Interaction studies of *Drosophila* transcription factors Daughterless and Schnurri

Master's thesis

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Drosophila transkriptsioonifaktorite Daughterlessi ja Schnurri interaktsioonide uurimine

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Declaration

Hereby I declare that I have compiled the paper independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not been previously been presented for grading.

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Contents

Abbreviations

AD1, AD2 and AD3	activation domain 1, 2 and 3
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
Brk	Brinker
CNS	central nervous system
Da	Daughterless
DMEM	Dulbecco's Modified Eagle Medium
Dpp	Decapentaplegic
E-box	Ephrussi box
Emc	Extramacrochaete
h	hour
HEK	Human embryonic kidney cells
HEK-FT	Human embryonic kidney cells
HIVEP	Human immunodeficiency virus type I enhancer-binding protein
HLH	helix-loop-helix
kDa	kilodaltons
Mad	Mothers against Dpp
MATH1	Mammalian atonal homolog 1
Med	Madea
MEM	Eagle’s Minimum Essential Medium
min	minute
NF-κB	Nuclear factor κB
NLS	nuclear localisation signal
PBS	phosphate-buffered saline
PBST	PBS and 0.1% Triton X-100
PEI	polyethyleneimine
PNS	peripheral nervous system
PTHS	Pitt-Hopkins syndrome
Put	Punt
S2	Schneider cells
Shn	Schnurri
SNP	single nucleotide polymorphism
SSTR2	Somatostatin receptor type 2
TCF4	Transcription Factor 4
Tkv	Thick veins
Ubx	Ultrabithorax

Introduction

Transcription factor 4 (TCF4) is a basic helix-loop-helix transcription factor that has a crucial role in nervous system development, including neural migration, differentiation of neurons, synapse formation and neurite branching. Human immunodeficiency virus type I enhancer-binding protein 2 (HIVEP2) is a zinc finger transcription factor that regulates the activity of many genes, linked to brain development. TCF4 and HIVEP2 have both homologs in *Drosophila melanogaster*. TCF4 and HIVEP2 are homologous to Daughterless (Da) and Schnurri (Shn), respectively. Mutations in *TCF4* cause Pitt-Hopkins syndrome and mutations in *HIVEP2* cause HIVEP2-related intellectual disability. Both diseases are characterized by intellectual disability, motor delay, delayed speech development, behavioral abnormalities, and dysmorphic features. Previous experiments have shown that TCF4 and HIVEP2 interact, suggesting that mutations within *TCF4* and *HIVEP2* may cause dysregulation of the same molecular pathways.

In this thesis, we aimed to study the interactions of TCF4 and HIVEP2 fly homologs Da and Shn using fruit fly as a model organism. Studying these proteins will help us better understand the connections between TCF4 and HIVEP2-associated diseases. Given this, the first aim of the thesis was to describe if Da and Shn are expressed at the same developmental time points in *Drosophila* nervous system, by performing Western blot analysis. The second aim of the thesis was to investigate if Da and Shn are also co-expressed in adult *Drosophila* brain by performing immunohistochemical staining. The third aim of the thesis was to study TCF4 and HIVEP2 as well as Da and Shn interactions in mammalian cell lines (HEK293) by performing co-immunoprecipitation analysis. For this, we needed to clone Shn mammalian expression vector. The final aim of the thesis was to investigate whether Shn affects the transcriptional ability of Da.

The thesis consists of the literature overview, materials and methods, results, and discussion. The literature overview gives information on TCF4 and HIVEP2, describing their structure, expression, function and associated diseases. In addition, the structure, expression, and functions of *Drosophila* homologs Da and Shn, and also a brief overview of *Drosophila melanogaster* as a model organism for Pitt-Hopkins syndrome are given.

In this Master's thesis, it was shown that Da and Shn are expressed at the same time period in 3rd instar larvae and adult *Drosophila* brain. We also showed that Da and Shn are partially co-expressed in adult *Drosophila* brain. We were able to clone mammalian Shn expression vector and validated its functionality in HEK293-FT cells. By studying TCF4 and HIVEP2 as well as Da and Shn interactions in HEK293 cells we were able to confirm that when overexpressed, TCF4 and HIVEP2 and their fruit fly homologs Da and Shn interact in HEK293 cells. Finally, we showed that overexpression of Shn causes a decrease in Da transcriptional activity in adult *Drosophila* brains.

Overview of the literature

1.1 Transcription factor 4

Transcription factor 4 (TCF4), also known as E2-2, Immunoglobulin transcription factor 2 (ITF2), or SL3-3 enhancer factor 2 (SEF2), belongs to a family of class I basic helix-loop-helix (bHLH) transcription factors, also called E-proteins (Murre et al., 1994). E-proteins also include Transcription factor 3 (TCF3 or E2A) and Transcription factor 12 (TCF12 or HEB), as well as the *Drosophila* homolog Daughterless (Da). E-proteins bind directly to the Ephrussi box (E-box) sequence CANNTG in the promoters and enhancers of specific genes, allowing them to regulate gene expression (reviewed by Massari and Murre, 2000).

1.1.1 TCF4 gene and protein structure

The human *TCF4* gene is located on chromosome 18 and encodes 18 transcripts with unique N-terminal sequences. The gene has 41 exons (20 alternative 5' exons, 20 internal coding exons, 1 3' terminal non-coding exon). The use of numerous alternative 5' exons leads to the expression of many transcripts of the *TCF4* gene, resulting in a variety of TCF4 protein isoforms (TFC4-A to TCF4-R). The diversity of isoforms is further increased by alternative splicing of multiple internal exons. All TCF4 isoforms contain transactivation domains (AD2 and AD3), a repression domain (Rep), and a C-terminal bHLH domain (Figure 1) (Sepp et al., 2011). The bHLH domain contains a region of basic residues that allows the protein to bind the E-box and a region of hydrophobic residues (HLH domain) that allows dimerization with other helix-loop-helix proteins (Figure 1) (Murre et al., 1989; Massari and Murre, 2000). Only the isoforms with the longest N-terminus have the transactivation domain AD1 and the nuclear localization signal (NLS) (Figure 1). Isoforms containing the NLS are localized in the nucleus, whereas isoforms lacking the NLS rely on binding to other bHLH proteins that contain the NLS (Figure 1) (Sepp et al., 2011). The bHLH domain has been shown to contain additional NLS domain and two nuclear export signals (NES) (Greb-Markiewicz et al., 2019). The region between AD2 and bHLH is critical for depolarization-mediated TCF4 activation in rat primary neuronal culture (Sepp et al., 2017). TCF4 isoforms also contain the conserved element (CE) domain, which represses AD1 activity and Rep domain that represses both AD1 and AD2 activity (Figure 1) (Markus et al., 2002; Herbst and Kolligs, 2008).

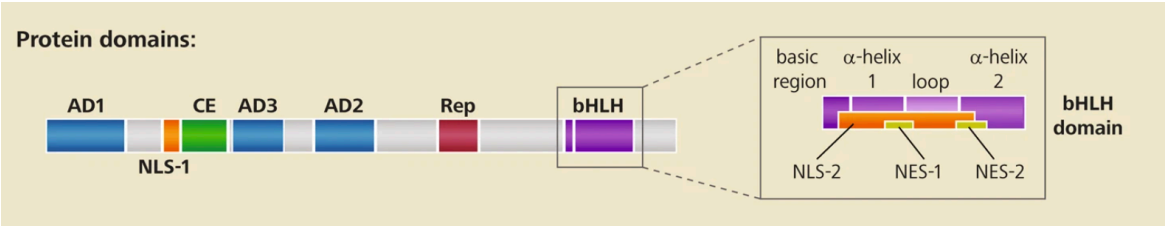

Figure 1. Schematic representation of the TCF4 protein domains. Blue boxes represent transactivation domains (AD1, AD2, AD3). Red box represents repression domain (Rep). Green box represents conserved element (CE). Orange box represents nuclear localization signal (NLS). Purple box represents basic helix-loop-helix domain (bHLH). Box on the right shows the composition of the bHLH domain, including the basic region helix-ling-helix domain, nuclear export signal (NES) (Teixeira et al., 2021).

1.1.2 Expression of *TCF4* mRNA and protein

In humans, *TCF4* transcripts are broadly expressed, but their expression levels vary greatly between tissues. *TCF4* expression is particularly high in the brain, being the highest in fetal brain and adult cerebellum (de Pontual et al., 2009; Sepp et al., 2011; Sirp et al., 2022). In the developing rodent brain, the expression of TCF4 protein expression is highest in the cerebral cortex and hippocampus around birth and in the cerebellum 1-2 weeks after birth (Sirp et al., 2022). In the adult mouse brain, high TCF4 protein expression can be detected in the hippocampus, cortex, and cerebellum (Brzózka et al., 2010; Chen et al., 2016; Jung et al., 2018; Sirp et al., 2022). In the juvenile and adult mouse brain TCF4 protein level is high in most cortical and hippocampal cells, including astrocytes, excitatory and inhibitory neurons, and oligodendrocytes (Kim et al., 2020).

1.1.3 Dimerization partners of TCF4

TCF4 forms heterodimers with class II bHLH transcription factors, including proneural proteins, to regulate gene expression during neurodevelopment. TCF4 forms a functional DNA-binding complex with Mammalian Atonal Homolog 1 (MATH1) to regulate differentiation of pontine neurons (Flora et al., 2007). Using neuroblastoma cell lines, it has been shown that TCF4 is the major interacting protein of Achaete-Scute homolog-1 (ASCL1) and this TCF4/ASCL1 complex binds to the E-box *in vitro* (Persson et al., 2000). TCF4 interacts with Neurogenic Differentiation factor 2 (NEUROD2), which plays an important role in neuronal differentiation and survival (Brzózka et al., 2010). The complex of TCF4 and Oligodendrocyte transcription factor 2 (OLIG2) regulates the differentiation of oligodendrocytes (Wedel et al., 2020). Inhibitor of DNA binding proteins (ID1 and ID2) are dominant-negative regulators of TCF4 that form inactive heterodimers with TCF4 and prevent it from binding E-boxes or interacting with other transcriptional activators (Massari and Murre, 2000).

1.1.4 Functions of TCF4

TCF4 is important for neural development but also it has functions in non-neural tissues. TCF4 plays an important role in immune system. TCF4 has shown to regulate plasmacytoid dendritic cell development and B and T lymphocyte development (Zhuang et al., 1996; Wikström et al., 2008). TCF4 is also important for sertoli cell development (Muir et al., 2006) and for the epithelial-mesenchymal transition (Sobrado et al., 2009). TCF4 is also involved in calcium signaling by interacting with calmodulin, which results in inhibition of TCF4 binding to DNA (Corneliussen et al., 1994). The role of TCF4 in brain development has been studied in mouse models, where it has been found that homozygous *Tcf4* null mutant mice do not survive longer than 1 week after birth (Zhuang et al., 1996). In addition, heterozygous and homozygous *Tcf4* mutations cause impaired hippocampal and cortical morphology (Crux et al., 2018; Schoof et al., 2020). More specifically, neural morphology of *Tcf4* knockout mice is affected, characterized by shorter apical dendrites and increased branching of dendrites (Crux et al., 2018; Schoof et al., 2020). *Tcf4* haploinsufficient mice exhibit deficits in social interaction, associative learning and memory formation, hyperactivity, reduced anxiety, motor and balance asymmetry, and enhanced long term potentiation (Kennedy et al., 2016; Thaxton et al., 2018; Li et al., 2019). Furthermore, adult mice moderately overexpressing TCF4 in the brain display deficits in sensory-motor gating and cognitive performance (Brzózka et al., 2010). In developing brain TCF4 is also important for migration of pontine nucleus and cortical neurons (Flora et al., 2007; Chen et al., 2016), differentiation of neurons (Forrest et al., 2013), cell

proliferation (Hill et al., 2017), formation of prefrontal cortical minicolumns (Page et al., 2018), synapse formation and neurite branching (D'Rozario et al., 2016). In adult brain, TCF4 is required for synaptic plasticity (Kennedy et al., 2016).

1.2 TCF4 associated diseases

Aberrations within *TCF4* cause Pitt-Hopkins syndrome (PTHS), mild-moderate intellectual disability (MMID) (Kharbanda et al., 2016; Maduro et al., 2016) and Fuchs' endothelial corneal dystrophy (FECD) (Wieben et al., 2012). In addition, small nuclear polymorphisms within *TCF4* are linked to the generation of schizophrenia (Stefansson et al., 2009), autism (Stessman et al., 2017), post-traumatic stress disorder (Gelernter et al., 2019), major depression (Wray et al., 2018), and cancer (Kolligs et al., 2002).

