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Identifying novel members of the Wntless interactome through genetic and candidate gene approaches.

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Abstract

Wnt signaling is an important pathway that regulates several aspects of embryogenesis, stem cell maintenance, and neural connectivity. We have recently determined that opioids decrease Wnt secretion, presumably by inhibiting the recycling of the Wnt trafficking protein Wntless (Wls). This effect appears to be mediated by protein-protein interaction between Wls and the mu-opioid receptor (MOR), the primary cellular target of opioid drugs. The goal of this study was to identify novel protein interactors of Wls that are expressed in the brain and may also play a role in reward or addiction. Using genetic and candidate gene approaches, we show that among a variety of protein, Wls interacts with the dopamine transporter (target of cocaine), cannabinoid receptors (target of THC), Adenosine A2A receptor (target of caffeine), and SGIP1 (endocytic regulator of cannabinoid receptors). Our study shows that aside from opioid receptors, Wntless interacts with additional proteins involved in reward and/or addiction. Future studies will determine whether Wntless and WNT signaling play a more universal role in these processes.

2. Introduction

Wnt signaling is a key regulator of many embryonic processes including cell fate determination, proliferation, cell polarity, and morphogenetic movements (Clevers and Nusse, 2012; Nusse and Varmus, 2012; Willert and Nusse, 2012). This pathway has also been shown to be involved in maintenance of adult tissues and its dysregulation has been implicated in several disease states including cancer, neurological disorders, neurodegenerative diseases, and addiction (Cuesta et al., 2016; Cuesta et al., 2017; Dias et al., 2015; Jenney et al., 2016; Jin et al., 2010a; Petko et al., 2013; Tacelosky et al., 2015).

Recently, it been shown that regulation of Wnt signaling occurs not only at the level of the cellular cascades triggered in signal receiving cells, but also by controlling the amount of

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Wnt ligand secreted from signal producing cells. Wnt proteins are post-translationally modified with palmitate and palmitoleate in the endoplasmic reticulum, and these additions are required for both secretion and the ability to activate Frizzled receptors (Takada et al., 2006; Willert et al., 2003; Zhai et al., 2004). Due to their hydrophobic nature, Wnt proteins require a specialized secretory pathway, and as a morphogen must also have a mechanism by which to diffuse through an aqueous environment to distant cells. Wntless (Wls) is a protein that interacts with lipid-modified Wnt ligands and traffics them from the Golgi to the plasma membrane for secretion (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006; Herr and Basler, 2012). In Drosophila cells, removal of Wls results in accumulation of Wnt proteins in the Golgi (Banziger et al., 2006). After Wnt release, Wls is recycled via clatherin-mediated endocytosis and retromer-mediated retrograde transport to the Golgi network (Belenkaya et al., 2008; Eaton, 2008; Franch-Marro et al., 2008; Harterink et al., 2011; Port et al., 2008; Yang et al., 2008). Blocking endocytosis of Wls in *C. elegans* or *Drosphila* leads to an accumulation of Wls at the plasma membrane and a decrease in the overall levels of Wnt secreted (Franch-Marro et al., 2008; Pan et al., 2008; Yang et al., 2008). Inhibiting retromer function does not alter endocytosis, but routes endosomal Wls to the lysosome for degradation, also leading to a decrease in Wnt secretion (Franch-Marro et al., 2008; Yang et al., 2008). These findings indicate that Wls must be recycled to participate in multiple rounds of Wnt secretion, and blocking this function is extremely deleterious to the developing organism.

We have previously demonstrated that morphine inhibits Wnt secretion from Wnt producing cells via direct interaction between the mu-opioid receptor (MOR) and Wls (Jin et al., 2010a). Electron microscopy of immunogold labeled rat brain indicates that morphine induces a shift in Wls localization from intracellular compartments to the plasma membrane of striatal dendrites (Reyes et al., 2012). The interaction between Wls and MOR is enhanced in the presence of morphine, and we, therefore, hypothesized that this interaction leads to the sequestration of the MOR/Wls protein complex at the plasma membrane (Jin et al., 2010a). This, in turn, inhibits the recycling of Wls, and ultimately reduces Wnt secretion and signaling. Wls is also known to interact with Kappa and Delta opioid receptors (Petko et al., 2013), but whether activation of these receptors alters Wls function and localization is currently unknown.

Several recent studies have shown that Wls protein levels are altered in animals that are exposed to morphine pellets (experimenter administered) or in rats that exhibit addiction-like behaviors for heroin in self-administration paradigms (Herrero-Turrion et al., 2014; Jenney et al., 2016; Petko et al., 2013; Tacelosky et al., 2015). These results provide support for the idea that opioids may serve to alter Wnt secretion and signaling, as well as some of the neural changes that accompany opioid addiction. It has been established that the canonical Wnt signaling pathway in the nucleus accumbens and prefrontal cortex are critical regulators of cocaine-induced neuroplasticity and sensitization (Cuesta et al., 2016; Cuesta et al., 2017; Dias et al., 2015). Taken together, these studies suggest that alterations in Wnt signaling may underlie general changes in cell structure and function that are associated with multiple drugs of abuse, including opioids and stimulants.

Studies from our group were among the first to show that Wls function and Wnt secretion may be regulated via interaction with G-protein coupled receptors. Based on these observations, we now seek to identify novel interacting proteins of Wls and to determine whether these binding partners play a role in mediating reward and/or addiction. Three yeast-2-hybrid screens using human fetal brain cDNA libraries were performed, and thirty-five potential interacting proteins for Wls were identified. The physical association of Wls with selected interactors identified in the screens, as well as several candidate GPCRs, were tested via coimmunoprecipitation and GST-pulldown approaches. Our results indicate that Wls interacts with several reward and addiction-related molecules including cannabinoid receptors, the adenosine A2a receptor, and the dopamine transporter. Future studies will be designed to test the ability of these interactors to regulate Wnt secretion in response to drugs of abuse.

3. Methods

3.1 Yeast 2-Hybrid Techniques

3.1.1 Classical yeast two-hybrid screen.—Classical yeast two-hybrid screens (Y2H) involve linking bait and prey proteins to the DNA-binding domain and activation domain, respectively, of the modular transcription factor GAL4 from yeast (Fields and Song, 1989). These fusion proteins must enter the yeasts nucleus and physically interact in order to activate yeast reporter gene (ex. LacZ, encodes β-galactosidase) expression (Fig. 1). The requirement for nuclear localization limits the size and hydrophobicity of the proteins that can be used in the classical Y2H screens. Since, as shown in Fig. 1, Wls is predicted to be an 8 transmembrane domain-containing protein (Jin et al., 2010b), we used short intracellular pieces of the human Wls protein including the third intracellular loop (Wls-IL3; amino acids 402–431) and the C-terminal tail (Wls-CT; amino acids 493–541) as baits by cloning the pieces individually into the pGBKT7 bait vector (Clontech, Palo Alto, CA) containing the sequence for the GAL4 DNA-binding domain (BD). Bait plasmids were transformed into the matchmaker gold yeast strain (Clontech) and then mated with yeast containing an empty pGADT7 (Clontech) prey plasmid. Both bait plasmids tested negative for auto-activation of β-galactosidase (β-gal) activity in a nitrocellulose lift assay. Yeast containing either Wls-IL3 or Wls-CT were then mated with yeast containing a cDNA library from human fetal brain (Clontech). Yeast were grown on double dropout (-Leu,-Trp) selection medium containing X-alpha-gal. Blue colonies (positive for Wls/protein interaction) were additionally tested for interaction by growth on quadruple dropout selection plates (-Leu,-Trp,-His,-Ade) containing 3-aminotriazole (3-AT). The identity of the prey cDNAs was determined by DNA extraction from yeast colonies, sequencing of the prey plasmids, and BLAST searches of the REF SEQ RNA database (www.ncbi.nlm.nih.gov).

