understand the properties and functions associated with these region, further investigations are required. Within the last two decades there has been an increase in literature describing the involvement of intrinsically disordered proteins in a variety of biological processes, however, complications due to their dynamic behaviour and the tendency to switch between order and disorder under different conditions make the study of these regions particularly difficult (Necci *et al.,* 2021). To expand on preliminary findings in chapter 2, investigations into the behaviour and function of disordered regions in *Drosophila* Wnt ligands is imperative. In order to achieve this experimentally, intrinsically disorder proteins can be identified by physiochemical methods such as NMR spectroscopy (Lieutaud *et al.,* 2016). Furthermore, computational analysis of intrinsic disorder, will allow for potential functional regions to be identified, a throughput predictor method known as DisoRDPbind was introduced for prediction of multiple functions of disordered regions that can be used to predict the RNA-

, DNA-, and protein-binding residues located in IDRs of the input protein sequences. If applied to Wnt ligands, this would aid in understanding protein interactions mediated by Wnt intrinsically disordered regions with other proteins (Peng *et al.,* 2017).

6.2. Chapter 4

In chapter two, I explored the capacity of Wnt6 to rescue Wg. These experiments showed that Wnt6 was unable to rescue Wg function in *Drosophila*, indicating even the most closely related Wnt subfamilies from the same species are functionally different and not just redundant or readily interchangeable. Furthermore, the Wg/Wnt6 chimeric experiments showed that these domains alone are not sufficient to confer Wg functionality on Wnt6.

6.2.1. Wnt6 cannot replace Wg in Drosophila

I have demonstrated that DWnt6 cannot functionally replace DWg even when it is expressed from the endogenous locus like the control NRT-Wg, which I corroborated could rescue Wg function even though it is tethered to the source cells (Alexandre *et al.,* 2012). The rescue using Wnt6 sought to attempt a global rescue of all DWg functions during *Drosophila* development. However, as previously mentioned, it remains possible that Wnt6 or the chimerics still rescue Wg function in specific tissues. I began to explore this but more time should be dedicated to this using specific GAL4 drivers to flip out *Dm-wg* in the leg or wing imaginal discs to see if any rescue by Wnt6 or the chimerics can be observed. An extension of this could include testing other *Drosophila* Wnts to see if any have the capacity to function "like" Wg in specific tissues. This rationale was used by Limargas and Lawrence (2001) in over-expression experiments to test if other Wnt ligands could substitute for Wg in the developing tracheal system. They found

that DWnt2 rescued some dorsal trunk trachea, but it could not rescue other embryonic defects in *wg* mutant embryos.

Findings from Limargas and Lawrence, (2001) combined with my attempted paralog rescue findings indicate that protein-protein interactions likely play a vital role in the ways Wg ligand specificity occurs. It is highly likely that certain interactions have evolved specific to individual Wnt ligands, and therefore when trying to replace one for another, rescues are not permissible. It is important to note that some *Drosophila* Wnts have almost identical patterns in several tissues (Janson *et al.,* 2001). Such as *Wnt6* and *wg* have identical expression patterns in the wing imaginal discs (Janson *et al.,* 2001). Furthermore*, wg, Wnt6, Wnt9* and *Wnt10* are found in an evolutionary conserved cluster (Nusse, 2001) that contains scattered cis-regulatory elements that are able to regulate distal and proximal genes in this cluster which suggests they have overlapping roles (Koshikawa *et al.,* 2015; Holzem *et al.,* 2020). Therefore, where different Wnts are expressed in the same cells of a tissue they could be involved in specific protein-protein interactions and it is the overall output of this 'Wnt landscape' that is required for correct development. However, there are examples of no detectable effects when removing most Wnts (e.g Limargas and Lawrence, 2001; Yu *et al.,* 2020; Ewen-Campen *et al.,* 2020).