1.2.1 Pitt-Hopkins syndrome

Rare mutations in *TCF4* cause Pitt-Hopkins syndrome (PTHS) (Amiel et al., 2007; Brockschmidt et al., 2007; Zweier et al., 2007). PTHS is a rare neurodevelopmental disorder first described in 1978 and is characterized by intellectual disability, developmental delay, breathing anomalies (hyperventilation, apnoea), microcephaly, absent or limited speech, motor delay, hypotonia, gastrointestinal problems, seizures, and distinct facial features (deep-set eyes, wide mouth, broad nasal base). Behavioral problems include anxiety, stereotypic movements, and agitation. Brain abnormalities include underdevelopment of the corpus callosum, small hippocampus, enlarged caudate nuclei, and frontal lobe hypoplasia (Zweier et al., 2008; Marangi et al., 2011; Whalen et al., 2012). PTHS is caused by *de novo* mutations in one allele of *TCF4*, resulting in TCF4 haploinsufficiency (Zweier et al., 2007). Some studies have also identified individuals carrying rare mosaic deletions of TCF4 (Kousoulidou et al., 2019).

A variety of mutations have been identified within the *TCF4* locus that cause PTHS, including missense mutations, splice site mutations resulting in frameshift, nonsense and small indel mutations resulting in premature stop codon, as well as large deletions and translocations in the *TCF4* gene (Whalen et al., 2012). Missense mutations generally occur within the bHLH domain and affect all isoforms. Missense mutations in the bHLH domain have shown to affect protein stability, DNA-binding activity, subnuclear localization, transactivation ability and dimerization preferences (Forrest et al., 2012; Sepp et al., 2012; Sirp et al., 2021). Deletions in the proximal region of the *TCF4* gene affecting only longer protein isoforms have been described in patients with mild nonsyndromic intellectual disability (Kharbanda et al., 2016).

1.2.2 Schizophrenia

Schizophrenia is a mental disorder characterized by delusions, paranoia, hallucinations, and cognitive defects (Lewis and Lieberman, 2000). Several single nucleotide polymorphisms (SNP) in or near *TCF4* have been associated with increased risk in schizophrenia (reviewed by Navarrete et al., 2013). The first SNP discovered in the *TCF4* gene was rs9960767, which is located in intron three of the gene (Stefansson et al., 2009). Later, several other SNPs have been discovered: rs17512836, rs4309482, rs2958182, rs147445499 (reviewed by Navarrete et al., 2013). TCF4 schizophrenia-risk

SNPs are also associated with early information processing and cognitive markers, some of which are endophenotypes of schizophrenia. For example, some schizophrenia-risk SNPs have been associated with poor verbal fluency and lower reasoning and problem-solving performance compared to controls (Lennertz et al., 2011; Albanna et al., 2014). Moreover, pre-pulse inhibition (PPI), the measure of sensorimotor gating, is significantly reduced in patients carrying the schizophrenia risk variant rs9960767 (Quednow et al., 2011). PPI and fear memory formation are also impaired in transgenic mice overexpressing *Tcf4* (Brzózka et al., 2010). Recent research suggests that rare variants at the *TCF4* locus may also contribute to schizophrenia risk, as some of the missense variations found in schizophrenia patients slightly increase TCF4 transcriptional activity in neurons (Basmanav et al., 2015; Sepp et al., 2017). *TCF4* transcript levels have been shown to be increased in blood cells and in neurons derived from induced pluripotent stem cells from schizophrenia patients (Brennand et al., 2011; Wirgenes et al., 2012). *TCF4* is also regulated by the microRNA miR-137, which has also been liked to schizophrenia (Wright et al., 2013).

1.3 Daughterless, the fruit fly homolog of TCF4

1.3.1 Gene and protein structure of Daughterless

Da is the only E-protein in *Drosophila melanogaster* that belongs to the family of class I bHLH proteins and is homologous to mammalian E-proteins, sharing the highest similarity with TCF4 (Tamberg et al., 2015). The *da* gene is located on the 2L chromosome and encodes three transcripts (Caudy et al., 1988; Cronmiller et al., 1988). All three transcripts encode a single polypeptide of 710 amino acid residues with a molecular weight of 74 kilodaltons (kDa) (Caudy et al., 1988; Cronmiller et al., 1988).

Da protein contains a basic region that allows the protein to bind to the E-box (CANNTG) target sequence and a HLH region that allows dimerization with other helix-loop-helix proteins (Figure 2) (Cronmiller et al., 1988). Amino acid identity between Da and human E-proteins is highest in the bHLH domain, 35,54% (Tamberg et al., 2015). Two activation domains important for peripheral neurogenesis have been described for Da: activation domain 1 (AD1), located in the N-terminus of the protein, and loop-helix domain (LH), located in the middle (Figure 2) (Zarifi et al., 2012). Da has also a repression domain (REP) that is shown to be required for Twist/Da-mediated transcriptional repression of myogenic genes (Wong et al., 2008). REP is located 21 amino acids N-terminal to the bHLH domain and shares 16% homology with the mammalian E-protein repression domain (Rep) (Wong et al., 2008).

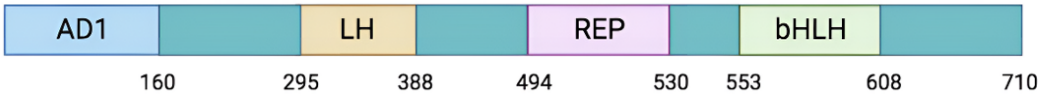

Figure 2. Schematic drawing of Da protein structure. Blue box represents the activation domain (AD1), yellow box loop-helix domain (LH), pink box repression domain (REP) and green box basic helix-loop-helix domain (bHLH).

1.3.2 Expression of Daughterless

Da is expressed at different levels throughout the development of the fruit fly (Cronmiller and Cummings, 1993; Tamberg et al., 2020). Da is present at the preblastoderm stage and disappears before blastoderm formation. Expression increases again before germband extension and reaches its maximum when most neural precursors form. At this stage, Da is present in ectodermal cells and in putative neuroblasts during delamination. At later stages, higher levels of Da protein are seen in ventral nerve cord of central nervous system (CNS), salivary glands, and parts of the gut and muscles (Vaessin et al., 1994). Postembryonically, Da expression is highest in the salivary glands, imaginal discs, and CNS. Tamberg *et al*. has characterized Da expression in third instar larval brain, where Da is ubiquitously expressed throughout the larval CNS, including the Kenyon cells of the mushroom body (Tamberg et al., 2020). In adult fruit flies, Da is found in the somatic component of the ovary and in adult male gonads in the testis (Cronmiller and Cummings, 1993; Cummings and Cronmiller, 1994). Da is also widely expressed in adult brain (Tamberg et al., 2020).

1.3.3 Functions of Daughterless

Da participates in numerous developmental processes, including transcriptional activation of Sex lethal (Sxl) to initiate sex determination (Cronmiller and Cline, 1987), myogenesis (Wong et al., 2008), development and differentiation of the photoreceptors within the developing eye (Brown et al., 1996), differentiation of the salivary gland (King-Jones et al., 1999) and cell cycle control (Hassan and Vaessin, 1997). Da also plays an important role in the development of the nervous system as Da is required for the formation of PNS (Caudy et al., 1988). Da is also shown to function in synapse formation, neurite branching, and in associative memory and learning (D'Rozario et al., 2016; Tamberg et al., 2020). In adult *Drosophila*, Da is required for ovarian follicle formation during oogenesis (Cummings and Cronmiller, 1994; Smith et al., 2002).

1.3.4 Dimerization partners and regulation of Daughterless

Like its mammalian homologs, Da can homodimerize or form heterodimers with class II bHLH proneural proteins. Da heterodimerizes with Achaete-Scute complex proteins (Achaete (Ac), Scute (Sc), and Lethal of scute (L'sc)) to enhance the expression of neural precursor genes, thereby allowing the initiation of neuronal development (Cabrera and Alonso, 1991; Vaessin et al., 1994). Da also heterodimerizes with proneural proteins Amos and Atonal, which are required for the formation of multiple dendritic neurons in the peripheral nervous system (PNS) and for the formation of chordotonal organs, respectively (Jarman et al., 1993; Huang et al., 2000). Da also interacts with the transcription factors Nervy and Senseless in a manner that does not involve HLH-HLH interactions (Wildonger and Mann, 2005; Jafar-Nejad et al., 2006). Nervy downregulates and Senseless increases the transcriptional activity of Da.

The Da/Ac-Sc protein complex is negatively regulated by Extramacrochaete protein (Emc; homologue to mammalian ID proteins), which forms heterodimers with Da that are unable to bind DNA due to the lack of basic region of Emc (Van Doren et al., 1991). Da has also been shown to regulate its own expression by enhancing Emc expression, making Emc a negative feedback

regulator that inhibits self-stimulation of *da* transcription (Bhattacharya and Baker, 2011). Other negative regulators are the Enhancer of Split (E(spl)) proteins, that interact with Da heterodimers through AD1 and Orange domains and inhibit Da/Sc activity (Zarifi et al., 2012). *da* regulation has also been studied in ovaries, where the authors have presented evidence of *da* autoregulation and described two cis-acting elements involved in positive and negative regulation of *da* (Smith and Cronmiller, 2001).

1.4 *Drosophila melanogaster* as a model for Pitt-Hopkins syndrome

Tamberg *et al*. has proposed *Drosophila* model for Pitt-Hopkins syndrome. They have shown that introducing PTHS-associated mutations into Da abolished Da transactivation capability *in vivo* resulting in hypomorphic to dominant negative effects, which were also observed when the corresponding mutations were introduced into *TCF4 in vitro* (Sepp et al., 2012). Expression of human TCF4 in *da* null embryos rescues embryonic nervous system development (Tamberg et al., 2015). Downregulation of *da* in larval mushroom bodies impairs memory and learning (Tamberg et al., 2020). Downregulation of its mammalian homolog *TCF4* in the mouse hippocampus has similar effects (Kennedy et al., 2016). In summary, *Drosophila* is a great model for PTHS, allowing us to study the functions of TCF4 using Da.

1.5 Human immunodeficiency virus type I enhancer-binding protein 2

Human immunodeficiency virus type I enhancer-binding protein 2 (HIVEP2) also known as Schnurri-2, MBP-2 (MHC enhancer binding protein), MIBP1 (C-myc intron binding protein), AT-BP1 (α1-antitrypsin binding protein 1), AGIE-BP1 (Angiotensinogen gene-inducible enhancer binding protein) is a zinc-finger transcription factor that binds to the Nuclear factor kB -like DNA sequence motifs (NF-kB , 5'-GGGG(N4-5)CC-3') of regulatory regions of several genes (Mitchelmore et al., 1991; Nomura et al., 1991; Ron et al., 1991; van 't Veer et al., 1992; Makino et al., 1994). HIVEP2 belongs to a family of large zinc finger proteins that includes three members in mammals (HIVEP1, HIVEP2, and HIVEP3) and one in *Drosophila* (Schnurri) (reviewed by Wu, 2018).

1.5.1 Gene and protein structure of HIVEP2

The human *HIVEP2* gene is located on the long arm of the sixth chromosome and encodes a 2500 amino acid protein with a molecular weight of 270 kDa. The HIVEP2 protein contains two clusters of C2H2-type (Cys_2His_2) zinc fingers near the N- and C-terminus of the protein, a serine/threonine-rich sequence, nuclear localization signal and a region of acidic amino acids (Figure 3) (Nomura et al., 1991; Sudo et al., 1992; Makino et al., 1994; Dörflinger et al., 1999).

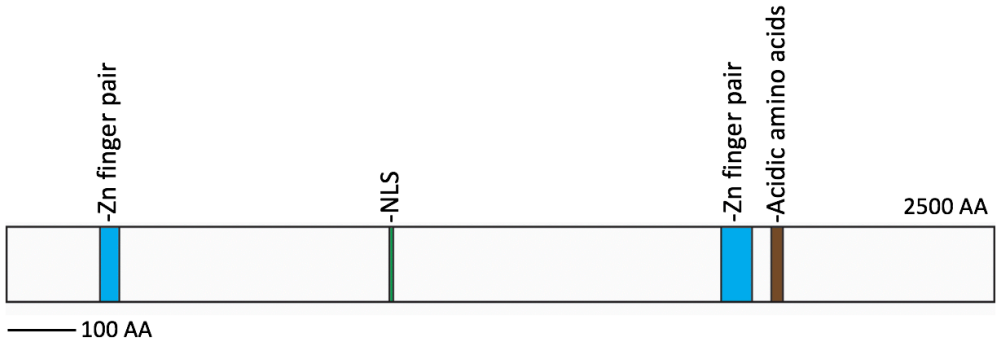

Figure 3. Schematic representation of HIVEP2 protein structure. Blue boxes represent C2H2-type zinc fingers, green box represents nuclear localization signal (NLS), brown box represents region of acidic amino acids.

