Evolutionary Genetics of CORL Proteins

by

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ABSTRACT

Transgenic experiments in *Drosophila* have proven to be a useful tool aiding in the determination of mammalian protein function. A CNS specific protein, dCORL is a member of the Sno/Ski family. Sno acts as a switch between Dpp/dActivin signaling. dCORL is involved in Dpp and dActivin signaling, but the two homologous mCORL protein functions are unknown. Conducting transgenic experiments in the adult wings, and third instar larval brains using mCORL1, mCORL2 and dCORL are used to provide insight into the function of these proteins. These experiments show mCORL1 has a different function from mCORL2 and dCORL when expressed in *Drosophila*. mCORL2 and dCORL have functional similarities that are likely conserved. Six amino acid substitutions between mCORL1 and mCORL2/dCORL may be the reason for the functional difference. The evolutionary implications of this research suggest the conservation of a switch between Dpp/dActivin signaling that predates the divergence of arthropods and vertebrates.

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INTRODUCTION

Transforming Growth Factor-β Signaling pathways

Intracellular signaling is a key factor that results in the proliferation and differentiation of different cell types in multicellular organisms. Without it, cells would not work cooperatively and perform the appropriate functions relative to their placement in the organism. The Transforming Growth Factor-β (TGF-β) signaling pathway has been thoroughly studied and plays major developmental roles in both vertebrates and invertebrates. Mutations in the genes that are in the TGF-β signaling cascade can lead to tumor growth.

Our lab uses Drosophila melanogaster, commonly known as a fruit fly, as our model organism to study developmental and evolutionary genetics. There are many benefits to using Drosophila as a model organism. They are cheap and easy to maintain. It only takes ten days, at room temperature, for the organism to become a reproductive adult from fertilization, so the life cycle is fast. It is easy to study a variety of tissues at different stages in development due to the compartmentalization of larval imaginal discs that eventually form the appendages, wings, and eyes. Our lab's research has traditionally focused on the embryonic stage, 3rd instar larva, and adults.

The development of the Drosophila wing is a classical model for studying appendage development. The adult wing has five lateral veins and an anterior and posterior cross vein. Vein pattern formation is determined in the developing 3rd instar larva wing disk. Mutations in genes involved in Drosophila's development can lead to a variety of phenotypes in the adult wing. Some mutant adult wing phenotypes from the

Decapentapalegic (Dpp) signaling pathway, which will be explained later, cause loss of vein tissue, ectopic vein tissue, abnormally large or small wings, and bristle transformations.

The two major subfamilies of TGF-β are Bone Morphogenetic Protein (BMP)/Dpp and TGF-β/dActivin. The ligands of these subfamilies are highly conserved across vertebrates and invertebrates in protein sequence and in function. The TGF-β ligand family tree (**Fig. 1**) shows the protein sequence homology between three species, mouse, fly, and worm. In the BMP/Dpp subfamily, the mouse BMP2 and BMP4 show highest sequence similarity to Dpp from the fly. In previous transgenic studies, BMP2 and BMP4 rescue Dpp mutant fly phenotypes (Padgett et al. 1993). Dpp can induce bone formation in mammalian cell culture like BMP2 and BMP4 (Sampath et al. 1993). The importance of these transgenic rescue studies show homologous proteins across species frequently hold a conserved function. It validates Drosophila as an ideal model organism to generate hypotheses about gene function to mammals.

Overexpression and ectopic expression of genes can be studied easily in flies and mammalian cell culture but it is often a painstaking process to study loss of function in mammals due to the higher maintenance costs and longer life cycles. The GAL4/upstream activating sequence (UAS) system is a useful tool in Drosophila genetics experiments that allows us to study overexpression and loss of function quickly and cheaply. The yeast Gal4 gene is inserted into the promoter region of a gene in the fly's genome that can have tissue specific expression. When the gene is expressed, the Gal4 is activated and becomes a transcription factor for the UAS.Gal4 lines are tissue specific so

if we want to over express or ectopically express a gene, for example in the wing, we use a Gal4 line that has been inserted in the promoter region of a gene specific for wing expression. The UAS line has a complimentary DNA (cDNA) sequence downstream of the UAS and is transcribed when the Gal4 protein binds to the UAS. The cDNA can be a wild type or mutated version of a gene from any organism and allows for transgenic studies in the fly. The UAS.cDNA can be inserted anywhere in the genome of the fly. It is best to have multiple UAS lines on different chromosomes to ensure that any phenotype observed is not due to the UAS line being inserted directly into a gene and disrupting its function. For the Gal4/UAS to work, one parent has the tissue specific Gal4 line and the other parent has the UAS.cDNA. When they mate, the progeny will have both Gal4 and UAS.cDNA in its genome. Transcription of mRNA occurs when the Gal4 is activated.