3.1.2 Membrane yeast 2-hybrid screen.—Membrane yeast 2-hybrid (MYTH) screens (also called split-ubiquitin screens) involve membrane-localizing bait and prey proteins that are fused to the C-terminal or N-terminal half of ubiquitin, respectively (Kittanakom et al., 2009; Snider et al., 2010). In addition, the bait protein is fused to a transcription factor such as LexA. Upon interaction, the ubiquitin molecule is reconstituted and an ubiquitin-specific protease cleaves and releases the transcription factor from the bait

protein (Fig. 1). The transcription factor is then free to enter the nucleus and activate the transcription of LacZ or other reporter genes. The full open reading frame of human Wls was cloned into the bait plasmid pCCW-STE (Dualsystems Biotech AG, Switzerland) and transformed into the THY.AP4 yeast strain. The bait tested negative for auto-activation of β-gal activity in a nitrocellulose lift assay when cotransformed with the empty prey plasmid pPR3-N (Dualsystems). The yeast bait strain was then cotransformed with a human fetal brain cDNA library (Dualsystems) and grown on quadruple dropout selection (-Leu,-Trp,-His,-Ura) plates containing 3-AT. Resulting colonies were regrown and subjected to nitrocellulose lift assays for interaction confirmation. 2.7×10^6 clones were screened and 89 transformants tested positive for β-gal activity. Prey plasmids were extracted, sequenced, and identified as in the classical Y2H screen.

3.1.3 Directed Yeast 2-Hybrid Assays.—Isolated prey plasmids from both Y2H screens were retransformed into the corresponding bait strains containing control bait plasmids or the original Wls bait plasmid. Empty pGBK plasmid served as a negative control for the classical Y2H screen, and pCCW-STE containing the full length mu-opioid receptor (MOR) was used as a negative control for the MYTH screen. Yeast were grown in serial dilutions (one colony resuspended in 100ul water and diluted 1:10, 1:100, and 1:1000) on quadruple dropout (-Leu,-Trp,-His,-Ade) plates containing 3-AT and on double dropout plates (-Leu,-Trp) for nitrocellulose lift assays. Lift assays were performed as described previously (Lin et al., 2005).

3.2 Interaction confirmation techniques

3.2.1 Cell Lines, transfections, and antibodies.—HEK-293T cells were obtained from ATCC. HEK-293 cell lines stably transfected with A2A adenosine receptor (A2A-293) or D2 Dopamine receptor (D2R-293) were a generous gift from Dr. Jurgen Zezula, Medical University Vienna/Center for Biomolecular Medicine and Pharmakology, Vienna, Austria and Dr. Mark van Zastrow, University of California San Francisco, respectively (Gsandtner et al., 2005; Vickery and von Zastrow, 1999). All cell lines were maintained in DMEM + 10% FBS + 1% Pen/Strep. Stably transfected cells were additionally treated with G418 to maintain the integrated plasmid. Transfection of 293T cells with plasmids was achieved using Effectene (Qiagen, Valencia, CA) according to manufacturer's protocol. For co-immunoprecipitation and GST pulldown studies, cells were lysed using lysis buffer (20 mM Tris-HCl, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1% Triton X-100, and 1mg/mL leupeptin) containing protease inhibitor cocktail (cOmplete MINI EDTA free: Roche, Indianapolis, IN). The following antibodies were used in this study: rat anti- dopamine transporter antibody (1:5000; Chemicon International Inc., Temecula, CA), chicken anti-Wls antibody (Jin et al., 2010a), rabbit anti-flag (1:10,000; Sigma-Aldrich, St. Louis, MO), HRP-conjugated S-tag (1:5000, Novagen, Madison, WI), mouse anti-HA (1:2500; Covance, Emeryville, CA), rabbit anti-A2A (Abcam, Cambridge, MA), and mouse anti-MYC (1:5000 dilution; Millipore, Billerica, MA). HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA) and used at a 1:20,000 dilution. Immunoreactivity was detected by enhanced chemiluminescence with an ECL2 kit (Pierce, Rockford, IL).

3.2.2 GST Pulldowns.—Bait constructs were generated by sub-cloning Wls-IL1 (residues 254–268), Wls-IL2 (residues 325–331), Wls-IL3 (residues 402–431), Wls-CT (residues 493–541), A2A-IL1 (residues 33–42), A2A-IL2 (residues 101–120), A2A-IL3 (residues199–234), A2A-CT (residues 291–412), A2A-CT1 (residues 291–312), and A2A-CT2 (residues 312–412) in to the pGEX-4T-1 bacterial expression plasmid (Amersham Biosciences, Piscataway, NJ). DAT-NT (residues 1–60), DAT-IL1 (residues 119–139), and DAT-CT (residues 575–620) in pGEX-4T-1 were a generous gift from Gonzalo Torres (University of Pittsburgh). S-tagged prey proteins for GST pulldowns were generated by sub-cloning directly from prey plasmids into the pET30 (Novagen, Madison, WI) bacterial expression plasmid and performing bacterial auto-inductions. Breifly, pGEX-4T-1 and pET30 plasmids were transformed into BL21 (DE3) E.coli cells and induced using ZYP-5052 auto-induction media as described previously (Studier, 2005). Bacteria were sonicated in PBS containing 1% Triton x 100 and protease inhibitors (cOmplete MINI EDTA free: Roche) and the resulting lysates were cleared by centrifugation. GST fusion proteins were bound to glutathione sepharose beads (GE Healthcare, Piscataway, NJ) and then incubated with either S-tagged interacting proteins or with lysates from transiently transfected 293T, stably transfected HEK-293 cells, or rat brain cell lysates. GST-only bound beads were used as a negative control. Proteins were eluted with loading dye containing SDS and β- mercaptoethanol, separated by SDS-PAGE, and analyzed by western blotting.

3.2.3 Co-immunoprecipitations.—For immunoprecipitations (IP) involving candidate Wls interactors, cDNA from prey plasmids were subcloned into pCMV-Tag3B containing a FLAG-tag (Stratagene, La Jolla, CA). DAT-Flag in pcDNA3 was provided by Gonzalo Torres (University of Pittsburgh, PA), while HA-tagged CB1 and CB2 cannabinoid receptors were a gift from Ken Mackie (Indiana University, Bloomington, IN). For Wls homodimerization studies, full length Wls was cloned into pEGFP (Clontech), and the Wls-CT was cloned into pCMV-Tag2B containing a myc tag (Stratagene). Constructs were transfected into 293T cells using Effectene (Qiagen), and cells lysed as described above. For IP experiments involving D2R or A2A, the stably expressing D2L-HEK-D2L or HEK-A2A cells were used to generate cell lysates. IPs were performed using Protein-G mag-sepharose beads (GE) and the antibodies indicated in Figures 3–5. Proteins were eluted with loading dye, separated by SDS-page, and analyzed by western blotting.

4. Results

4.1 Yeast two-hybrid screens for Wls interacting proteins

To identify novel interactors of Wls that may play a role in addiction or reward, three Y2H screens were performed using human fetal cDNA libraries. Two of the screens utilized classical Y2H methods with either the Wls-IL3 or the Wls-CT as bait. The Wls-IL3 screen yielded 16 colonies: 9 of which were identified as phosphoglucomutase 1 (PGM1) and 6 of which were identified as HCLS1 Associated Protein X-1 (HAX1). These proteins were also identified in the Y2H screen performed with the Wls-CT. PGM1 and HAX1 each auto-activated growth on quadruple dropout nutrient selection plates and demonstrated β-galactosidase activity when transformed into yeast containing empty bait plasmid (data not

shown). We conclude, therefore, that each of the positive clones from the Wls-IL3 screen represented false positives.