1.5.2 Expression of *HIVEP2* mRNA and protein

HIVEP2 mRNA expression has been studied in the developing mouse brain, where *HIVEP2* expression restricts to the dorsal telencephalon, with strong expression in cortical plate and subplate. In the early postnatal brain, *HIVEP2* expression has been observed in both telencephalon and thalamus (Campbell and Levitt, 2003). In adult mice, *HIVEP2* is expressed in the brain, heart, spleen, lung, skeletal muscle, liver, kidney, testis, and prostate (Ron et al., 1991; Dörflinger et al., 1999). *HIVEP2* expression is particularly high in the brain (Makino et al., 1994; Campbell and Levitt, 2003). In the adult rat brain, *HIVEP2* mRNA is strongly expressed in the olfactory bulb, cerebral cortex, hippocampus, granule cell layer of the dentate gyrus, and cerebellum (Fukuda et al., 2002). *HIVEP2* is expressed mainly in mature neurons and less in immature neurons and glial cells (Fukuda et al., 2002). In rat embryos, *HIVEP2* expression is also detectable mainly in postmitotic neurons (Fukuda et al., 2002). Zhao and colleagues have shown that HIVEP2 is expressed in both the cytoplasm and nuclei of dopaminergic neurons (Zhao et al., 2019).

1.5.3 Functions of HIVEP2

HIVEP2 has been shown to regulate the activity of many genes that have been linked to brain development. For example, HIVEP2 represses the expression of C-myc, which regulates apoptosis, growth, differentiation, and replication (Fukuda et al., 2002; reviewed by Pelengaris et al., 2002). In addition to C-myc, HIVEP2 represses genes in the NF-kB signaling pathway (Iwashita et al., 2012). NF-kB is a widely expressed transcription factor involved in processes such as immunity, inflammation, and also in various brain functions such as synaptic transmission, neural development, and neural plasticity (O'Neill and Kaltschmidt, 1997; Kaltschmidt and Kaltschmidt, 2009). HIVEP2 has also been shown to interact with TCF4 and activate the Somatostatin receptor type 2 (SSTR2) in neural cell lines (Dörflinger et al., 1999). SSTR2 belongs to a family of G protein-coupled receptors (GPCRs) that mediate the actions of the regulatory peptide Somatostatin (SST) (reviewed: Patel, 1999). During development, SSTR2 regulates neural migration and axonal growth (Le Verche et al., 2009). HIVEP2 expression has been shown to overlap with SSTR2 and TCF4 expression in the hippocampus and frontal cortex (Dörflinger et al., 1999). One study also showed that HIVEP2 binds to the intron 1 of dopamine transporter SLC6A3 and regulates its activity (Zhao et al., 2019). Outside of the nervous system, HIVEP2 plays an important role in cellular immunity by

regulating T cell development (Kimura et al., 2005, 2007; Nakayama and Kimura, 2010). HIVEP2 is required for positive selection of T cells in the thymus (Takagi et al., 2001). In mature T cells, HIVEP2 controls cell survival to support the generation of memory Th1/Th2 (T helper) cells (Kimura et al., 2007). HIVEP2 also regulates NK (natural killer) cell function and is involved in T cell lymphoma development (Yamashita et al., 2012). In addition, HIVEP2 has been shown to regulate bone development (Jones et al., 2010). HIVEP2 is also involved in the bone morphogenic protein (BMP) signaling pathway, for example regulating adipocyte differentiation (Jin et al., 2006).

1.6 HIVEP2-assosiated diseases

1.6.1 HIVEP2-related intellectual disability (*autosomal dominant 43*)

HIVEP2-related intellectual disability is caused by *de novo* mutations in the *HIVEP2* gene (Srivastava et al., 2016; Steinfeld et al., 2016). HIVEP2-associated disease is characterized by hypotonia, developmental delay, intellectual disability, behavioral abnormalities, motor delay, seizures, gastrointestinal problems and dysmorphic features (Quental et al., 2022). Whole genome sequencing has identified 17 patients with 5 frameshift variants, 9 nonsense variants and 2 missense variants. Most of the variants occur in exons 5, 10, and 9. Early symptoms of the disease include weak muscle tone (hypotonia) and delayed development of motor skills. Many patients have difficulty sitting, standing, and walking during their first years of life. Even after learning to walk, patient's gait is clumsy. Various dysmorphic features such as widely spaced eyes (hypertelorism), a broad nasal bridge, a high forehead and narrow fingers have also been observed in many patients. In addition, patients also have delayed speech development and behavioral problems such as hyperactivity, aggression, anxiety, and autism. Symptoms such as microcephaly, seizures, and hyperphagia are less common (Srivastava et al., 2016; Steinfeld et al., 2016; Park et al., 2019; Quental et al., 2022).

1.6.2 Schizophrenia

HIVEP2 is also associated with schizophrenia. *HIVEP2* expression has been shown to be reduced in the brains of schizophrenia patients (Volk et al., 2015). *HIVEP2* knockout mice are used as a model for schizophrenia and mental retardation (Takagi et al., 2001; Takao et al., 2013; Kobayashi et al., 2018). Several schizophrenia-like phenotypes have been observed in *HIVEP2* knockout mice, such as schizophrenia-like behavioral phenotypes, thinner cerebral cortex, chronic cerebral inflammation, and schizophrenia-like gene expression (Takao et al., 2013). Behavioral phenotypes in mice include hyperactivity, anxiety disorders, memory defects, reduced PPI and impaired socialization. Hippocampal dentate gyrus granule cells fail to mature in *HIVEP2* knockout mouse, which is considered an endophenotype of neuropsychiatric disorders (Takagi et al., 2006; Hagihara et al., 2013; Takao et al., 2013). Morphological phenotypes in *HIVEP2* knockout mice include an immature dendritic spine morphology, abnormal mitochondrial morphology and increased neuronal density (Nakao et al., 2017).

1.7 Schnurri, the *Drosophila* homolog of HIVEP2

1.7.1 Gene and protein structure of Schnurri

schnurri (shn) encodes a zinc finger transcription factor that is homologous to HIVEP family of mammalian transcription factors. The *shn* gene is located on the second chromosome and encodes a polypeptide of 2527 amino acid residues (Nüsslein-Volhard et al., 1984; Arora et al., 1995; Staehling-Hampton et al., 1995). Schnurri (Shn) protein contains eight C2H2-type (Cys_2His_2) zinc fingers, that are structurally related to human HIVEP1 and HIVEP2 proteins (Arora et al., 1995). The Zn fingers are clustered in two pairs and one triplet. Between the first and second Zn finger pairs, there is a region, C-C-X_{13}-H-C, that forms a single Zn finger. Shn also contains S/TPXK/R (serine/threonine-proline-X-lysine/arginine) motifs that bind to DNA and two regions of acidic amino acid residues (Figure 4) (Nüsslein-Volhard et al., 1984; Arora et al., 1995; Staehling-Hampton et al., 1995).

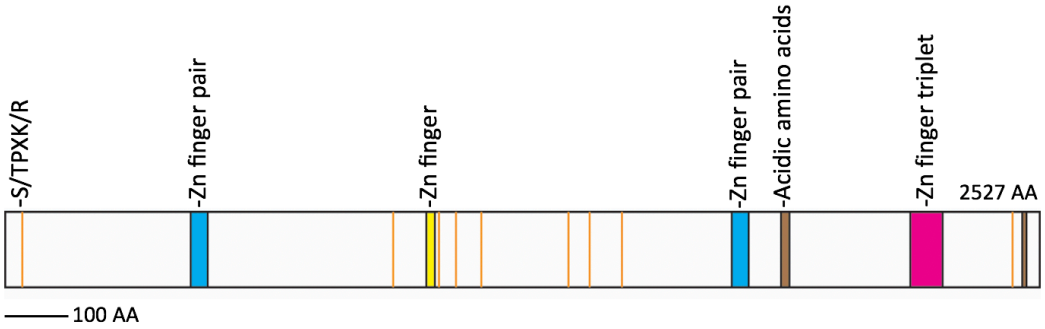

Figure 4. Schematic drawing of Shn protein structure. Light blue boxes represent pairs of zinc fingers, and the pink box represents a triplet of zinc fingers, which functions as a repression domain. The yellow box represents a single zinc finger. Dark brown boxes represent domains rich in acidic amino acids, and thin orange boxes represent DNA binding domains, S/TPXK/R repeats of amino acids (Bachelor's thesis of Mikk, 2021).

1.7.2 Expression *of schnurri* mRNA and protein

The expression of *shn* has been studied mainly in the embryonic stages of fruit flies, in imaginal discs, and in ovaries (Arora et al., 1995; Staehling-Hampton et al., 1995). At early blastoderm stage, *shn* is expressed in dorsal cells of the blastoderm embryo, and at stage 5, expression can be detected in ventral cells of the future mesoderm. During germband extension, *shn* is expressed in the mesoderm, dorsal ectoderm, and midgut. At stage 14 embryo, *shn* is enriched in sites such as the gastric caeca and visceral mesoderm. At stage 16, expression persists in the gut and ectoderm. During larval development, *shn* is expressed in the imaginal discs. In adult *Drosphila*, *shn* expression can be detected in the germanium, and from stage 6 it can be observed in the nurse cells and in the developing oocyte (Arora et al., 1995; Staehling-Hampton et al., 1995). Marleen Mikk has studied Shn protein expression in *Drosophila* embryos using a fly line where Shn is tagged with HA-tag using CRISPR-Cas9 system (Bachelor's thesis of Mikk, 2021). At stage 9, Shn protein is expressed in the dorsal amnioserosa and in the developing gut. At stage 12, Shn expression can be seen in the foregut, midgut and hemocytes. By the 16th stage, expression is evenly distributed over the entire gut region, with a weak signal also in ventral nerve cord (Bachelor's thesis of Mikk, 2021).

1.7.3 Functions of Schnurri

Shn plays a vital role in mediating Decapentaplegic (Dpp) signaling during embryogenesis (Torres-Vazquez et al., 2001). *shn* was first identified in a screen for zygotic embryonic lethal mutations that cause defective dorsal closure phenotype (Nüsslein-Volhard et al., 1984). It has been shown that Shn prevents N-terminal kinase induced apoptosis by ensuring the survival of dorsal edge cells during dorsal closure (Beira et al., 2014). Shn is also required for anterior/posterior patterning of the imaginal wing discs and vein differentiation in pupal development of wings (Torres-Vazquez et al., 2000). Shn has also shown to function independently of the Dpp signaling pathway like in cellular response to DNA damage (Kelsey et al., 2012). In the nervous system, Shn is required for dorsal and lateral PNS formation, as *shn* homozygous mutants show severe defects in PNS development (Rusten et al., 2002). Shn is also required for dendrite morphogenesis (Iyer et al., 2013), motor axon guidance and synaptogenesis (Kraut et al., 2001) and in associative memory and learning of *Drosophila* larvae (Bachelor's thesis of Loviisa Pihlas, 2021). Shn is also required in spermatogenesis (Matunis et al., 1997), and oogenesis (Charbonnier et al., 2015).

1.7.4 Schnurri in Dpp signaling pathway

Shn carries out its functions in the Dpp signaling pathway, where it plays an important role acting as both a transcriptional activator and a repressor, regulating the expression of Dpp target genes (Torres-Vazquez et al., 2000). Dpp is a morphogen, that belongs to the TGF-β (transforming growth factor beta) superfamily of signaling molecules. Dpp is similar to vertebrate bone morphogenic proteins 2 and 4 (BMP2 and BMP4) (Padgett et al., 1987). Dpp plays a vital role in embryonic development of the fruit fly. Dpp is required for the dorsal/ventral patterning of the embryo (Wharton et al., 1993). Dpp also induces the formation of visceral mesoderm and the heart (Frasch, 1995). Later in embryogenesis Dpp is required for dorsal closure (Reiesgo-Escovar et al., 1997). Post embryonically, Dpp is needed for *Drosophila* wing development (Khalsa et al., 1998). Dpp acts through the transmembrane-receptor serine/threonine kinases: Punt (Put) and Thick veins (Tkv) (Nellen et al., 1994; Letsou et al., 1995). The binding of Dpp to its receptors triggers the phosphorylation of Tkv by Punt and in turn enables Tkv to phosphorylate the cytoplasmic protein Mothers against Dpp (Mad). Phosphorylation enables Mad to form a complex with Medea (Med) and translocate to the nucleus, where they can bind to cis-acting elements in target genes and activate or repress transcription (Wisotzkey et al., 1998; reviewed by Affolter et al., 2001). One way to activate Dpp target genes is that Dpp signalling must suppress the transcription of a repressor Brinker (Brk) (Jaźwińska et al., 1999).