To expand this work further, it will be important to better understand how Wnt ligands interact with other proteins. Protein interactions play a significant role in finding the molecular function of a protein, other proteins associated with the target protein and cluster of similar function genes or proteins (Dahiya *et al.,* 2019). The work of Dahiya *et al.,* (2019) investigated Wnt3A, Wnt5A and Wnt7B and associated proteins involvement in neural tube development and defects to identify protein complexes. They identified Wnt3A, Wnt5A and Wnt7A were

predicted to be interacting with 18, 17 and 11 proteins, respectively, and that the identified hub proteins can thus be projected at drug targets for different neural development disorders like autism and learning disorders. Tsting protein- protein interactions of other pairs of Wnt ligand beyond Wnt6 and Wg in *Drosophila* such as Wnt9 (DWnt4) and Wnt10, would aid in understanding the complexity of Wnt protein interaction behaviour. To further investigate protein-protein interactions experimentation using the following methods could be considered: protein affinity chromatography, affinity blotting, co-or immunoprecipitation (Lin and Lie, 2017; Jarmoskaite et al., 2020). Vallon *et al.,* (2018) used chemical cross-linking and affinity chromatography to characterise the binding partners Wnt7. They were able to demonstrate that canonical Wnt7 signalling is specifically regulated by interacting with the RECK complex.

6.2.2. *Drosophila* Wnt ligands interacting with Frizzled proteins

frizzled (fzd) genes *encode* seven-pass transmembrane G-protein coupled receptor proteins that are essential Wnt receptors for beta-catenin dependent and independent signalling (MacDonald *et al.,* 2012). Fzd receptors contain an N-terminal cysteine rich domain that is involved in Wnt ligand recognition (Schenkelaars *et al.,* 2015). Ten FZDs have been identified in most vertebrates, whereas *Drosophila* has four Fzds (Wu & Nusse, 2002; Schenkelaars *et al.,* 2015).

Janda *et al.,* (2012) demonstrated how *Xenopus* Wnt8 forms a complex with mouse Fzd8CRD, via palm domain (see Chapter 1) extending to hold onto the Fzd8CRD globular structure. Janda *et al.,* (2012) found evidence that specific Wnt/Fz affinity differences may exist, - therefore a given Fzd could respond with different signalling amplitudes to different Wnt ligand with different affinities, therefore demonstrating binding preferences among receptor and ligands.

Wu and Nusse, (2002) demonstrated that different binding affinities exist between *Drosophila* Fzds and different Wnt ligands. They showed that Wg binds to three of the Fzd proteins: Fz, Dfz2, and Dfz3, but has highest affinity for Dfz2. Interestingly, Wnt7 (DWnt2) also binds to the same three Fzd proteins- Fz, Dfz2, and Dfz3 but with approximately the same affinity. Whereas, Wnt9 (DWnt4) binds to Fz, Dfz2, and Dfz4, and Wnt5 (DWnt3) does not bind to any Fzds. The different affinities further evidence that Wnts act in a specific manner. Furthermore, Boutros *et al.,* (2002) demonstrated that although Fz and Fz2 can both activate the beta-catenin and planar polarity pathways, they do so with different efficiencies. This indicates that differences in signalling efficiency among receptors is also a mechanism for generating signalling specificity. More recently, using both structural knowledge and experimental data Agostino *et al.,* (2017), determined binding affinities for a selection of Wnt-Frizzled CRD interactions. This was achieved by generating homology models of Wnt-Frizzled CRD interactions. Future work could therefore strive to investigate the specificity between Wnt, Fzd and the LRP5/6 complex among a wider array of Wnt orthologs, due to the combinatorial nature of potential Wnt-Fzd interactions Agostino *et al*., (2017). In addition to this, a more systematic approach to produce chimeric ortholog proteins could be implemented. In future chimeric designs it will be essential to consider the addition of suitable linkers, as often is it observed in nature that protein domains connect through short stretches of amino acids referred to as linkers (Kortt *et al.,* 2001). The presence of a domain linker would allow suitable space between protein domains, which will decrease their intrusion, and improve folding and enhance the activity.

6.3. Chapter 5

In chapter five, I explored the rescue capacity of Wg orthologs from other species in *Drosophila*. This suggested that orthologs of Wg are unable to rescue Wg function, and there has been evolution of protein- protein interactions. However, this requires further testing to confirm the capacity for rescue and perhaps also testing orthologs from other species even more closely related to *D. melanogaster* such as other Diptera. This could be investigated through the use of ancestral protein reconstruction. For example, Liu *et al.,* (2018) reconstructed ancestral forms of Bcd to dissect the genetic and evolutionary trajectory of Bcd's functional evolution. This strategy allowed the change in Bcd function to be mapped to a specific phylogenetic branch, quantify the biochemical and developmental aspects of that change, and characterize the extent to which historical amino acid substitutions were necessary and sufficient to account for the evolution of Bcd-specific functions.