The Wls-CT screen yielded 110 colonies, all of which turned blue on X-α-Gal plates. Those prey that were identified as PGM1 or HAX1 were not pursued further. The identities of the remaining positive clones are presented in Table 1. These proteins have a wide variety of functions including cell structure/adhesion (CDH10, KRT222), cell signaling regulation (CAP1, SGIP1), protein folding (HSP40, PPIG), ubiquitination (CUL1, HECTD1), and active transport of ions (ATP1B1, ATP1B3). Prey plasmids were retransformed into yeast containing the Wls-CT bait plasmid or with empty bait plasmid to confirm the interaction and to test the prey protein for auto-activation. Several clones exhibited auto-activation of both growth and β-gal activity

These include the two β subunits of the sodium-potassium ATPase, CDH10, DDX40, and CUL1 (Fig. 2A), and thus are likely false positives. Some clones auto-activated in the growth assay, but not in the β-gal activity assay. The Wls-CT bait did not grow or show auto-activation of β-gal in the presence of empty prey vector demonstrating that the bait does not auto-activate growth on selection media or exhibit autonomous β-gal activity.

In addition to the classical Y2H screen described above, we also performed a MYTH screen to identify membrane bound interactors of full length human Wls (Table 1). Prey plasmids were retested for their interaction with Wls in directed Y2H assays. Each prey demonstrated growth on nutrient selection and β-gal activity in the presence of full length Wls. However, none of the positive clones showed auto-activation when tested with the mu-opioid receptor as bait (Fig. 2B). Clones identified in the screen encode proteins of a variety of functions including trafficking (TVP23B, YIP1, CNIH4), post-translational modification (PIGW), and dopamine reuptake (DAT).

4.2 Validation of the Wls/protein interactions

Several full length positive clones identified in the MYTH screen were tested for interaction with Wls in mammalian cells. This was achieved by tagging the full length interactors with a FLAG tag, expressing them in HEK293T cells, followed by co-immunoprecipitation. As shown in Figure 3A, Wls co-immunoprecipitates with FLAG-tagged EBP, TVP23b, and DAT. Several of the FLAG-tagged proteins were not detectable in cell lysates suggesting that they did not express after transfection (Fig. 3A, top panel).

GST pulldowns were used to determine whether there was a direct interaction between Wls-CT and several proteins from the classical Y2H screen including C7ORF35, KRT222, PEX11b, PPIG, and SGIP. C7ORF and SGIP both successfully pulled down with Wls-CT (Fig. 3B) while KRT222, Pex11b and PPIG did not (data not shown). GST pulldowns were also used to map the interaction between Wls and the dopamine transporter (DAT). DAT is responsible for presynaptic reuptake of dopamine and is the primary molecular target of cocaine in the brain. To map site/s of interaction between Wls and DAT, the intracellular portions of Wls were separately fused to glutathione S-transferase (GST) and tested for their interaction with DAT endogenously expressed in rat brain lysates. As shown in Figure 3C, Wls-IL3 was able to precipitate DAT (band of 80 kDa) from whole brain lysates, although

DAT is undetectable in whole brain lysates, most likely due to low levels of expression. The largest intracellular portions of DAT, including the N-terminus (NT), the first intracellular loop (IL1) and the C-terminus (CT), were fused to GST and used to precipitate endogenous Wls from HEK-293T cell lysates. As shown in Figure 3D, only DAT-IL1 was able to precipitate full length Wls. These results indicate that the third intracellular loop of Wls physically interacts with the first intracellular loop of the DAT.

4.3 Wls heterodimerizes with cannabinoid receptors and the A2A adenosine receptor

We have previously shown that Wls can interact with each of the three opioid receptors, mu, kappa, and delta (Jin et. al., 2010). We hypothesized that other GPCR proteins might interact with Wls and regulate its function, however, no additional GPCRs were identified in our subsequent Y2H screens. Because our laboratory is interested in GPCR involvement in addiction and reward, we chose three candidate proteins including the cannabinoid receptors (CB1 and CB2; the cellular targets of THC) and the A2A adenosine receptor (A2A; target of caffeine) to test for interaction with Wls. Figure 4A shows that, as previously reported, Wls does not interact with the D2-dopamine receptor and, therefore, is not likely to be universally regulated by all GPCRs. Both HA-tagged CB1 and CB2 cannabinoid receptors co-immunoprecipitated with FLAG-tagged Wls from 293T cells exogenously expressing these proteins (Fig. 4B). Endogenous Wls co-immunoprecipitated with FLAG-tagged A2A from stably expressing A2A-293 cells using rabbit A2A specific antibodies (Fig. 4C). These results demonstrate that Wls interacts with several additional GPCRs besides the family of opioid receptors. GST-pulldowns using the intracellular portions of Wls were used to map the interaction site of Wls with the A2A adenosine receptor. A2A-FLAG from A2A-293 cells was pulled down by the Wls-CT but not by any other segment of Wls or GST alone. To map the portion of A2A that interacts with the Wls-CT, the intracellular portions of the A2A were fused to GST and incubated with S-tagged Wls-CT produced in bacteria. Figure 4G shows that the Wls- CT is pulled down with the N-terminal half of the A2A-CT. These results are similar to those observed with MOR. Like the Wls/A2A interaction the Wls/MOR interaction was mediated by reciprocal interactions between the C-terminal tail of each protein.

4.4 Homodimerization of Wls

We used an antibody that specifically recognizes the Wls-CT (Jin et al., 2010) to assess successful production of the GST-tagged Wls-CT fusion protein. As shown in Fig. 5A, the antibody detected two bands from glutathione-purified bacterial lysates containing Wls-CT-GST; one at the predicted size of 36 kDA, and another with a predicted size of ~ 70 kDa. Many transmembrane proteins are known to function as homo- or heterodimers. Based on our results, we hypothesized that Wls may form homodimers through its C-terminal tail. To test this hypothesis, 293T cell lysates were subjected to GST pulldown analysis using the intracellular portions of Wls bound to glutathione sepharose beads as bait. Endogenous Wls from the mammalian cell lysates was only detected in pulldowns containing the Wls-CT (Fig. 5B). To test whether Wls also forms homodimers in mammalian cells, 293T cells were co-transfected with FLAG-tagged Wls and GFP-tagged Wls. As shown in Figure 5C, the FLAG-tagged Wls was immunoprecipitated using a FLAG antibody, and both the IP of Wls-FLAG and the co-IP of Wls- GFP was detected using an antibody specific to the CT of Wls.

Wls-FLAG was detected as a doublet at about 50 kDA, while Wls-GFP was detected at 75 kDA due to the substantial size of the GFP tag., Wls-GFP is detected when an IP is performed with rabbit anti-FLAG, but not with a rabbit IgG control (Fig 5C). Finally, the CT of Wls was MYC-tagged and transfected into 293T cells along with full length Wls-FLAG. As shown in Fig. 5D, Wls-FLAG co-immunoprecipitated with Wls-CT-MYC. Together, these results support the hypothesis that Wls forms homodimers and that homodimerization occurs through the C-terminal tail of Wls.

5. Discussion

5.1 Summary of findings

Evidence is mounting that altered Wnt signaling is involved in neuroadaptations to cocaine (Cuesta et al., 2016; Cuesta et al., 2017; Dias et al., 2015). We have previously shown that for opioid drugs, this may be due to dysregulated Wnt secretion from Wnt producing cells. As shown previously, (Jin et al., 2010a; Reyes et al., 2012) morphine promotes the interaction of MOR and Wls, which in turn increases the level of Wls at the plasma membrane of striatal rat neurons and causes a decrease in Wnt secretion in cultured cells.