Shn functions as a cofactor for Mad and Med, inhibiting transcription of the transcriptional repressor *brk* in response to Dpp signaling (Dai et al., 2000; Marty et al., 2000). Zinc fingers 6 to 8 of Shn are required to interact with the Mad/Med complex and a repression domain (RD) in the C-terminal part of Shn is required for *brk* repression (Figure 4) (Müller et al., 2003). This RD functions through interaction with two corepressors, *Drosophila* homolog of the C-terminal-binding protein 1 (dCtBP) and *Drosophila* homolog of the SIN3 Transcription Regulator Family Member A (dSin3A) (Cai and Laughon, 2009). In addition, a silencing element (brkSE) has been found in the regulatory part of the *brk* gene that corresponds to Dpp signaling and binds the Shn/Mad/Med complex (Figure 5A) (Müller et al., 2003). Several additional repression domains located in the N-terminal half of the Shn protein have been discovered (Cai and Laughon, 2009). These repression domains function by

interacting with multiple corepressors, such as Groucho, dCtBP, dSin3A, and Smarter (Cai and Laughon, 2009). In addition to regulating Dpp target genes through *brk*, Shn also binds directly to the regulatory regions of Dpp target genes. For example, functional Mad/Med/Shn-dependent silencing elements have also been found in other fruit fly genes such as *bag of marbles*, *gooseberry and, intermediate neuroblasts defective* (Pyrowolakis et al., 2004; Stathopoulos and Levine, 2005). In addition to this repressive function, Shn also acts as a transcriptional activator, by binding directly to the regulatory regions of Dpp target genes (Torres-Vazquez et al., 2001). For example, Shn is required for Ultrabithorax (Ubx) expression in the midgut and binds to the *Ubx* enhancer together with Mad and Med. Shn can also directly bind to the *Ubx* enhancer using NF-kβ-like DNA sequence motifs (Figure 5B) (Dai et al., 2000). Shn also harbors a region capable of transcriptional activation (Cai and Laughon, 2009).

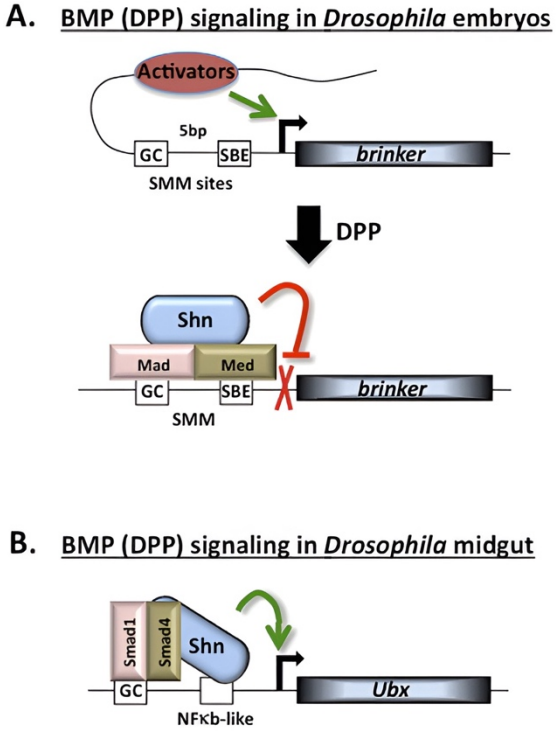

Figure 5. Shn and Dpp signaling. (A) In the absence of Decapentaplegic (Dpp) signaling, Brinker (Brk) is expressed and Dpp target genes are silenced. In Dpp signaling, Mothers against Dpp (Mad) binds to a GC-rich sequence and Madea (Med) binds to a Smad binding element (SBE) in the regulatory region (brkSE) of the *brinker* (*brk*) gene. Shn is recruited to brkSE by the Mad/Med complex and expression of Dpp target genes occurs. (B) Shn binds to the *ultrabithorax* (*Ubx*) enhancer through two interactions. First, it binds to Mad and Med (human homologues Smad1 and Smad4), which in turn also binds to the *Ubx* enhancer. Second, Shn also binds directly to the *Ubx* enhancer via NF-kB-like DNA sequences (Blitz and Cho, 2009).

Aims of the thesis

The aims of the thesis are:

1. Investigate expression and possible co-expression of Shn and Da in adult *Drosophila* brain.

2. Investigate whether Shn and Da and their mammalian homologs HIVEP2 and TCF4 interact in HEK293 cells.

3. Investigate if Shn affects the transcriptional activity of Da in adult *Drosophila* brain.

Materials and methods

3.1 *Drosophila* strains and maintenance

All *Drosophila* stocks were kept at 25°C with 60% humidity and a 12 h light and dark daily rhythm and fed malt and semolina-based substrate.

Following *Drosophila* strains were used in this study: 3xFLAG-*da* (Tamberg et al., 2020), 2xHA-*shn* (Bachelor's thesis of Marleen Mikk, 2021), *white** (Fly Facility, GReD, Clermont-Ferrand, France), UAS-*shn* (Gift from prof. M. Affolter (Marty et al., 2001)), UAS-*da* (BDSC, 37291), *elav*-Gal4 (BDSC, 8760, (Luo et al., 1994)), E-box-Luc (Master's thesis of Käthy Rannaste, 2021), Min-Luc (Master's thesis of Käthy Rannaste, 2021).

3.2 Sample preparation for Shn and Da expression analysis

Brains of 8 days old adult flies, 0-24 h old adult flies, and 3rd instar larvae where dissected in phosphate-buffered saline (PBS) (14 brains per sample, 7 females and 7 males). Brains were lysed in RIPA buffer (20 mM Tris ph 7.5, 400 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1x Protease inhibitor (Roche)) and homogenized with Kontes Pellet Pestle homogenizer. Protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientiffic). Laemmli sample buffer (125 mM Tris-HCl, pH 6.8; 5% SDS; 15% volume-by-volume glycerol; 0.025% Bromophenol Blue; 5% β-mercaptoethanol (Roth)) was added to the samples and heated at 95°C for 10 minutes.

3.3 Immunohistochemistry of adult brains

Adult flies (0-24 h old) were fixed in 4% paraformaldehyde in PBS for 1.5 h at room temperature. Brains were washed with PBST (PBS+ 0.1% TritonX-100) and dissected in PBS, followed by blocking at room temperature for 1 h in blocking buffer (0.1M TrisHCl pH 7.5, 0.15M NaCl, 0.1% TritonX-100, 10% Normal Goat Serum (Invitrogen)). The brains were incubated with primary antibodies in blocking buffer for 48 h at 4°C while swinging. Brains were washed three times with PBST before secondary antibody labeling. Secondary antibody labelling was done in the dark for 3 h at room temperature in blocking buffer followed by washing with PBST. Labeled brains were mounted using Vectashield mounting medium (Vector Laboratories). Image collection was performed using a Zeiss LSM 900 confocal microscope with a Pln Apo 20X (NA 0.8) objective. ImageJ software was used to select suitable layers from collected images. Following antibodies were used: mouse anti-FLAG M2 (Sigma-Aldrich, 1:2000), rat anti-HA 3F10, Roche (1:50), goat anti-mouse 488-conjugated secondary antibody (Jackson Immuno Research, 1:100), goat anti-rat 594-conjugated secondary antibody (Jackson Immuno Research, 1:100).

3.4 Molecular cloning of HA-Shn expression vectors

For creating HA-Shn expression vector pUC-Act-HA-Shn for *Drosophila* cell lines, cDNA from 2xHA-Shn, pOT2-IP16071 (Drosophila Genomic Research Center, DGRC), pUC-Act-GFP (DGRC, #1219)

were used as templates to generate fragments containing the beginning of 2xHA-Shn coding region, the ending of Shn coding region and plasmid backbone, respectively. Gibson assembly was used to connect these fragments. Overlapping fragments were generated using PCR and Gibson assembly primers (listed in Supplementary table 1). The PCR protocol was the following: 5 min 95°C followed by 35 cycles of 30s 95°C, 30s 60°C and 1.5 min 72°C, after which came 10 min 72°C and cooling down to 4°C. Purified fragments were combined in HiFi assembly mix (NEB) and the mixture was heated at 50°C for 15 minutes. The Shn expression vector puC-Act-HA-Shn was transformed and multiplied in *E. coli* strain TOP10 (Invitrogen). For obtaining larger quantities of plasmid, PureLink HiPure Plasmid Midiprep Kit (Invitrogen) was used.

Mammalian HA-Shn expression vector was created by restriction enzyme mediated cloning - HA-Shn coding region was obtained from puC-Act-HA-Shn vector using PacI (Thermo Scientific) and PsiI (Thermo Scientific) in PacI buffer (Thermo Scientific) (1h 37°C). PacI creates TA-overhangs, witch where removed by blunting using T4 polymerase (Thermo Scientific) (10 min 75°C). Mammalian expression vector backbone was obtained from pcDNA-EF1a-Da-FLAG (Tamberg et al., 2020) using EcoRV (Thermo Scientific) restrictase in R buffer (Thermo Scientific) (1 h 37°C). Fast AP (Thermo Scientific) was used to remove phosphate groups from DNA ends to avoid self-ligation. Ligation was performed using T4 DNA ligase (Thermo Scientific) in T4 ligase buffer (Thermo Scientific). Plasmid was produced in E. coli strain TOP10 (Invitrogen) bacterial cells. Colony PCR was used to verify that insertion was successful. Primers used for the colony PCR are listed in Supplementary table 1. For obtaining larger quantities of plasmid, PureLink HiPure Plasmid Midiprep Kit (Invitrogen) was used.

3.5 Transfection for validating expression of HA-Shn

Schneider (S2) cells were cultured in 75cm^2 tissue culture flasks, passaging the cells after reaching a visually high enough cell density (>2x10^7 cells per ml). Cells were kept at room temperature. Schneider's *Drosophila* medium (Biowest) with added penicillin (100 U/ml, PAA Laboratories), streptomycin (0.1mg/ml, PAA Laboratories) and fetal bovine serum (10%, PAA Laboratories) was used for culturing. For transfection, cell count was obtained with a Neubauer chamber (Paul Marienfeld GmbH) and cells were plated in 800 µl at a density of 10^6 cells per ml in 12-well format (Greiner). Cells were transfected with 800 ng of plasmids pUC-Act-HA-Shn, pUC-Act-FLAG-Da (Master's thesis of Käthy Rannaste, 2021) or pUC-Act-GFP (DGRC) in 12-well format (Greiner). In addition, some cells were co-transfected with 400 ng of pUC-Act-HA-Shn and 400 ng of pUC-Act-FLAG-Da plasmids in 12-well format (Greiner). 42 µl of unsupplemented Schneider's *Drosophila* medium was added to the DNA and incubated for 15 minutes. Transfection was performed with Lipofectamine 2000 (Thermo Scientific) (DNA to reagent ratio 1:3) and with Lipofectamine 3000 (Thermo Scientific) (DNA to reagent ratio 1:2) separately (to test the transfection efficiency) in unsupplemented Schneider's *Drosophila* medium. 40 µl of Lipofectamine mix was added to the DNA mix and incubated for 20 minutes. The conditioned medium was collected and replaced with unsupplemented Schneider's Drosophila medium. 80 µl of transfecting medium was added to the wells and placed on slow shaker for 4 h. At the end of the transfection, transfection medium was replaced with previously collected conditioned medium. Cells were lysed 24 h after transfection in 1x Laemmli buffer + 5% 2-mercaptoethanol (Roth).

Human embryonic kidney cells (HEK-FT) where cultured in Dulbecco's Modified Eagle Medium (DMEM) (Corning) with 10 % fetal bovine serum (PAA Laboratories), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco) added. Cells At the time of transfection, the cells were at 50-70% confluency on 12 well plate (Greiner). HEK-FT cells were transfected with 1 µg of pcDNA-ef1α-HA-Shn, pcDNA-ef1α-FLAG-Da (Tamberg et al., 2020) or pPGK-GFP. In addition, some cells were co-transfected with pcDNA-ef1α-HA-Shn (0.70 µg or 0.9 µg) and pcDNA-ef1α-FLAG-Da (0.30 µg or 0.1 µg) in different ratios again with a total DNA mass of 1ug. 1 µg DNA was added to 42.9 µl serum free DMEM. Transfection master mixes where incubated for 15 minutes. 2.2 µl of polyethyleneimine (PEI 1000 ng/ µl) (Sigma-Aldrich) was mixed with 37.8 µl serum-free DMEM per well. 40 µl PEI mix was added to the DNA master mix and incubated for 20 minutes. 80 µl of transfection master mix was added to each well. Cells were lysed 24 h after transfection in 1x Laemmli buffer + 5% 2-mercaptoethanol (Roth).