6.3.1. Future directions to understand Wnt ligand specificity and functionality

Drosophila has proved to be an efficient and economic model organism for studying Wnt ligand functionality, due to the ability to manipulate genes and re-introduce foreign genes and chimeras, as well as the ability to test rescue in a tissue specific context. However, during the course of this work there are several areas that could be addressed to provide a better understanding of the mechanism that allow for Wnt ligand specificity and evolution. The findings indicate paralogous and orthologous Wnt ligands are not capable of rescuing Wg function. This highlights there must be other components that are at play that are essential for Wg function. Therefore, further detailed dissection of the amino acids and sequences that are unique to *Drosophila* Wg should be carried out. For example, through the use of a new descriptor described by Abo-Elkhier et al., (2019), which allows for an alignment free method

for protein sequences, by representing amino acids as numbers, this method eliminates degeneracy and prevents loss of information. This would allow for a breakdown of the key features of Wg and coupled with methods to map protein-protein interactions this would facilitate treading into the realms of designing synthetic Wnts to test predictions. Machine learning approaches are being used to understand protein structure evolution, probability density models that use evolutionary data can be used to model and predict how to modify proteins to improve function (AlQuraishi, 2021). This approach could be used in combination with functional analysis to help identify specific domains required for signalling ligand function.

Wnt signalling is implicated in most physiological processes, but also plays a role in various cancers in which Wnt signalling is perturbed. By understanding which domains are key to Wnt function, there is great opportunity to design synthetic Wnt inhibitors that could be utilised in the treatment of diseases including cancers.

6.3.2. Conclusions

My investigation has broadly contributed to our knowledge of Wnt protein structure and functionality, and in my opinion has highlighted some key ideas for future Wnt protein studies:

- Alphafold modelling provides more in-deepth protein models when compared to modelling with Swiss-mod.
- Modelling more closely related species to *Drosophila* would help to reveal the emergence of the disordered protein region.
- Analysing protein-protein interactions could greatly improve our knowledge on Wnt disorder region function.
- Finally, the ultimate goal would be to find the bare minimum requirements of Wnt ligands and to construct synthetic constructs and test them in vivo.
- The work undertaken in this investigation sheds light on the structure and function of *Drosophila* Wnt ligands, but highlights that in order to understand Wnt functionality in greater depth a greater exploration of protein domain function is essential.

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Appendix

Table 2: Summary of Wnt sequences and accession numbers

Species	Accession number	Annotated Wnt
Drosophila melanogaster	NM_078778.3	*Wnt1 (Wg)*
Drosophila melanogaster	NM_057462.3	*Wnt7 (DWnt2)*
Drosophila melanogaster	NM_057576.3	*Wnt5 (DWnt3)*
Drosophila melanogaster	NM_135264.2	*Wnt6*
Drosophila melanogaster	NM_135264.2	*Wnt 8 (DWnt8)*
Drosophila melanogaster	NM_057624.2	*Wnt9 (DWnt 4)*
Drosophila melanogaster	NM_135265.2	*Wnt10*
Nematostella vectensis	AY530300.1	*Wnt1*
Nematostella vectensis	AY725201.1	*Wnt2*
Nematostella vectensis	DQ492689.1	*Wnt3*
Nematostella vectensis	AY725203.1	*Wnt6*
Nematostella vectensis	AY687350.1	*Wnt7*
Nematostella vectensis	AY792510.1	*Wnt8*
Nematostella vectensis	AY725205.1	*Wnt8b*
Nematostella vectensis	AY530301.1	*Wnt10*
Parasteatoda tepidariorum	AB167808.1	*wg*
Parasteatoda tepidariorum	AB167813.1	*Wnt2*
Parasteatoda tepidariorum	HQ650544	*Wnt4*
Parasteatoda tepidariorum	AB167810.1	*Wnt5*
Parasteatoda tepidariorum	HQ650545	*Wnt6*
Parasteatoda tepidariorum	AB167809.1 AB167811.1	*Wnt7-1* *Wnt7-2*
Parasteatoda tepidariorum	FJ013049.1	*Wnt8*