To identify novel, brain-related regulators of the Wnt secretion process, we used both genetic and candidate gene approaches to identify protein interactors of Wls. Three Y2H screens were performed which resulted in identification of a new cohort of potential Wls interactors. In addition, three candidate GPCR proteins were tested for their interaction with Wls due to their involvement in THC and caffeine reward processing. Finally, the homodimerization of Wls was assessed. In all, nine interactions were confirmed by either co-immunoprecipitation from cultured mammalian cells or GST-pulldowns. These include DAT, EBP, TVP23b, CB1, CB2, A2A, SGIP, C7ORF35, and Wls itself. The distinct interaction sites were mapped for three of these protein-protein interactions. Through its CT, Wls interacts with the CT of other Wls molecules, and with the CT of the A2A adenosine receptor. This is consistent with our previous observation that another GPCR, the mu opioid receptor, interacts with Wls through their respective CTs (Jin et al., 2010a). In contrast, it appears that the third intracellular loop of Wls interacts with first intracellular loop of the DAT, a 12 membrane spanning domain containing protein.

5.2 The current Wls interactome.

Our results have led us to propose a putative Wls interactome which is shown in Figure 6. Wls interacting proteins are sorted according to their various functions. While the confirmation of these interactions in mammalian tissues are an essential next step, the current state of the Wls interactome allows for some speculation on the functional significance of these interactions. Many Wls interacting proteins function in protein modification and trafficking. These may be general regulators of Wntless in its cycling to and from the plasma membrane or may play a more specific role in regulating WNT secretion in response to environmental triggers. A number of the newly identified interactors modulate cellular signaling, particularly the signaling of GPCRs. In addition, we demonstrate that Wls interacts with a number of GPCRs directly. We have previously shown that Wls function is regulated by MOR (a GPCR) activation. It will be of interest to

determine whether other GPCRs also affect Wnt secretion or whether reciprocal regulation exists. In other words, does Wls serve to regulate the signaling processes of GPCRs? While many of the interactors identified in this study may function in Wnt processes related to development or cancer, the remainder of this discussion will focus on the potential role of Wls/protein interactions in regulating the reward/addiction process.

5.3 Potential importance of newly identified Wls interacting proteins in addiction.

5.3.1 Dopamine transporter.—The dopamine transporter (DAT) is a presynaptic reuptake molecule that clears dopamine from the synaptic cleft. Inhibition of DAT by cocaine, or reversal of the transporter by methamphetamine, leads to sustained dopamine signaling in the nucleus accumbens and underlies the rewarding effects of these drugs (Gether et al., 2006). In addition, alterations in dopamine clearance by DAT have also been implicated in autism and attention deficit/hyperactivity disorder (Bowton et al., 2010; Hamilton et al., 2013; Mazei-Robinson and Blakely, 2006; Sakrikar et al., 2012; Vaughan and Foster, 2013). Changes in Wnt signaling have been detected in the nucleus accumbens and prefrontal cortex of cocaine consuming rats, and these changes lead to cocaine-induced neuroplasticity and sensitization (Cuesta et al., 2016; Cuesta et al., 2017; Dias et al., 2015). DAT internalization is constitutive but enhanced by exposure to PKC activators or substrates including dopamine, cocaine, methamphetamine, and amphetamines (Melikian, 2004; Torres et al., 2003). It will be interesting to determine whether the trafficking of DAT alters the intracellular localization of Wls and whether the Wls/DAT interaction is responsible for the alterations in Wnt signaling and the concomitant changes in plasticity and sensitization observed with cocaine use.

5.3.2 Cannabinoid receptors.—The cannabinoid receptors, CB1 and CB2 are GPCRs that regulate mood, appetite, memory, pain-sensation, and immune function (Lu and Mackie, 2016). CB1 is found in many tissues throughout the body with highest levels in specific areas of the brain (cortex, basal ganglia, nucleus accumbens, and cerebellum), and it is thought to be responsible for the majority of the rewarding effects of exogenous cannabinoids such as tetrahydrocannabinol THC, the psychoactive compound of cannabis (Katona et al., 1999; Katona et al., 2000; Katona et al., 2001; Marsicano and Lutz, 1999; Matyas et al., 2006; Matyas et al., 2007; Nyiri et al., 2005a; Nyiri et al., 2005b; Suarez et al., 2008; Tsou et al., 1998; Tsou et al., 1999). CB2 is much more restricted in its distribution and can be found in low levels in some neuronal populations and higher levels in microglial cells and cells of the immune system (Klein et al., 1998; Walter et al., 2003). In the nervous system, endogenous cannabinoids act as presynaptic neuromodulators and include the membrane lipid derivatives 2-arachidonoyl glycerol (2-AG) and anandamide (Kano et al., 2009). Dysfunction of this signaling system has been implicated to contribute to the etiology of schizophrenia (Bae et al., 2014; Ortega-Alvaro et al., 2011). Similarly, Wnt signaling has also been linked to neurodevelopmental processes and dysfunction of the Wnt signaling pathway may contribute to schizophrenia, bipolar disorder, and autism (Mulligan and Cheyette, 2017). In mammalian brains, both cannabinoid and Wnt signaling pathways have been shown to contribute to synaptic plasticity (Chen et al., 2006; Dickins and Salinas, 2013; Rosso and Inestrosa, 2013). *In vitro* studies have linked the endocannabinoid anandamide to inhibition of canonical Wnt signaling in breast cancer cells and the activation

of non-canonical Wnt signaling in cholaniocarcinoma (DeMorrow et al., 2008; Laezza et al., 2013). Whether the effects of cannabinoids on Wnt signaling also occur in the brain, and whether these effects rely on an interaction between CB1 and Wls remains to be determined.

5.3.3 A2A adenosine receptor.—A2A adenosine receptor is a neuromodulatory GPCR that plays an important role in neuroprotection, memory, and sleep. A2A interacts with the D2 dopamine receptor, a key receptor that regulates reward and motor function, and antagonizes D2R function (Fuxe et al., 2007). The A2A/D2R interaction has become a target for novel therapeutics aimed at treating Parkinson's disease (Jorg et al., 2014). Caffeine is an A2A antagonist that, while not considered addictive, does trigger the reward system within the brain by blocking the antagonistic effects of A2A on D2R. A2A, like MOR, interacts with Wls through its CT and is internalized upon agonist activation (Zezula and Freissmuth, 2008). Future studies will be necessary to determine whether A2A agonists and antagonists can modulate Wls trafficking and Wnt release.

5.3.4 SGIP1.—SGIP1 is an endocytic regulator that is expressed almost exclusively in the CNS (Trevaskis et al., 2005). SGIP was originally identified as an interactor of endophilin and a modulator of cellular energy homeostasis. More recently, SGIP1 has been shown to inhibit the endocytic trafficking of CB1 in response to agonist treatment (Hajkova et al., 2016). This regulation occurs through an interaction of SGIP1 with the CT of CB1. Because the CT of Wls is involved in the control of Wls trafficking, and appears to contain the SGIP1 binding site (present study), we hypothesize that SGIP1 may restrict the intracellular trafficking of Wls. Recent studies have shown that variants of SGIP are associated with specific brain wave patterns (reduced p300 amplitudes) that are often observed in alcoholics and their close non-alcoholic relatives (Chwedorowicz et al., 2016). It will be interesting to determine whether these SNPs in SGIP1 alter its trafficking functions for cell surface proteins such as CB1 and possibly Wls, and whether Wnt signaling is altered in individuals with a predisposition to alcoholism.

6. Conclusion

In summary, we have defined a novel cohort of Wls interacting proteins that together present an initial picture of the Wls interactome. Most importantly, many of these Wls interacting proteins function in processes that are also regulated by Wnt signaling such as reward, neurodevelopment, and motor function. It will clearly be of interest to characterize the significance of these Wls/protein interactions not only in the context of Wnt secretion and possibly Wnt-independent functions of Wls, but also whether these interactions contribute to the development of substance abuse, addiction, and relapse.