3.6 Transfection for co-immunoprecipitation

Human embryonic kidney cells (HEK293) were cultured in Eagle's Minimum Essential Medium (MEM) (Corning) with 10 % fetal bovine serum (PAA Laboratories), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco) added. Cells At the time of transfection, the cells were at 50-70% confluency on 10 cm plate (Greiner). HEK293 cells were transfected with 6.7 µg of pcDNA-ef1alpha-FLAG-HIVEP2 (Cloned previously in Neurobiology laboratory) or pcDNA-ef1alpha-HA-Shn expression plasmid and 3.3 µg of pcDNA-ef1alpha-TCF4-A, pcDNA-ef1alpha-TCF4-B (Sepp et al., 2017) or pcDNA-ef1alpha-FLAG-Da (Tamberg et al., 2020) plasmid. All the plasmids were also co-transfected together with pcDNA-ef1alpha plasmid (3.3 µg or 6.7 µg). For control 10 µg of pcDNA-ef1alpha was added. Following co-transfections were also conducted: pcDNA-ef1alpha-TCF4-A and pcDNA-ef1alpha-FLAG-HIVEP2, pcDNA-ef1alpha-TCF4-B and pcDNA-ef1alpha-FLAG-HIVEP2, pcDNA-ef1alpha-FLAG-Da and pcDNA-ef1alpha-HA-Shn. DNA was mixed with 240 µl serum-free MEM per well and incubated for 10 minutes. All together 10 µg of plasmid was used. 24 µl of polyethyleneimine (PEI 1000 ng/µl) was mixed with 526 µl serum-free MEM per well. PEI and expression mixtures were incubated for 15 minutes. 1 ml of the mixture was then pipetted into each well and incubated for 24 h.

3.7 Co-immunoprecipitation

Dynabeads Protein G beads (Invitrogen) were used to bind antibodies. Beads were first washed with 1xPBS-0.05% Tween20 (Amresco) and then incubated in 1xPBS-0.05% Tween20 solution with antibody on a rotator for 4 h at 4°C (antibody concentrations listed in supplementary table 2). HEK293 cells which had grown 24 h post transfection were lysed in lysis buffer (20 mM Tris-HCl ph 7.5, NaCl 400 mM, 1 mM EDTA, 0.5 mM NP-40 (BioChemica), 0.01 mM Zn-acetate, 1x protease inhibitor (PI)) (Roche) and incubated 30 minutes at 4°C on a rotator. Cells were then sonicated with 30% power, 3 seconds (Ultrasonic cell disruptor, Torbeo), and centrifuged (15 min, 16 000 g, 4°C). Supernatant was transferred to new tubes and protein concentrations were measured using Pierce BCA Protein Assay Kit (Thermo Scientiffic). Input sample was taken before diluting the IP samples and diluted with IP buffer (200 mM NaCl, 20 mM Tris-HCl ph, 1 mM EDTA, 0.5 mM NP-40, 0.01 mM Zn-acetate, protease inhibitor (PI, Roche)) to 50 ml. For IP 600 mg protein was taken. The samples

were then diluted twice: first in lysis buffer (400 mM NaCl, 20 mM Tris-HCl ph, 1 mM EDTA, 0.5 mM NP-40, 0.01 mM Zn-acetate, protease inhibitor (PI, Roche)) to 500 ml and then in dilution buffer (20 mM Tris-HCl ph, 1 mM EDTA, 0.5 mM NP-40 (BioChemica), 0.01 mM Zn-acetate, and protease inhibitor (PI) (Roche)) to a total volume of 1 ml. Beads were washed with 1xPBS-0.05% Tween20 and 20 µl of beads was added to each IP sample and incubated on a rotator for overnight at 4°C. Next day, 80 µl of sample (Flowthrough) was taken before washing the beads. Beads were washed three times with IP buffer and then lysed in 1x Laemmli buffer + 2-mercaptoethanol (Roth). Laemmli buffer + 2-mercaptoethanol (Roth) to a final concentration of 5% was also added to the input and flow through samples. Samples were then heated at 95°C for 10 minutes.

3.8 Western blot analysis

Samples were electrophoretically separated by SDS-Polyacrylamide gel (Shn and Da expression analysis (gradient gel: 4% to 12%), co-immunoprecipitation (5%, 8%, 10%), HA-Shn plasmid expression (5%)) at 100 V and transferred to Immobilon-P transfer membrane (Millipore) in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol pH 8.3) at 100 V for 100 minutes. Membranes were blocked with 5% skimmed milk powder (PanReac AppliChem) dissolved in 1x Tris Buffered Saline with 0.1% Tween-20 (TBST, Sigma Aldrich) solution for 30 minutes at room temperature. Primary and secondary antibodies were diluted in 2% skimmed milk-TBST and membranes were incubated in the solutions overnight at 4°C and 1 h at room temperature, respectively. Antibodies used are listed in Supplementary table 3. Membranes were washed with TBST solution 3 times for 10 minutes, followed by chemiluminescence signal detection with SuperSignal West Atto (Thermo Scientific). Femto Chemiluminescence Substrate (Thermo Scientific) was to visualize Mouse anti-FLAG M2-peroxidase (HRP) signal. The chemiluminescence signal was visualized with ImageQuant LAS 4000 bioimager (GE Healthcare). The membrane staining with Coomassie Blue R-250 (0.1% Coomassie Brilliant Blue R-250, 25% ethanol, 7% acetic acid) was used as a loading control. PageRuler Prestained Protein Ladder Plus (Thermo Scientific) or PageRulerTM Prestained Protein Ladder (Thermo Scientific) was used as a reference to determine the molecular weight of the separated proteins.

3.9 *in vivo* E-box reporter assay

Fly lines used here were generated previously by Laura Tamberg. Three experiments were performed – one by Laura Tamberg and two by the author. Following lines were used:
elav(C155)-Gal4/+;min-Luc/+;UAS-*da*, *elav*(C155)-Gal4/+;+;UAS-*da*/E-box-Luc,
elav(C155)-Gal4/+;+;UAS-*shn*/E-box-Luc, *elav*(C155)-Gal4/+;+;UAS-*da*,UAS-*shn*/E-box-Luc,
elav(C155)-Gal4/+;+;+/E-box-Luc

Brains of 0-24 h old flies were dissected in PBS (16 brains per sample). Brains were lysed with Passive Lysis Buffer (Promega) and homogenized with Kontes Pellet Pestle homogenizer. Protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientiffic). Luciferase reporter assay was performed using the Dual-Glo Luciferase assay kit (Promega) according to the protocol provided by the manufacturer. Luciferase signal was measured using GENios Pro Multifunction Microplate Reader (Tecan Group). Data was log-transformed, mean-centered, and

autoscaled for calculating means, statistical significance and upper and lower errors. Statistical analysis was performed using one-way ANOVA (Analysis of variance) with post hoc Tukey's test. For graphical representation the data was back-transformed.

Results

4.1 Shn and Da are expressed in *Drosophila* third instar larval and adult brain

HIVEP2 and TCF4 are associated with severe mental retardation syndromes that are symptomatically similar (Zweier et al., 2008; Marangi et al., 2011; Whalen et al., 2012; Srivastava et al., 2016; Steinfeld et al., 2016). In this thesis, we decided to use the fruit fly as a model to investigate whether HIVEP2-related intellectual disability and PTHS are caused by dysregulation of molecular pathways that are regulated by HIVEP2 and TCF4 together. Previously it has been shown that HIVEP2 and TCF4 interact in *in vitro* pulldown experiments and activate the Somatostatin receptor type 2 (SSTR2) expression in neural cell lines (Dörflinger et al., 1999). Therefore, we set out to investigate whether the *Drosophila* homologs Shn and Da interact in the fruit fly nervous system. In order to study this, we first needed to confirm that Shn and Da are expressed at the same time in *Drosophila* larval and adult brain. For this purpose, we collected the brains of 3rd instar larvae, 0-24 h old adult flies, and 8 days old adult flies and performed Western blot analysis. Fly lines used for this study were: HA-Shn and FLAG-Da and HA-Shn+FLAG-Da. In these fly lines, endogenous Shn is tagged with 2xHA tag (Bachelor's thesis of Marleen Mikk 2021) and endogenous Da with 3xFLAG epitopes (Tamberg et al; 2020) since no commercial antibodies are available for either of the proteins. Western blot analysis revealed that both Shn and Da are expressed in all studied developmental stages. The expression of both Shn and Da decreased during development: was highest in the 3rd instar larval brains and lowest in the 8 days old adult brains (Figure 6A, B). The expected molecular weights of Shn and Da are 270 kDa and 74 kDa, respectively (Cronmiller et al., 1988; Arora et al., 1995). Although tagged proteins could have reduced mobility, our results still showed Shn signal just above the 250 kDa and Da signal below the 100 kDa protein marker.

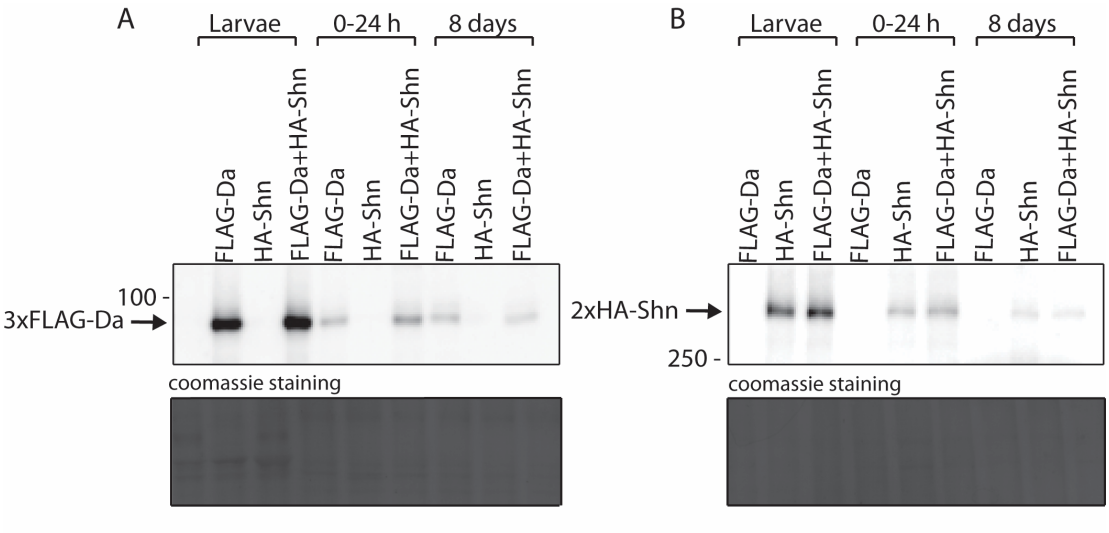

Figure 6. Da and Shn expression analysis in *Drosophila* third instar larval and adult brains. Western blot analysis of FLAG-Da (A) and HA-Shn (B) protein expression in brains of three different fruit fly developmental stages: 3rd instar larvae (Larvae), 0-24 hours old adult flies (0-24h) and 8 days old adult flies (8 days). FLAG-Da - brains from 3xFLAG-Da fly line, HA-Shn - brains from 2xHA-Shn fly line, FLAG-Da+HA-Shn - brains from 3xFLAG-da,2xHA-Shn fly line. Coomassie membrane staining is

shown at the bottom of the figure A as a loading control. Numbers on the left of each figure indicate molecular weight in kilodaltons (kDa) according to the protein marker.

4.2 Shn and Da partially co-express in adult *Drosophila* brain

Western blot analysis showed that the expression of Shn and Da is strongest in the larval brain followed by young adult brain. Since Shn and Da were both expressed in the brains of 0-24 h old adult flies at higher levels than in the older *Drosophila* brains, we wanted to see if Shn and Da are also co-expressed in the same brain regions of 0-24 h old adult flies. For this, we performed immunohistochemical staining using HA-Shn+FLAG-Da flies and anti-HA and anti-FLAG antibodies. HA-Shn and FLAG-Da fly brains were used as antibody specificity controls. Results showed that Shn and Da are indeed co-expressed in adult *Drosophila* brain (Figure 7A, B, C). *Drosophila* brain regions are presented in Figure 7D. Shn and Da co-expression can be seen in different brain regions including mushroom bodies, optic lobes, and antennal lobes (Figure 7A, B, C). We saw strong Shn and Da specific signal in many cells in adult fly brains (Figure 7E, G), even though the used anti-HA and anti-FLAG antibodies gave some unspecific signal (Figure 7F, H). Our results show that Shn and Da are potentially capable to interact in the 0-24h old fly brain, since these proteins exhibit overlapping expression patterns.