Dpp signaling establishes the dorsal/ventral axis of developing embryos in the fly. Dpp is found in its highest concentration in the dorsal most portion of the developing embryo. Dpp is released from the cells that produce the protein and it forms a concentration gradient in the extracellular matrix that weakens as it travels ventrally. The dorsal tissue with the highest concentration of Dpp becomes ectoderm, which will eventually form the exoskeleton. Lateral tissue receives a medium concentration of Dpp and becomes neural tissue. Ventral tissue receives the lowest concentration of Dpp and becomes mesoderm.

In the fly, TGF-β signal transduction has two pathways, Dpp and Drosophila Activin (dActivin), that lead to different subsets of transcription factors being activated

downstream in the nucleus. This project focuses on a Smad cofactor in the TGF-β/dActivin signal transduction pathway.

The Dpp signal transduction pathway (**Fig. 2**), homologous to the mammalian BMP pathway in mammals, begins with the ligand, Dpp, being secreted from a cell. At this point dpp is floating through extracellular space until it binds to a type II cellular membrane receptor, Punt. Punt recruits Thickveins (TKV), a type I receptor, and forms a heterodimer becoming an active receptor serine/threonine kinase capable of phosphorylating Mothers Against Dpp (Mad), a receptor smad, within the cells cytoplasm. Phosphorylated Mad heterodimerizes with Medea (Med), a co-smad, and translocates to the nucleus facilitating transcription to a variety of target genes downstream. Dpp/BMP signaling is usually associated with cell differentiation.

The dActivin signal transduction pathway (**Fig. 2**), homologous to TGFβ/activin in mammals, begins with the ligand, dActivin, binding either punt or wit, a type II serine/threonine kinase membrane receptor. The type II receptor can then bind the type I receptor, baboon, activating its kinase ability. The receptor Smad phosphorylated by baboon is dSmad2. Once dSmad2 is phosphorylated, the receptor Smad forms a complex with the same Co-Smad that Mad associates with, Med. The dSmad2/Med heterodimer translocates to the nucleus effecting the transcription of a different set of genes downstream. TGFβ/dActivin signaling is usually associated with cell proliferation (Brummel et al. 1999). One gene that requires dActivin signaling to promote its transcription is Ecdysone Receptor (EcR; Zheng et al. 2003).

The ligands and ligand receptors are not the only homologous proteins within the

TGF-β family. Smad proteins have protein sequence and functional homology across species also (**Fig. 3**). Mad, from the fly, is homologous in sequence and function to the mammalian Smad1 and Smad5 proteins. These are the receptor Smads associated with BMP/Dpp signaling. Drosophila Smad2 (dSmad2) is homologous to mammalian Smad2 and Smad3 and are the receptor Smads for TGFβ/activin signaling. The Co-Smad Medea is homologous to mammalian Smad4. Anti Smads in the Smad family tree repress the signaling pathway by preventing the receptor Smads from forming a complex with the Co-Smads. Mammalian Smad4 rescues mutant Medea in the embryo (Hudson et al. 1998). This shows that the entire pathway is highly conserved.

dSno Is a switch between Dpp/dActivin Signaling

Earlier studies show SnoN, a mammalian homolog to Drosophila Sno (dSno) antagonizes TGF-β/BMP signaling by interacting with Smad4, a Medea homolog, repressing transcriptional activity when overexpressed (Luo et al. 1999). All of the findings with SnoN are based on overexpression studies, but loss of function experiments on Sno/Ski oncogenes had yet to be performed.

Using Drosophila, our lab was able to study loss of function of dSno using a null mutant. Mutants for dSno were lethal in early pupal stages. The optic lobe of 3rd instar larval brains did not develop a normal abundance of photoreceptor neurons, a phenotype observed with mutant baboon and mutant dSmad2 flies. BMP's regulate the growth of motor neurons in the CNS and the amount of phosphorylated Mad did not seem effected in the CNS in dSno mutants, so dSno does not effect BMP signaling in motor neurons. To

test that loss of dSno was responsible for the mutant phenotypes, heat shocking larva to activate the Gal4/UAS system at a specific stage in development was the experimental technique employed. In a dSno mutant background, rescue was achieved to adulthood by expressing UAS.dSno.

Overexpression analysis of dSno in wings supports the mammalian studies that conclude Sno antagonizes Dpp/BMP signaling (**Fig. 6**). When expressing UAS.dSno with A9.Gal4, a wing disc specific Gal4, loss of vein tissue and undersized wings result with 100% penetrance. Additive effects were seen when expressing UAS.dSno with Dpp mutants. Expression of UAS.dSno with constitutively activated form of TKV (Haerry et al. 1998) does not rescue the dSno overexpression phenotype. The results of the epistatic experiment place the repressive effects of dSno downstream of TKV in the Dpp/BMP signaling pathway. Coexpression of dSno and Medea or Mad resulted in a rescue of the dSno overexpression phenotype, the wings were normal size and vein truncations were lessened. Coexpression of dSno and dSmad2 had no greater or lesser effect on the overexpressed dSno phenotype.