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Highlights

- Three yeast 2-hybrid screens elucidated 34 potential protein interactors of Wntless.
- Wls interacts with multiple regulators of signaling pathways involved in reward and addiction.
- DAT, CB1, CB2, and A2A receptor interact with Wls in mammalian cells.

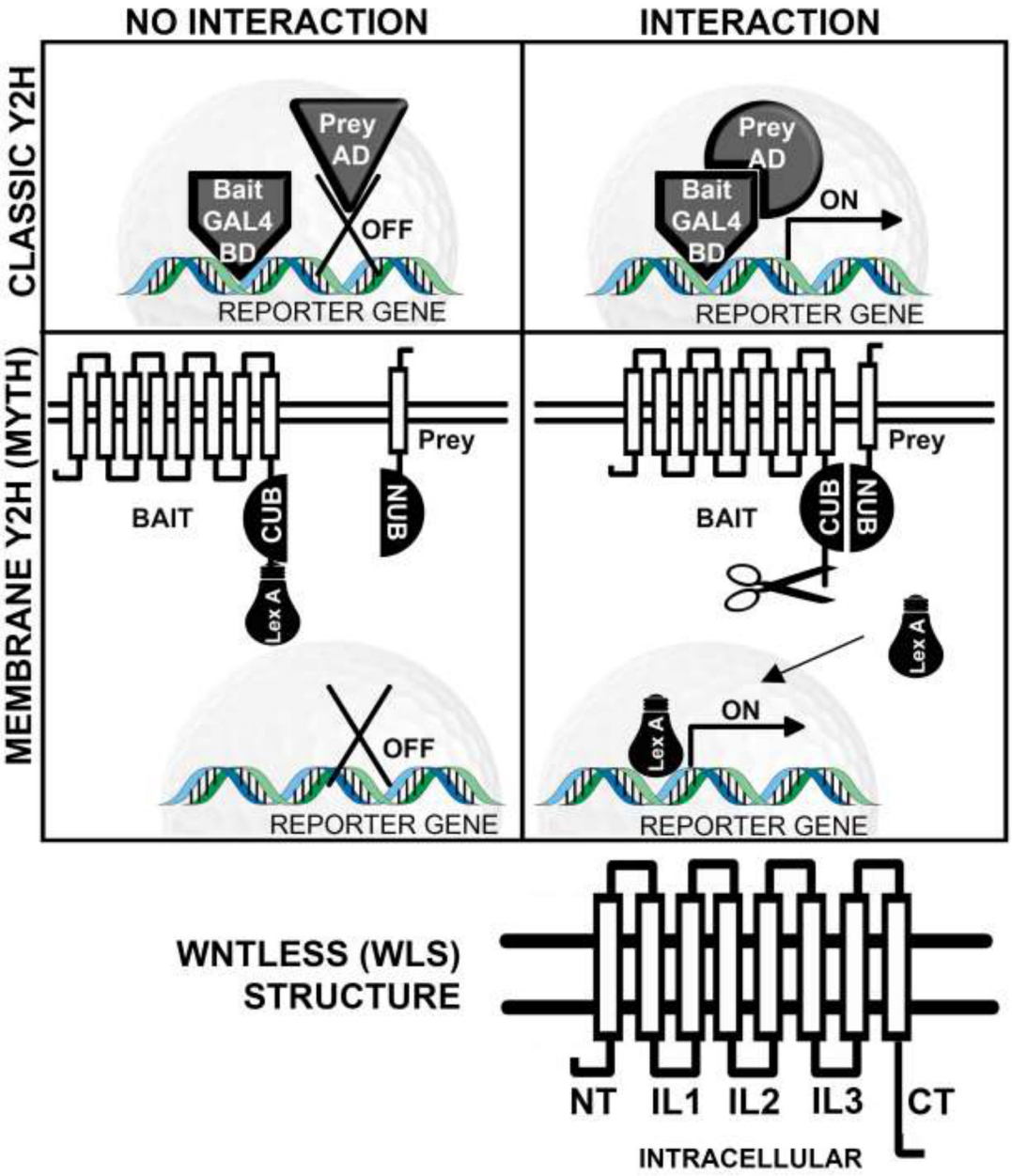

Figure 1. Yeast 2-hybrid screening.

Yeast 2-hybrid screens employ the reconstitution of the transcription factor, GAL4 (top panels), or the liberation of a membrane-restricted transcription factor, LexA (bottom panels), to activate reporter gene expression. Reporter genes allow synthesis of essential amino acids for growth on nutritional selection plates or production of the β-galactosidase enzyme and metabolism of X-gal into a blue pigment. The proposed transmembrane structure of Wls at the plasma membrane is depicted at the bottom of the figure (Jin et al., 2010b). Wls-IL3 and CT were used in classical screens, while the entire eight transmembrane structure was used as bait in the MYTH screen. In all three screens, prey constructs consisted of human fetal brain cDNA libraries. CUB – C-terminus of ubiquitin;

NUB – N-terminus of ubiquitin; BD – DNA binding domain; AD – transcriptional activating domain; NT – N-terminus; IL – intracellular loop; CT – C-terminus.

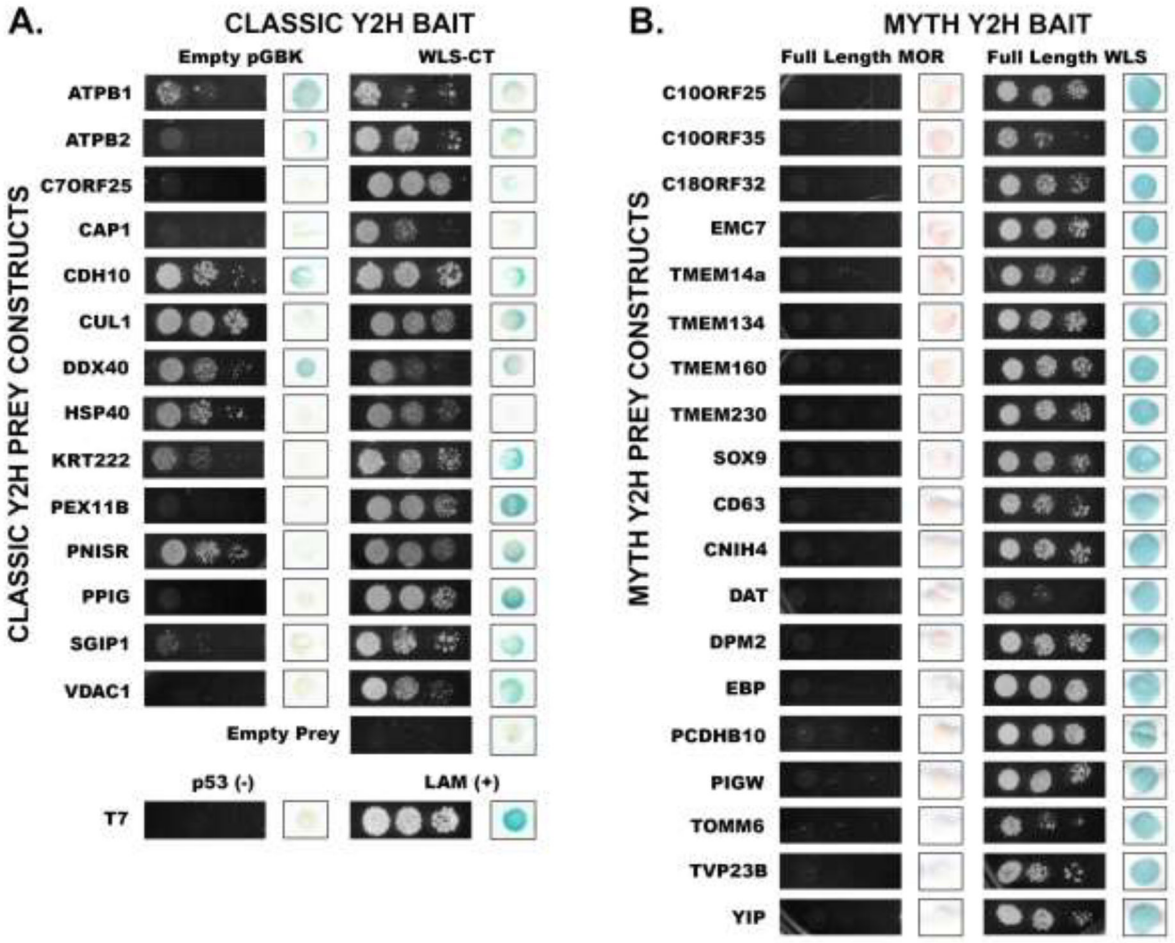

Figure 2. Directed Y2H of Wls/protein interactors.