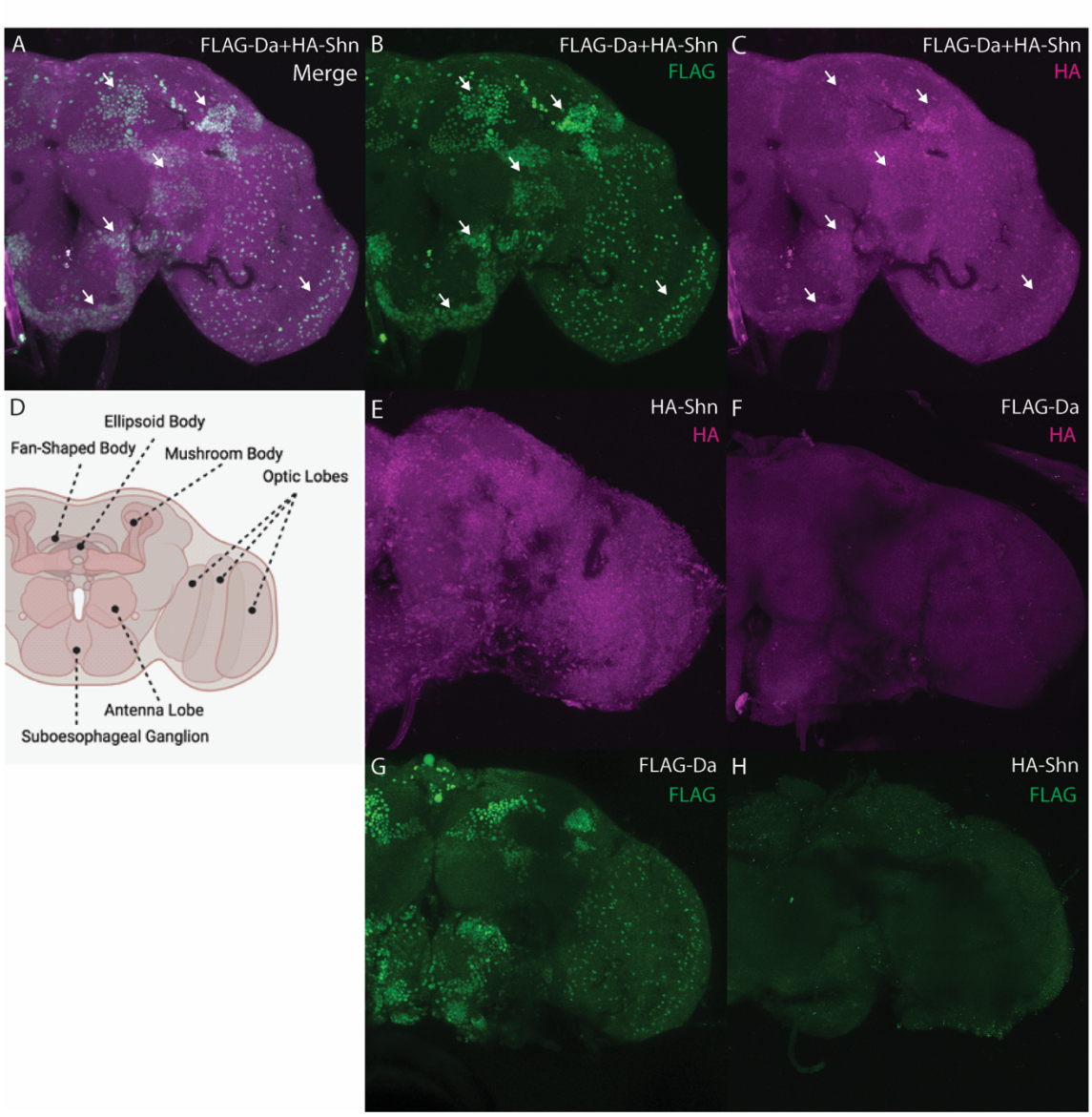

Figure 7. Shn and Da expression in adult *Drosophila* brain. (D) Schematic representation of *Drosophila* brain regions. Specific regions of the adult fly brain with key structures are labeled - the mushroom bodies, the ellipsoid body, the fan-shaped body, the antenna lobes, the optic lobes, the protocerebrum, and the suboesophogael ganglia (Nichols, 2006). (A-C) Immunohistochemical staining was performed in adult fly brains of HA-Shn+FLAG-Da fly lines aged 0-24 h. HA-Shn+FLAG-Da stained with anti-HA and anti-FLAG antibodies, white arrows showing parts of Da and Shn co-expression (A, B, C). Brains of HA-Shn and FLAG-Da fly lines, stained with anti-HA (E) and anti-FLAG (G) antibodies, respectively. Antibody specificity was tested by staining HA-Shn (F) and FLAG-Da (H) fly lines with anti-FLAG and anti-HA antibodies, respectively. Magenta shows the expression of HA-Shn (C, E, F), and green the expression of FLAG-Da (B, G, H). Images were obtained with a Zeiss LSM 900 confocal microscope with a Pln Apo 20× (NA 0.8) objective.

4.3 Cloning of Shn expression vector pcDNAef1α-HA-Shn

In order to study Shn and Da interactions, we decided to first investigate the interaction in over-expressed conditions in cultured cells. For that, we cloned Shn expression vector for *Drosophila* cell lines (pUC-Act-HA-Shn). Unfortunately, transfection of the *Drosophila* cell line S2 was not

successful, so we continued with mammalian cell lines. For this, we cloned mammalian Shn expression vector (pcDNAef1α-HA-Shn). Da expression vector (pcDNA-ef1α-FLAG-Da) was previously cloned by Tamberg et al 2020. To confirm the protein expression from the newly cloned pcDNAef1α-HA-Shn vector and previously cloned pcDNA-ef1α-FLAG-Da, we transfected the vectors into human embryonic kidney (HEK-FT) cells and performed Western blot analysis. HEK-FT cells were used because they are very easy to grow and with high reproducibility. We also co-transfected pcDNA-ef1α-HA-Shn and pcDNA-ef1α-FLAG-Da in different ratios, to test whether HA-Shn expression vector should be added in excess, since HA-Shn expression vector is very large (13 900 bp) and could be more difficult to transfect. Results showed that the Shn and Da expression vectors are functional and that Shn (Figure 8A) and Da (Figure 8B) are expressed in HEK-FT cells. We could also conclude that Shn vector should be added in excess (7:3) during transfection (Figure 8A, B) since this ratio ensures better expression of Shn. These results showed that the cloning of pcDNA-ef1a-HA-Shn was successful and that we were able to proceed with the interaction experiments.

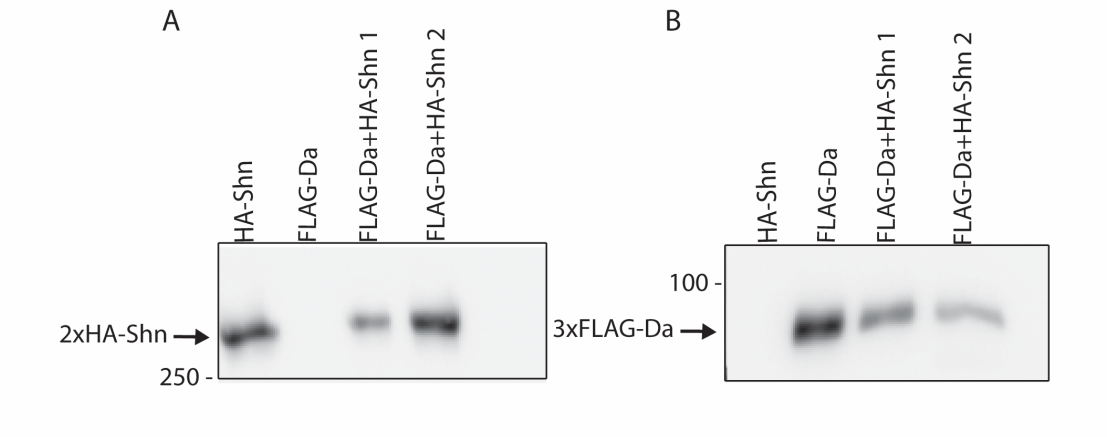

Figure 8. pcDNAef1a-HA-Shn expression in HEK-FT cells. HEK-FT cells were transfected with pcDNAef1α-HA-Shn and pcDNA-ef1α-FLAG-Da alone or together. The cell lysates were analyzed using Western blot analysis with an anti-HA (A) and anti-FLAG (B) antibody. FLAG-Da+HA-Shn 1 – HA-Shn and FLAG-Da expression plasmids transfected in 7/3 ratio. FLAG-Da+HA-Shn 2 – HA-Shn and FLAG-Da expression plasmids transfected in 9/1 ratio. Numbers on the left of each figure indicate molecular weight in kilodaltons (kDa) according to the protein marker.

4.4 Overexpressed human TCF4-A and FLAG-HIVEP2, and their fruit fly homologs FLAG-Da and HA-Shn interact in HEK293 cells

To investigate whether Shn and Da or HIVEP2 and TCF4 interact, we performed a co-immunoprecipitation analysis (co-IP). For this purpose, we co-transfected pcDNA-ef1alpha-FLAG-HIVEP2 and pcDNA-ef1alpha-TCF4-A or pcDNA-ef1alpha-TCF4-B, and pcDNA-ef1alpha-FLAG-Da and pcDNA-ef1alpha-HA-Shn into HEK293 cells. Anti-FLAG, anti-HA and anti-TCF4 antibodies were used for co-IP and Western blot analysis. FLAG-HIVEP2, TCF4-A, TCF4-B, FLAG-Da, and HA-Shn expression was confirmed in input samples with Western blot analysis (Figure 9A, B). For co-IP analysis, overexpressed TCF4-A or TCF4-B were co-immunoprecipitated with overexpressed FLAG-HIVEP2 and *vice versa* and the results were visualized using Western blot analysis. Results showed that pulldown of TCF4-A brings along FLAG-HIVEP2 and pulldown of FLAG-HIVEP2 brings along TCF4-A (Figure 9A). We also saw that pulldown of TCF4-B brings along FLAG-HIVEP2, but not the other way around (Figure 9A). For *Drosophila* co-IP analysis, overexpressed FLAG-Da was co-

immunoprecipitated with overexpressed HA-Shn or *vice versa* and the results were visualized in Western blot analysis. We were able to pull down HA-Shn when immunoprecipitating FLAG-Da, but the co-IP was unsuccessful when HA-Shn was immunoprecipitated (Figure 9B). Despite the fact that the co-IP with the fly proteins was only successful when FLAG-Da was pulled down, we showed that both the mammalian proteins TCF4 and HIVEP2 and their *Drosophila* homologs Da and Shn interact in overexpressed conditions in HEK293 cells.