To study dSno loss of function, imprecise exisions of a homozygous lethal transposable element insertion in the dSno promotor region were created. Imprecise excisions were determined by their ability to not complement a large deletion near the dSno coding region. Once the proper lines were identified, tests were done to ensure that loss of dSno was the reason for lethality, which occurs after pupation. Ectopic expression of UAS-dSno in early developing embryos results in lethality so rescue experiments had to be performed with heat shock Gal4 lines. Rescue was achieved to adulthood when

expressing UAS-dSno 10 hours after egg lay (AEL) and then again at 4 days AEL. The significance of this rescue experiment is it eliminates the possibility that another unknown gene that could have been mutated during the excision was not the reason for the lethality. The rescue experiment attributes the lethality to our gene of interest. A phenotype of dSno mutants is located in the optic lobes of the third instar larval brains (**Fig. 7**). The optic lobes have reduced photoreceptor innervation of the lamina and medulla. The lethality after pupation and optic lobe phenotypes in dSno mutants suggest that dSno mediates dActivin signaling rather than just repressing Dpp signaling.

Biochemical analysis shows that dSno has an affinity to bind Medea. Since our lab had shown that overexpression of dSno antagonizes Dpp signaling, loss of dSno represses activin signaling, and dSno has an affinity for Medea, our lab had concluded that dSno acts as a switch. dSno switches Medea's affinity away from forming a complex with Mad and increases its affinity to form a complex with dSmad2 under normal conditions. If cells are receiving signals from both Dpp and dActivin, cells that are expressing dSno will give preference to dActivin signaling and silence Dpp signaling.

dCORL Is Required for dSmad2 Mediated Activation of Ecr-B1

There are two other sub-families of genes in Drosophila that are similar to Sno, Dachshund and CORL (**Fig. 5**). Our lab focused on dCORL because little was known about it and the possibility that it might also play a role in the TGF-β signaling pathway was of interest. Our hypothesis was that CORL would act as a switch between Dpp and dActivin signaling. After beginning our research on dCORL, Mouse CORL1 (mCORL1)

was found to have neural specific expression during development that eventually become purkinje cells in the cerebellum. Mouse CORL2 (mCORL2) had later been cloned and found to express at embryonic day 10.5 in the Purkinje cell progenitors and in the adult Purkinje cells, also CNS specific expression (Minaki et al. 2008; Miyata et al. 2010).

RNA *in situ* hybridization of dCORL showed its expression to be limited to the central nervous system in embryonic development. The expression of dCORL is also prominently found near the Mushroom Body (MB) of the third instar larval brain located in the dorsal/anterior region, an area where dSno expression is absent. No dCORL expression was detected in the optic lobes of the brain or in any other larval tissue outside of the central nervous system.

dCORL was cloned and a mutant was made via intra chromosomal recombination named Df(4)dCORL. The deletion that resulted from the intra chromosomal recombination also includes three other genes not including dCORL. Rescue experiments conducted in the Df(4)dCORL background using UAS-dCORL are vital because we cannot conclude that our loss of function experiments are due to loss of dCORL until we rescue the mutant phenotype using a transgenic UAS-dCORL.

In dCORL mutants, there is reduced viability in larval and pupal stages compared to wild type flies. There is a developmental delay. Third instar larva exhibited prematurely everted spiricals and enter into the wandering stage early. Since the RNA in situ hybridization of dCORL showed expression to be near the MB of 3rd instar larva, we decided to look for a phenotype in the MB of the adult brain. The adult brains exhibited a number of structural defects including misshapen dorsal lobes, β neurons crossing the

midline and incorrect pruning of γ-lobes. All of these phenotypes exhibited were similar to loss of Ecdysone Receptor (EcR-B1) expression (Zheng et al. 2003). EcR-B1 is a downstream target in dActivin signaling.

The hypothesis that dCORL acts as a switch between the Dpp and activin signaling pathways in the MB is still a likely possibility with that data that we had collected thus far. Ectopic expression of dCORL in the wing results in loss of vein tissue. The effect is not as great compared to overexpression of dSno, but it still shows a repressive effect on Dpp signaling. UAS-dCORL with constitutively activated TKV resulted in loss of vein tissue and a normal size wing placing dCORL's repressive functions on Dpp signaling downstream of the TKV similar to dSno.

Loss of function analysis resulted in loss of EcR-B1 expression in late 3rd instar larval brains in the Df(4)dCORL background. Loss of EcR-B1 expression in the MB is associated with baboon and dSmad2 mutants (Zheng 2003) but not with dSno mutants. Rescue of EcR-B1 expression occurred when expressing UAS-dCORL through a MB specific 238Y.Gal4 in a Df(4)dCORL background, proving that dCORL is the gene that is responsible for the mutant phenotype.