Growth of serial dilutions of yeast expressing either bait or control plasmids along with prey plasmids from (A) classical or (B) MYTH screens on quadruple dropout nutritional selection plates (left panels – black background). β-galactosidase activity was assessed in the same yeast grown on double dropout selection media (right panels – white background). Blue staining indicates a positive interaction. Interaction with negative control indicates auto-activation. Wls C-terminus (CT) or empty pGBK bait vector (negative control) were used for classical Y2H analyses, while full length Wls or full length MOR were used in MYTH analyses.

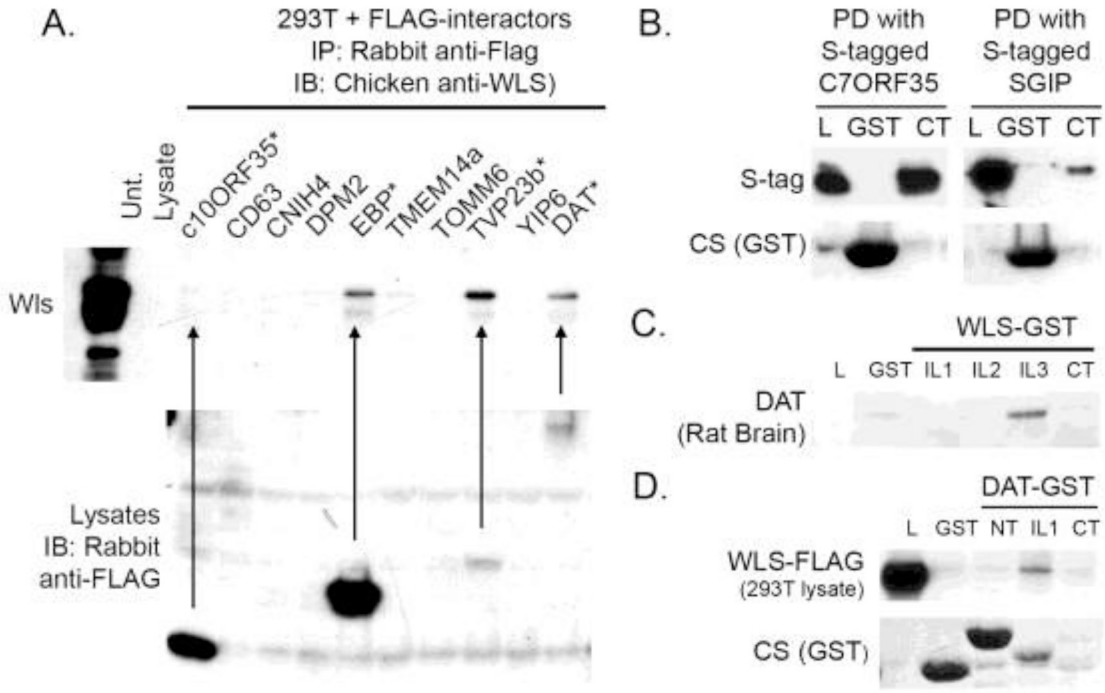

Figure 3. Confirmation and mapping of Wls interaction with the DAT(Dopamine Transporter).

(A) Top blot: Co-immunoprecipitation of Wls with FLAG-tagged interactors from transfected 293T cells. Bottom blot shows expression of the FLAG-tagged interactors in cell lysates used in the immunoprecipitations (IP). Proteins with detectable expression upon transfection are marked with an * and arrows are provided to show corresponding lysate and IP samples. (B) GST pulldowns of interactors from the classical Y2H screen (S-tagged C7ORF35 and SGIP) with the GST-tagged Wls-CT or GST alone. (C,D) GST pulldowns were used to map the interacting intracellular loop (IL) domains of Wls (C) and dopamine active transporter (D, DAT/SLC6A3) using GST-tagged and S-tagged proteins, produced in and isolated from E. coli. CS indicates coomassie staning of gels to show pulldown of GST tagged proteins. L – lysates; GST – glutathione S transferase; IP – immunoprecipitate; IB – immunoblot.