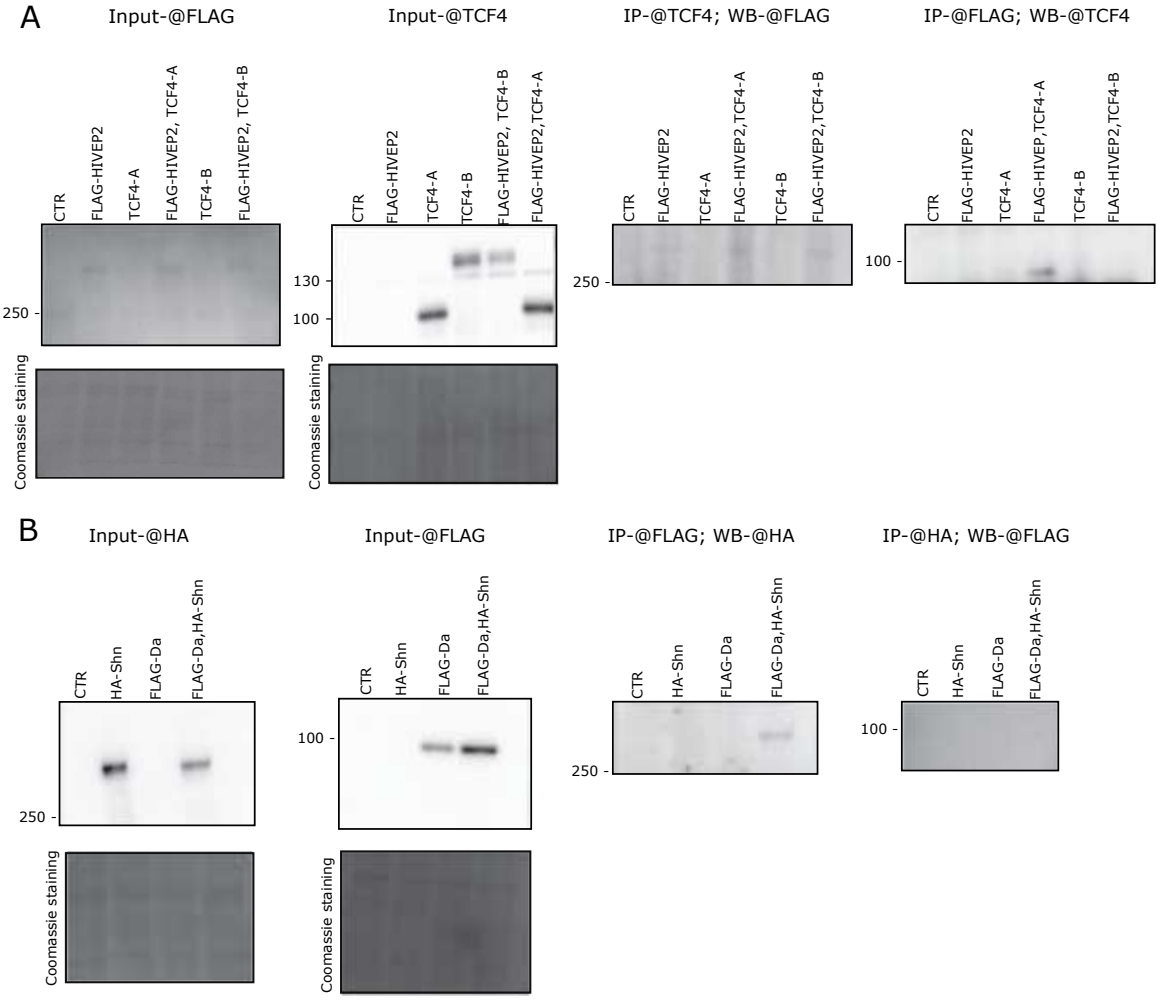

Figure 9. Overexpressed human TCF4 and HIVEP2 and *Drosophila* homologs Da and Shn co-immunoprecipitate in HEK293 cells. (A) Western blot analysis of mammalian co-IP samples. HEK293 cells were transfected with FLAG-HIVEP2, TCF4-A and TCF4-B encoding constructs alone or in combination with FLAG-HIVEP2 and TCF4-A, and FLAG-HIVEP2 and TCF4-B. Anti-FLAG antibody was used for co-IP and Western blot analysis of FLAG-HIVEP2 and anti-TCF4 antibody was used for co-IP and Western blot analysis of TCF4-A and TCF4-B. Empty vector transfected cells were used as a negative control (CTR). (B) Western Blot analysis of *Drosophila* co-IP samples. HEK293 cells were transfected with FLAG-Da and HA-Shn encoding constructs alone or in combination. Anti-FLAG antibody was used for co-IP and Western blot analysis of FLAG-Da and anti-HA antibody was used for co-IP and Western blot analysis of HA-Shn. Empty vector transfected cells were used as a negative control (CTR). Coomassie membrane staining is shown on the bottom of the figure and used as a loading control. Numbers on the left of each figure indicate molecular weight in kilodaltons (kDa) according to the protein marker.

4.5 Overexpression of Shn causes a decrease in Da transcriptional activity in adult *Drosophila* brains

Human TCF4 and HIVEP2 and fruit fly Da and Shn interact in overexpressed conditions in cultured mammalian cells and it has been shown in Neurobiology lab that HIVEP2 potentiates the transcriptional ability of TCF4-A, and Shn can inhibit the transcriptional ability of Da in rat primary neurons (unpublished data, Alex Sirp). To investigate whether Shn affects the transcriptional ability of Da *in vivo*, we performed *in vivo* luciferase reporter assay in 0-24h old adult *Drosophila* brains. For this we used fly lines carrying a transgene of firefly luciferase under the control of 12x (CAGCTG) E-boxes and *Drosophila* minimal promoter. In addition, these flies had transgenes of *elav*-Gal4;UAS-*shn* and/or *elav*-Gal4;UAS-*da* that drives either Shn or Da overexpression or both in neurons. Fly line carrying firefly luciferase construct and *elav*-Gal4 heterozygously was used as a control. In order to confirm that the transcriptional activation of luciferase gene by Da was E-box specific, we used fly line carrying firefly luciferase under the control of the minimal promoter only and *elav*-Gal4;UAS-*da* that drives Da overexpression in neurons. Results showed that transcriptional activation of luciferase by Da is indeed E-box specific (Figure 10). We also confirmed that overexpressed Da is capable of activating E-box controlled luciferase gene transcription while overexpressed Shn has no effect on luciferase activity (Figure 10). Lastly, our results showed that overexpression of Shn lowers Da transcriptional ability significantly (Figure 10). In conclusion, our results indicate that Da and Shn interact *in vivo* in 0-24 h old adult *Drosophila* brains.

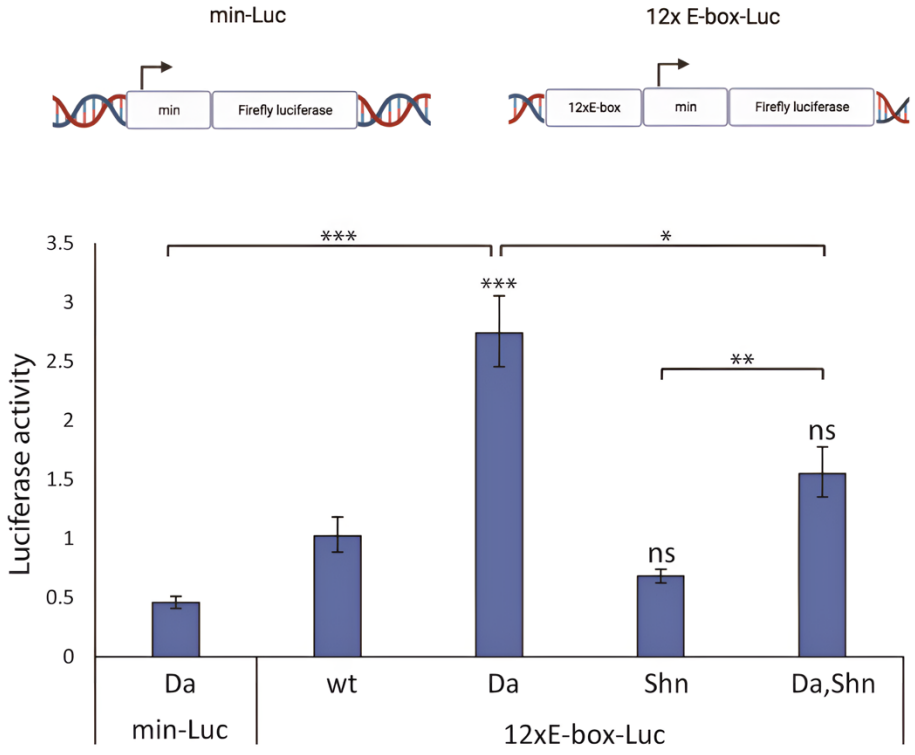

Figure 10. Shn decreases transcriptional ability of Da in 0-24h old adult *Drosophila* brains. *In vivo* luciferase reporter assay with fly lines carrying firefly luciferase under the control of 12x (CAGCTG) E-boxes and minimal promoter and *elav*-Gal4;UAS-*shn* (12x E-box; Shn) or *elav*-Gal4;UAS-*da* (12x E-box-Luc; Da) or both (12x E-box-Luc; Da, Shn). Control line carrying firefly luciferase under the control of 12x (CAGCTG) E-boxes (12x E-box-Luc; wt). Second control line carrying firefly luciferase under the control of the minimal promoter and *elav*-Gal4;UAS-*da* (min-Luc; Da). n=3, statistical

significance is shown with asterisks relative to “wt” or between the groups connected with lines. *p<0.05, **p<0.01, ***p<0.001, ns- nonsignificant, one way ANOVA with Tukey.

Discussion

Transcription factor 4 (TCF4) is a basic helix-loop-helix transcription factor, that has a crucial role in nervous system development, including proliferation, differentiation, migration, and synaptogenesis (Forrest et al., 2013; Chen et al., 2016; D'Rozario et al., 2016; Hill et al., 2017). Human immunodeficiency virus type I enhancer-binding protein 2 (HIVEP2) is a zinc finger transcription factor that regulates the activity of many genes and is linked to brain development (Dörflinger et al., 1999; Fukuda et al., 2002; Iwashita et al., 2012). Mutations in *TCF4* and *HIVEP2* can cause diseases with very similar outcomes. Mutations or deletions in one allele of *TCF4* result in Pitt-Hopkins syndrome (PTHS) described by severe developmental delay and mental retardation (Amiel et al., 2007; Brockschmidt et al., 2007; Zweier et al., 2007). In addition, large deletions affecting only longer TCF4 protein isoforms cause intellectual disability (Kharbanda et al., 2016). Mutations in one allele of *HIVEP2* cause HIVEP2-related intellectual disability (Srivastava et al., 2016; Steinfeld et al., 2016). HIVEP2 and TCF4 are also associated with schizophrenia (Stefansson et al., 2009; Volk et al., 2015). Both TCF4 and HIVEP2 have homologs in *Drosophila melanogaster*. Daughterless is a TCF4 homolog and Schnurri is HIVEP2 homolog in fruit fly (Caudy et al., 1988; Arora et al., 1995). *Drosophila* has been validated as a model for Pitt-Hopkins syndrome allowing researchers to study TCF4 functions using Da (Tamberg et al., 2015).

Previously, it has been shown that HIVEP2 and TCF4 interact in *in vitro* pulldown experiments and activate the Somatostatin receptor type 2 (SSTR2) expression in neural cell lines and that HIVEP2 expression overlaps with SSTR2 and TCF4 expression in the hippocampus and frontal cortex- brain regions associated with memory and learning (Dörflinger et al., 1999). Transcriptome analyses through RNA-sequencing have revealed that TCF4 regulates HIVEP2 mRNA levels (Mesman et al., 2020). In our lab it has been shown that HIVEP2 potentiates the transcriptional ability of TCF4-A upon depolarization in rat primary neurons (unpublished data, Alex Sirp). In this thesis, we decided to use the fruit fly as a model to investigate whether HIVEP2 and TCF4 fruit fly homologs Shn and Da also interact in *Drosophila* nervous system to further validate the interaction between TCF4 and HIVEP2 and to better understand the connection between HIVEP2-related intellectual disability and PTHS.

Da has shown to be expressed in all developmental stages of the fruit fly, expression being most abundant at 5-12 h of the development (Cronmiller and Cummings, 1993; Tamberg et al., 2020). In the heads of adult flies, Da expression has shown to be highest in 1-day-old females and decreases thereafter (Tamberg et al., 2020). So far there is no information about Shn protein expression in larvae or adult *Drosophila* nervous system. Based on *shn* mRNA sequencing analyses, *shn* is expressed in all developmental stages of the fruit fly, with higher expression level in some of the pupal stages and in 1-day-old females (Brown et al., 2014). We also saw that Shn and Da expression decreased during development, being highest in the 3rd instar larval brain and lowest in 8 days old adult fly brain. Thus, our results were consistent with previous data, showing higher expression levels of Da and Shn in the brains of young adults and lower levels in 8 days old adult flies. Immunohistochemical analysis of Da expression has shown that Da is widely expressed in the adult *Drosophila* brain including the mushroom bodies and optic lobes (Tamberg et al., 2020). We saw very similar expression pattern of Da. We showed for the first time that Shn is also widely expressed in the adult *Drosophila* brain. Although there is no information about HIVEP2 protein expression in

rodent or human nervous system, it has been shown that *HIVEP2* mRNA expression in adult rat is particularly high in the brain (Makino et al., 1994; Campbell and Levitt, 2003). In the adult rat brain, *HIVEP2* mRNA is strongly expressed in the olfactory bulb, cerebral cortex, hippocampus and, cerebellum (Fukuda et al., 2002). In adult *Drosophila* brain, Shn is also expressed in brain regions like mushroom bodies, antenna lobes which share similarities to mammalian hippocampus and olfactory bulb. Although we could see strong Shn specific signals in many cell nuclei, the anti-HA antibody also gave some unspecific signal. Thus, further experiments are needed to get a full understanding of the expression pattern of Shn. We also saw that Da expression overlaps with Shn expression in many cells in different brain regions, including the mushroom bodies. Downregulation of Shn or Da in larval mushroom bodies has shown to impair larval memory and learning (Bachelor's thesis, Loviisa Pihlas 2021; Tamberg et al., 2020). Thus, Shn and Da may be involved in similar functions in adult nervous system. Overall, these results indicate that Shn and Da potentially interact in the 0-24 h old fly brain.