Platynereis dumeilii	AJ491796.1	*wg*
Platynereis dumeilii	AJ491797.1	*Wnt2*
Platynereis dumeilii	AJ491798.1	*Wnt4*
Platynereis dumeilii	HM179275.1	*Wnt5*
Platynereis dumeilii	HQ413681	*Wnt6*
Platynereis dumeilii	HQ413682	*Wnt7*
Platynereis dumeilii	HQ413682	*Wnt8*
Platynereis dumeilii	AJ491799.1	*Wnt9*
Platynereis dumeilii	AJ491800.1	*Wnt10*
Tribolium castaneum	NM_001114350.1	*wg*
Tribolium castaneum	XM_969591.1	*Wnt5*
Tribolium castaneum	NM_001170666.1	*Wnt6*
Tribolium castaneum	XM_968066.1	*Wnt7*
Tribolium castaneum	XM_966346.1	*Wnt8*
Tribolium castaneum	XM_962805.1	*Wnt9*
Tribolium castaneum	XM_963117.2	*Wnt10*
Bicyclus anynana	BANY.1.2.t03758	*Wnt1*
Bicyclus anynana	BANY.1.2.t05290	*Wnt7*
Bicyclus anynana	BANY.1.2.t00712	*Wnt5*
Bicyclus anynana	BANY.1.2.t03759	*Wnt6*
Bicyclus anynana	nBa.0.1-t07006-RA	*Wnt9*
Bicyclus anynana	BANY.1.2.t03771	*Wnt10*
Mus Musculus	NM_021279.4	*Wnt1*
Mus Musculus	NM_023653.5	*Wnt2*
Mus Musculus	NM_009521.2	*Wnt3*
Mus Musculus	NM_009523.2	*Wnt4*

Mus Musculus	XM_006495887.4	*Wnt6*
Mus Musculus	22421 22422	*Wnt7a* *Wnt7b*
Mus Musculus	14370	*Wnt8*
Aedes aegypti	EAT37515.1	*wg*
Aedes aegypti	XP_021700182.1	*Wnt5*
Aedes aegypti	XP_021703000.1	*Wnt6*
Aedes aegypti	XP_021702998.1	*Wnt9*
Aedes aegypti	XP_001649191.2	*Wn10*
Drosophila hydie	XM_023319349.2	*wg*
Drosophila simulans	XP_016023932.1	*wg*
Drosophila mauritiana	XP_033152227.1	*wg*
Musca_domestica	XP_058976755.1	*wg*
Drosophila_pseudoobscura_	XP_001356396.2	*Wg*

Table 3:Primer sequences used for genomic verification of plasmid integration

62	**Fwd noncod wg locus**		**ATATAGCGGTGCTCTTCTG**
63	Rev RIV8 start		aataggaacttcgtCGTACG
64	Rev RIV8 middle		ttggccttagtcgggtac
65	Fwd RIV8 end		gtcgtattgagtctgagtgagacag
66	Rev Intron1 wg locus		GCTTCCGCATTTGGTGCGTG
67	Rev Exon2 wg locus		GTTCGCCGACCTTGGCAATG
68	M13 Fwd long		tgtaaaacgacggccagtgtcg
69	Fwd RIV8 middle		cgaacggattttcgtagaccct

A: Cross 1

X	MKRS	x	+	KB19-FLP ♂
CyO	TMB6		+	KB19-FLP

X	KB19-FLP
+	TM6B

B: Cross 2

X	KB19-FLP	x	if	MKRS
+	TMB6		CyO	TM6B

X	MKRS
CyO	TM6B

C: Cross 3

X	MKRS	x	X	MKRS
CyO	TM6B		CyO	TM6B

X	MKRS
CyO	TM6B

Supplementary Figure 43:Genetic Crossing scheme to flip DWg out with the desired transgenic.

All the crosses need to be completed to attempt a rescue with the desired transgenic**. A) X** = the desired construct that will be rescuing DWg, such as NRT-Wg, Wnt6 or any of the Wg orthologs is crossed to males of the germ line flippase line KB19-FLP. **B)** In the second cross the progeny is then crossed to a double balancer, this yields the transgenic over the CyO marker, with a double balancer located on the third chromosome. The presence of the desired transgenic can be checked via the presence of the mcherry marker. **C)** The final cross requires siblings from cross two that both express mCherry to be crossed to establish a stock. Once a stock is established the sibling parents can be genetically verified for the Wg flip.