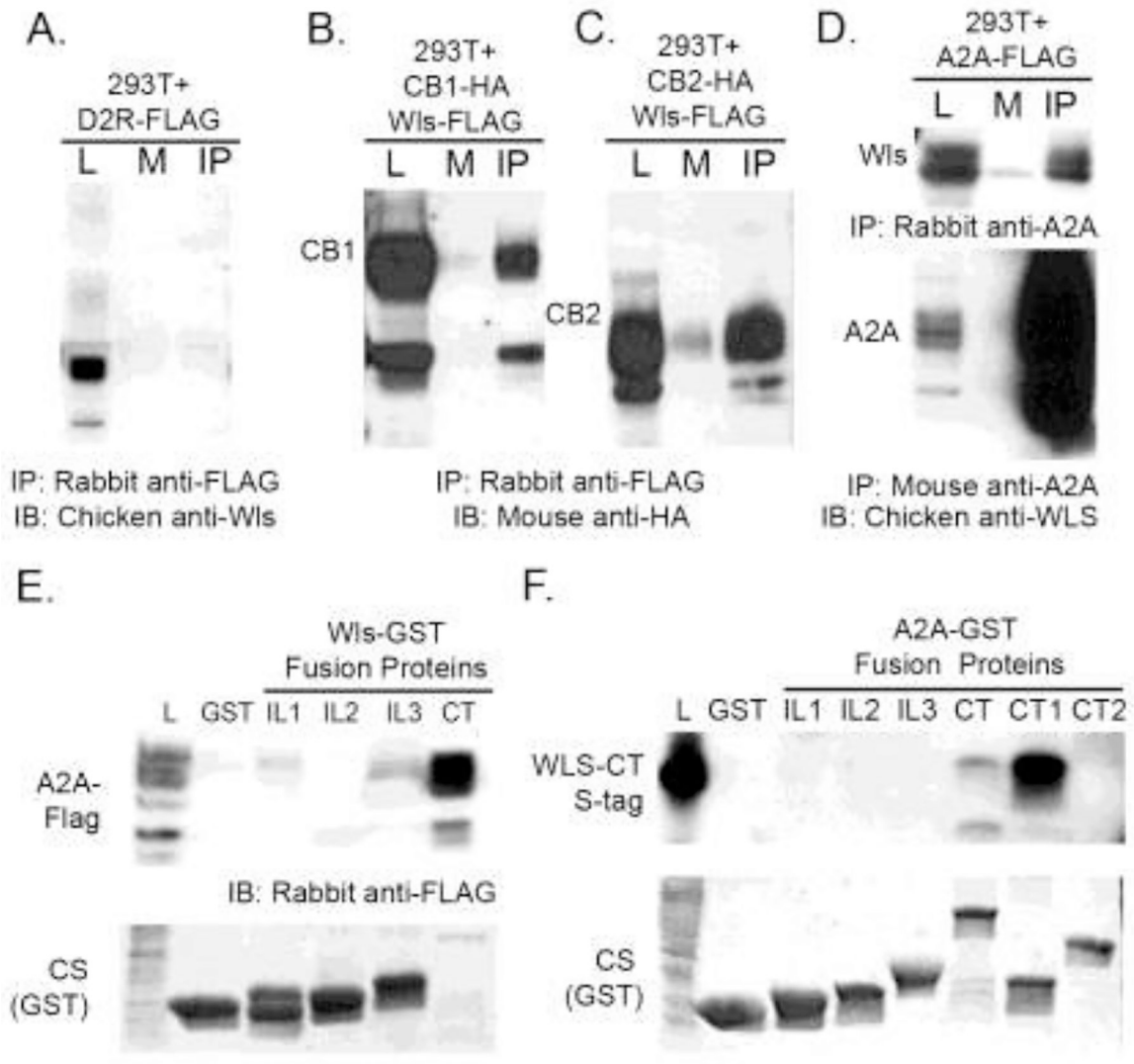

Figure 4. Wls interacts with Cannabinoid and A2A Adenosine Receptors.

Co-immunoprecipitation/western blotting analysis of Wls and (A) Flag-tagged D2-dopamine receptor from stably expressing HEK-293 cells, (B,C) HA-tagged cannabinoid receptors (CB1 and CB2) from transfected 293T cells, or (D) Flag-tagged adenosine A2A receptor from stably expressing HEK-293 cells. (E,F) GST pulldowns were used to map the interacting domains of Wls and A2A. (E) The intracellular portions of Wls were tagged with GST and used to precipitate Flag-tagged A2A from stably expressing HEK-293 cells. (F) Intracellular portions of A2A were tagged with GST and used to precipitate S-tagged Wls-CT. CT1 refers to the N-terminal half of the A2A-CT and CT2 refers to the C-terminal half of the A2A-CT. GST-tagged and S-tagged proteins were produced in and isolated from *E. coli*. L – lysate; M – mock; IP – immunoprecipitation; IB – immunoblot.

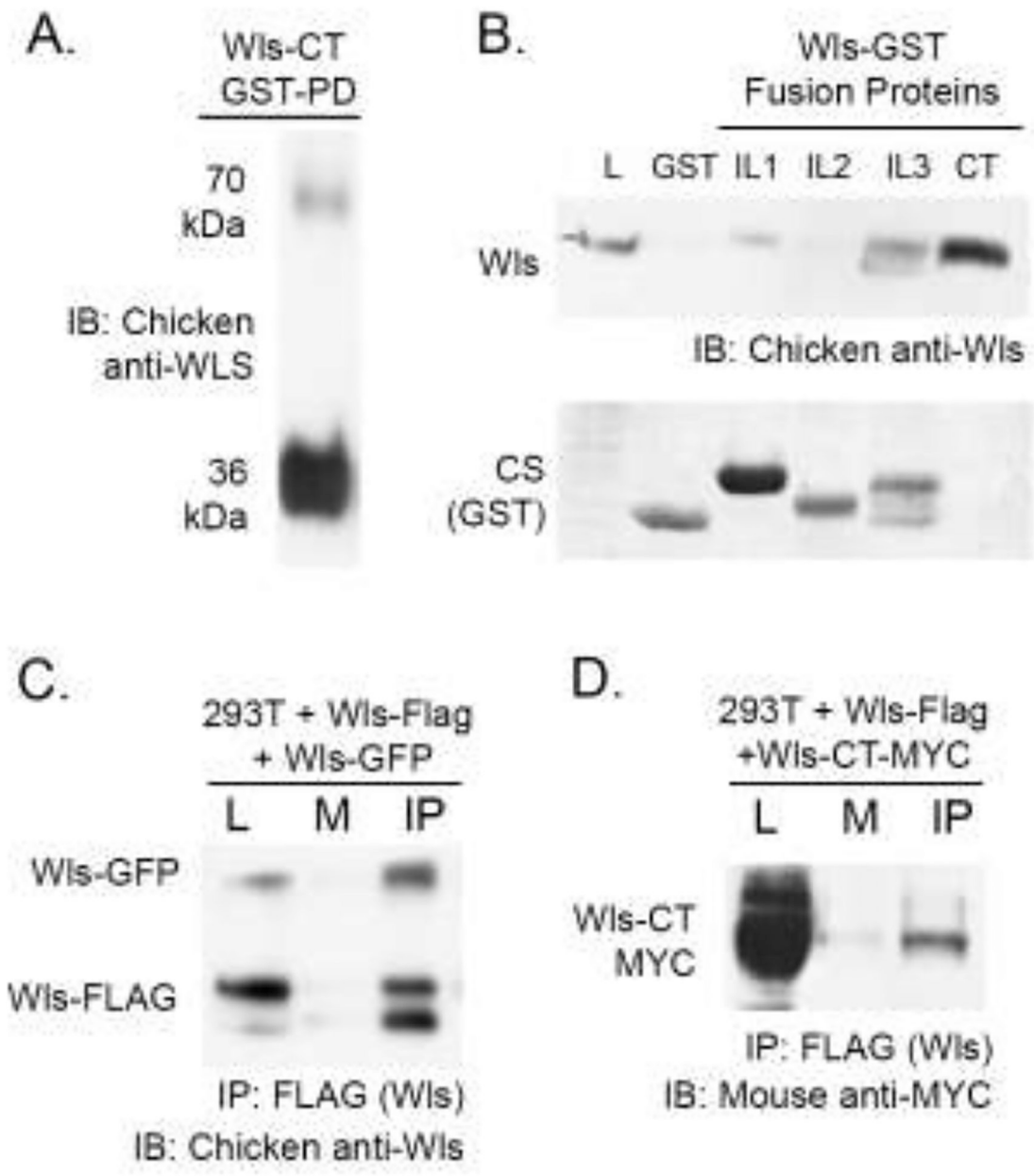

Figure 5. Wls homodimerizes through its C-terminal tail.

(A) Glutathione bead purified Wls- CT-GST from E.coli lysates. (B) GST-pulldown of endogenous Wls from 293T cell lysates with GST-tagged Wls truncations. (C) Immunoprecipitation of Wls-FLAG and co-precipitation of Wls- GFP from transfected 293T cell lysates. (D) Co-immunoprecipitation of Wls-CT-MYC with Full length Wls-FLAG from transfected 293T cell lysates. L – lysate; M – mock IP; IP – immunoprecipitation; IB – immunoblot.