We showed that overexpression of Shn significantly lowers Da transcriptional ability in 0-24 h old adult *Drosophila* brains. This is confirmed by an earlier study which shows that Shn acts mainly as a repressor when regulating the expression of Dpp target genes (Torres-Vazquez et al., 2000). It has been shown that Shn also inhibits Da transcriptional activity upon depolarization in rat primary neurons (unpublished data, Alex Sirp), which further supports our results, indicating that Da and Shn potentially interact *in vivo* in 0-24 h old adult *Drosophila* brains. The effect of Shn on Da can also be indirect. The activity of Da can also be affected by some transcription factor regulated by Shn, which is then either silenced or activated upon overexpression of Shn. It is interesting that the human homologues of these proteins, HIVEP2 and TCF4, act differently. It has been shown in our neurobiology lab that HIVEP2 potentiates the transcriptional activity of TCF4-A upon depolarization in rat primary neurons (unpublished data, Alex Sirp), functioning as an activator of TCF4 in this context.

To further validate the interaction between TCF4 and HIVEP2 and to see whether Da and Shn also interact in mammalian cell lines we performed co-immunoprecipitation analysis (co-IP) by overexpressing these proteins in HEF293 cells. TCF4-A and TCF4-B isoforms were chosen, because these are the most studied isoforms of TCF4. As TCF4-B is one of the longest isoforms of TCF4 and TCF4-A is one the shortest, we could also investigate whether there is a difference between the isoforms when it comes to interaction with FLAG-HIVEP2 (Sepp et al., 2011). We found out that co-IP with HIVEP2 and TCF4-A was a success, showing that TCF4-A and HIVEP2 interact. Co-IP with HIVEP2 and TCF4-B only worked one way– we were able to pull down HIVEP2 when immunoprecipitating TCF4-B. This indicates that the interaction between TCF4-B and HIVEP2 is weaker compared to interaction between TCF4-A and HIVEP2. In our neurobiology lab, Alex Sirp has done the same experiment with TCF4 and HIVEP2 in HEK293 cell line (unpublished results, Alex Sirp). His results showed that co-IP was only successful with TCF4-A and HIVEP2. The finding that TCF4-B also interacts with HIVEP2 is novel. In co-IP analysis of *Drosophila* homologs, we saw that co-IP worked only one way- we were able to pull down Shn when immunoprecipitating Da. This could mean that the anti-HA antibody used for co-IP gave weaker interactions with Shn or the interaction between Shn and Da could have been lost during the washing steps of the protocol. Despite the fact that the co-IP with the fly proteins was only successful when Da was pulled down,

our results showed that both the mammalian proteins TCF4 and HIVEP2 and their *Drosophila* homologs Da and Shn interact in overexpressed conditions in HEK293 cells.

In conclusion, we show that Shn and Da are expressed at the same time period in 3rd instar larvae and adult *Drosophila* brain. We also show that Shn and Da are partially co-expressed in adult *Drosophila* brain. We were able to clone *shn* to mammalian expression vector and validated its functionality in HEK-FT cells. By studying HIVEP2 and TCF4 as well as Da and Shn interactions in HEK293 cells we were able to confirm that overexpressed HIVEP2 and TCF4 and their fruit fly homologs Shn and Da interact in HEK293 cells. Finally, we showed that overexpression of Shn causes a decrease in Da transcriptional ability in adult *Drosophila* brains. These results indicate that Shn and Da interact both *in vitro* and *in vivo* in adult *Drosophila* brain, making *Drosophila* a good model organism to study the connections between HIVEP2 and TCF4-associated diseases.

Conclusions

The main results from this thesis are as followed:

1. Da and Shn are expressed at the same time period in 3rd instar larvae and adult *Drosophila* brain.

2. Da and Shn are partially co-expressed in adult *Drosophila* brain.

3. TCF4 and HIVEP2 and their fruit fly homologs Da and Shn interact in HEK293 cells.

4. Overexpression of Shn causes a decrease in Da transcriptional ability in adult *Drosophila* brains.

As a follow up to this study, we should also perform chromatin immunoprecipitation (ChIP) assay with sequencing to find direct targets of Shn and compare these to the targets of Da.

Abstract

Human immunodeficiency virus type I enhancer-binding protein 2 (HIVEP2) and Transcription factor 4 (TCF4) are transcription factors that are important in the development of the brain and in mature nervous system. Both HIVEP2 and TCF4 are associated with severe mental retardation syndromes that are symptomatically similar. In our laboratory and in other scientific articles it has been shown that HIVEP2 and TCF4 are interacting, suggesting that these diseases might be caused by dysregulation of molecular pathways that are regulated by HIVEP2 and TCF4 together. HIVEP2 and TCF4 have both homologs in *Drosophila melanogaster*. In this thesis, we used the fruit fly as a model to better understand the connections between HIVEP2-related intellectual disability and PTHS by investigating the interactions between HIVEP2 and TCF4 fruit fly homologs Schnurri (Shn) and Daughterless (Da) in the nervous system of *Drosophila melanogaster*.

The aims of the current thesis were to describe if Shn and Da are expressed at the same time point in 3rd instar larvae and Adult *Drosophila* brain, by performing Western blot analysis. The second aim of the thesis was to investigate if Shn and Da are also co-expressed in adult *Drosophila* brain, by performing immunohistochemical staining. The third aim of the thesis was to study HIVEP2 and TCF4 as well as Shn and Da interactions in mammalian cell lines (HEK293) by performing co-immunoprecipitation analysis. For this, we cloned Shn mammalian expression vector and validated its expression in HEK-FT cells. The final aim of the thesis was to investigate whether Shn affects the transcriptional ability of Da *in vivo*.

The results of the present study showed that Shn and Da are expressed at the same time period in 3rd instar larvae and adult *Drosophila* brain. We also show that Shn and Da are partially co-expressed in adult *Drosophila* brain. We were able to clone mammalian Shn expression vector and validated its functionality in HEK-FT cells. By studying HIVEP2 and TCF4 as well as Shn and Da interactions in HEK293 cells we were able to confirm that overexpressed HIVEP2 and TCF4 and their fruit fly homologs Shn and Da interact in HEK293 cells. Finally, we showed that overexpression of Shn causes a decrease in Da transcriptional ability in adult *Drosophila* brains.

In conclusion these results indicate that Shn and Da interact both *in vitro* and *in vivo* in adult *Drosophila* brain, making *Drosophila* a good model organism to study connections between HIVEP2-related intellectual disability and PTHS.

Annotatsioon

Human immunodeficiency virus type I enhancer-binding protein 2 (HIVEP2) and *Transcription factor 4* (TCF4) on transkriptsioonifaktorid, mis on olulised nii aju arengus kui ka närvisüsteemi toimimises. Nii HIVEP2 kui ka TCF4 on seotud raskete vaimse alaarengu sündroomidega, mis on sümptomaatiliselt sarnased. Nii meie laboris kui ka teistes teaduslikes artiklites on näidatud, et HIVEP2 ja TCF4 interakteeruvad, mis viitab sellele, et need haigused võivad olla põhjustatud samade signaalradade düsregulatsioonist, mida reguleerivad HIVEP2 ja TCF4. Käesolevas töös kasutasime äädikakärbest mudelina, et uurida HIVEP2 ja TCF4 äädikakärbse homoloogide Schnurri (Shn) ja Daughterlessi (Da) interaktsiooni äädikakärbse närvisüsteemis, et paremini mõista HIVEP2-seotud vaimse alaarengu ja Pitt-Hopkinsi sündroomi (PTHS) vahelisi seoseid.

Käesoleva töö eesmärk oli *Western blot* analüüsi abil kirjeldada, kas Shn ja Da ekspresseeruvad samaaegselt 3. staadiumi vaklade ja täiskasvanud äädikakärbse ajus. Töö teiseks eesmärgiks oli immunohistokeemilise värvimise abil uurida, kas Shn ja Da ekspresseeruvad täiskasvanud äädikakärbse ajus samades rakkudes. Töö kolmandaks eesmärgiks oli uurida HIVEP2 ja TCF4 ning nende kärbse homoloogide Shn ja Da interaktsioone imetajate rakuliinides (HEK293), kasutades ko-immunosadestamise analüüsi. Selleks tuli kloneerida Shn imetaja ekspressioonivektor ning testida selle funktsionaalsust HEK-FT rakkudes. Töö viimaseks eesmärgiks oli uurida, kas Shn mõjutab Da transkriptsioonivõimet *in vivo*.

Käesoleva uuringu tulemused näitasid, et Shn ja Da ekspresseeruvad samal ajal 3. staadiumi vaklade ja täiskasvanud äädikakärbse ajus. Samuti näitasime, et Shn ja Da ekspresseeritakse osaliselt täiskasvanud äädikakärbse aju samades rakkudes. Töö tulemusena klooneerisime ka imetaja Shn ekspressioonivektori ja kinnitasime selle funktsionaalsuse HEK-FT rakkudes. Lisaks leidsime, et üleekspresseeritud HIVEP2 ja TCF4 ning nende äädikakärbse homoloogid Shn ja Da interakteeruvad HEK293 rakkudes. Lõpuks näitasime, et Shn üleekspressioon põhjustab Da transkriptsioonivõime vähenemist täiskasvanud äädikakärbse ajus.

Kokkuvõttes näitavad need tulemused, et Shn ja Da interakteeruvad täiskasvanud äädikakärbse ajus nii *in vitro* kui ka *in vivo*. Seega saab kasutada äädikakärbest hea mudelorganismina uurimaks HIVEP2-seotud vaimse alaarengu ja PTHS vahelisi seoseid.

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Supplementary material

Supplementary table 1. Praimers for Colony PCR and Gibbson assembly

Praimers for Colony PCR	
EF1a-S	5’ GTTCATTCTCAAGCCTCAGACAG 3’
20rev	5’ GTCGTTAACGATTGGCAGCA 3’
Praimers for Gibson assembly	
pUC-Act FWD	5' cagcagcggcaagcaactgataactcgaccggcatgcaag3'
pUC-Act REV	5' tgcgtcgcgccatcattttgtctctggattagacgactgc 3'
POT2 FWD	5' agctcgaaccaccaaatggggtggttaaaaggaatgcctataagcc 3'
POT2 REV	5' cttgcatgccggtcgagttatcagttgcttgccgctgctg 3'
HA-Shn FWD	5' gcagtcgtctaatccagagacaaatgatggcgcgacgca 3'
HA-Shn REV	5' taggcattccttttaaccaccccatttggtggttcgagct 3'

Supplementary table 2. Co-immunoprecipitation antibodies

Antibody	Samples used for	Dilution in Western Blot	Amount used in co-IP
Mouse anti-FLAG M2, Sigma-Aldrich	Mammalian and *Drosophila*	1:5000	5 µg per IP
Mouse anti TCF4 (Santa Cruz Biotechnolgy)	Mammalian	1:1000	4 µg per IP
Rat anti-HA 3F10, Roche,	*Drosophila*	1:10000	4 µg per IP

Supplementary table 3. Western blot antibodies

Shn and Da expression analysis	**Primary antibodies**	**Secondary antibodies**
Shn	Rat anti-HA 3F10, Roche, 1:1000	Goat anti-rat IgG HRP secondary antibody Invitrogen, 1:5000
Da	Mouse anti-FLAG M2-peroxidase (HRP), Sigma-Aldrich, 1:5000	
Validating expression of HA-Shn expression vector	**Primary antibodies**	**Secondary antibodies**
Shn	Rat anti-HA 3F10, Roche, 1:2000	Goat anti-rat IgG HRP secondary antibody Invitrogen, 1:5000
Da	Mouse anti-FLAG M2-peroxidase (HRP), Sigma-Aldrich, 1:5000	
Co-immunoprecipitation	**Primary antibodies**	**Secondary antibodies**
TFC4	Mouse monoclonal IgG2a 1:1000 (Santa Cruz Biotechnology)	Goat anti-mouse, HRP, Invitrogen 1:5000
HIVEP2/Da	Monoclonal anti-FLAG M2 antibody, Sigma-Aldrich, 1:5000	Goat anti-mouse, HRP, Invitrogen 1:5000
Shn	Rat anti-HA 3F10, Roche, 1:2000	Goat anti-rat IgG HRP secondary antibody Invitrogen, 1:5000

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