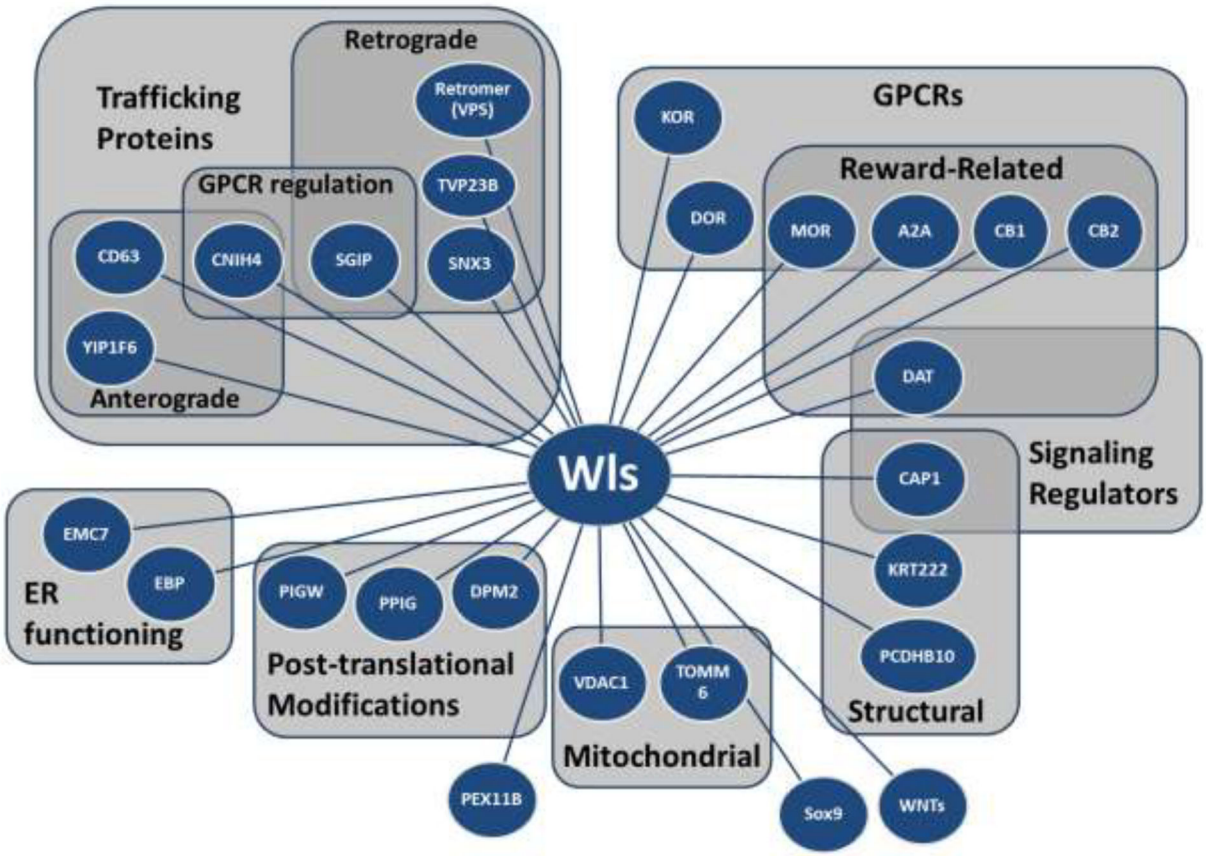

Figure 6. Putative Wls interactome.

Known and newly identified interactors of Wls categorized by function.

Table 1.

Putative Wls Interacting Proteins Identified by Y2H Screening

Abbrev	Full name	Bait	AA	Gene ID	Protein Function	Reference*	# hits
ATP1B1	ATPase Na+/K+ transporting subunit beta 1	Wls c-tail	102–304	481	Na^+/K^- pump	–	7
ATP1B3	ATPase Na+/K+ transporting subunit beta 3	Wls c-tail	231–280	483	Na+/K-pump	-	4
Aqp4	Aquaporin 4	FL-Wls	1–184	361	Brain water homeostasis	-	1
C7orf25	Chromosome 7 open reading frame 25	Wls c-tail	298–436	153792790	Unknown	-	5
C10orf25	Chromosome 10 open reading frame 25	FL-Wls	284–422	220979	Unknown	-	2
C10orf32	Chromosome 10 open reading frame 32	FL-Wls	1–77	119032	Unknown	-	1
C10orf35	Chromosome 10 open reading frame 35	FL-Wls	1–366	219738	Unknown	-	1
CAP1	Adenylate cyclase associated protein 1	Wls c-tail	411–475	10487	Regulates actin filament dynamics and RAS- dependent cAMP activation	(Hubberstey and Mottillo, 2002)	2
CD63	CD63 antigen	FL-Wls	1–197 123–238	967	TIMP1 surface receptor and marker for exosomes		2
CDH10	Cadherin 10	Wls c-tail	657–787	1008	Calcium-dependent cell adhesion protein	-	1
Chordc1	Cysteine and histidine rich domain containing 1	Wls c-tail	63–657	26973	Heat-shock protein 90 (HSP90) co-chaperone	(Gano and Simon, 2010)	1
CUL 1	Cullin 1	Wls c-tail	639–777	8454	E3 ubiquitin ligase	–	1
CNIH4	Cornichon family AMPA receptor auxiliary protein 4	FL-Wls	1–138	29097	GPCR trafficking protein	(Sauvageau et al., 2014)	1
DAT	Dopamine transporter	FL-Wls	1–343	6531	Dopamine transporter, target of cocaine and methamphetamines		1
DPM2	Dolichyl-phosphate mannosyltransferase subunit 2	FL-Wls	1–88	8818	Mannosyl residue donor for biosynthesis of glycosylphosphatidylinositol and N-glycan	(Maeda et al., 1998; Maeda et al., 2000; Watanabe et al., 2000)	1
EBP	Emopamil binding protein	FL-Wls	1–231	10682	Sterol isomerase	(Becker et al., 2001)	2
EMC7	ER membrane protein complex subunit 7	FL-Wls	37–243	56851	ERAD, ER - mitochondria transport	(Wideman, 2015)	1
KMT2c	Lysine methyltransferase 2C	Wls c-tail	4926–4964	58508	Histone methyltransferase	–	1
KRT222	Keratin 222	Wls c-tail	116–888	56881558	Intermediate filament protein	-	1
PCDHB10	Protocadherin beta 10	FL-Wls	682–801	56126	Neural cell adhesion	-	2
PEX11B	Peroxisomal biogenesis factor 11 beta	Wls c-tail	243–738	8799	Peroxisome proliferation	(Schrader et al., 1998)	1
PIGW	Phosphatidylinositol glycan anchor biosynthesis class W	FL-Wls	417–505	284098	Inositol acyltransferase	(Murakami et al., 2003)	1
PNISR	PNN interacting serine and arginine rich protein	Wls c-tail	621–806	1016841184	Splicing	(Zimowska et al., 2003)	1
PPIG	Peptidylprolyl isomerase G	Wls c-tail	606–739	9360	PPI family of protein folding proteins	–	1

Abbrev	Full name	Bait	AA	Gene ID	Protein Function	Reference*	# hits
SGIP1	SH3 domain GRB2 like endophilin interacting protein 1	Wls c-tail	203–429	8158909 77	Regulate endocytosis, regulates CB1R, polymorphism linked to alcoholism	(Chwedorowicz et al., 2016; Dergai et al., 2010; Hajkova et al., 2016)	1
SOX9	Sry-Box9	FL-Wls	381–458	6662	Transcription factor	–	1
TMEM14a	Transmembrane protein 14a	FL-Wls	1–100	28978	Unknown	-	6
TMEM134	Transmembrane protein 134	FL-Wls	1–165	80194	Unknown	-	1
TMEM160	Transmembrane protein 180	FL-Wls	17–187	103252561	Unknown	-	1
TMEM230	Transmembrane protein 230	FL-Wls	6–121	29058	Unknown	-	1
TOMM6	Translocase of outer mitochondrial membrane 6	FL-Wls	1–160	100188893	Mitochondrial protein transport	-	2
TVP23B	Trans-Golgi Network Vesicle Protein 23 Homolog B	FL-Wls	34–142	51030	Retrograde endosome to golgi vesicle trafficking in yeast	(Stein et al., 2009)	1
VDAC1	Voltage dependent anion channel 1	Wls c-tail	34–95	2099	Mitochondrial voltage-gated ion channel	–	2
YIP1F6	YIP1 family member 6	FL-Wls	1–237	853082	Implicated in intestinal inflammation, member of a family that regulates ER to Golgi protein transport	(Brandl et al., 2012)	2

*Proteins with unknown function or with functions that are considered general knowledge were not referenced.