When overexpressing UAS-dCORL with 238Y.Gal4 in the late 3rd instar larval brain in a wild type background. Rather than amplification of EcR expression in the MB, there was loss of EcR-B1 expression. This suggests that dCORL has a dosage dependent effect on dActivin signaling to promote EcR-B1 expression. These results do not support the hypothesis that dCORL acts as a switch between Dpp and dActivin signaling.

In flip out clone analysis, EcR-B1 expression was lost in large clones of CORL-

RNAi, dSmad2-RNAi and Medea-RNAi experiments. AY-Gal4 has a stop cassette within two FRT sites in the promoter region preventing the Gal4 from being activated (Struhl G et al. 1993). When heat shock is induced, flippase is transcribed and removes the stop cassette that is blocking the Gal4's ability to become active by causing recombination at the FRT sites. In every cell where the stop cassette is removed, AY-Gal4 is then active from that point on including every daughter cell derived from that cell. Cells that have the stop cassette flipped out express GFP to enable detection of the clones. Each cell expressing the UAS.GFP is also expressing any UAS.cDNA inserted in the genome, enabling comparison of cells expressing the transgenic lines next to wild type cells in the developing fly. EcR-B1 expression was lost in large clones expressing UAS.dCORL-RNAi, but EcR-B1 expression was present in small clones suggesting that dCORL is required to activate EcR-B1 expression but it is not responsible for maintenance of EcR-B1 expression in the MB. In the Df(4)dCORL background, the large clones expressing UAS.dCORL expressed EcR-B1 while the surrounding neurons did not. Small clones in the Df(4)dCORL background did not express EcR-B1 adding additional evidence that dCORL is required to activate EcR-B1 expression at an earlier time but is not necessary for maintaining expression.

Biochemical analysis shows that mCORL1 binds Smad3, the dSmad2 homolog, rather than Smad4, the Medea homolog. If dCORL acts as a switch between Dpp and activin pathways, then it would likely bind Smad4. Our lab determined that dCORL is required to facilitate dSmad2 signaling when expressed normally in the MB, but represses dActivin and Dpp signaling when expressed ectopically.

Evolutionary Genetics of CORL Proteins

The purpose of my Masters thesis is to determine which mCORL is most similar to dCORL in function. Multiple transgenic experiments have shown functional conservation of proteins within the TGF-β family. It is likely that one or both mCORL proteins have a conserved function with dCORL. We hypothesize that mCORL1 and dCORL are more similar in function than mCORL2 due to known common involvement with the TGF-β/activin signaling pathway. I will try to determine which mCORL protein is most similar to dCORL by repeating some of the experiments in the Takaesu et al. 2012 paper using transgenic mCORLs via the UAS/Gal4 system. Ectopic expression in the wings, mutant rescue in the third instar larval brain, and overexpression in the third instar larval brain will be the assays that I perform.

I show distinct functional differences between mCORL1 and mCORL2 and that a small number of amino acid substitutions within a highly conserved region of these proteins can be the cause for that functional difference. The implications of our results bring back the possibility of dCORL acting as conserved switch between Dpp and Activin signaling that predates the divergence of arthropods and vertebrates.

Materials and Methods

Drosophila Genetics

MS1096.Gal4 was used to ectopically express UAS.dCORL, UAS.mCORL1 and UAS.mCORL2. MS1096-Gal4 was chosen because its expression is specific to the entire pouch of the wing disc during larval development. The pouch of the wing disc contains the progenitor cells that become the adult wing. MS1096-Gal4 is located on the X chromosome and only female wings were scored in the experiment. Hemizygous male wings were excluded because they exhibit their own phenotype of chemosensory to mechanosensory bristle transformation without expression of a UAS line.

Fly lines used for ectopic expression experiments in wings are as follows: w P[w+; Gal4] MS1096 (Milan et al. 1998, Marquez et al. 2001), w; P[w+; Gal4]T80/CyO (Marquez et al. 2001), y w; P[w+; UAS.dCORL]7A (Takaesu et al. 2012), y w; P[w+; UAS.dCORL]17A (Takaesu et al. 2012), y w; P[w+; UAST.mCORL2]35.5, y w; P[w+; UAST.mCORL1]3.1, y w; P[w+; UAS.dCORL]7A/CyO.wg; P[w+; UAS.CG11093]17A

Fly lines used for flip out clones are as follows hs.flp; AY.Gal4 UAS-GFP; Df(4)dCORL (T. Orenic), y w; P[w^+; UAS.CG11093]7A; Df(4)dCORL, y w; P[w^+; UAST.mCORL1]1; Df(4)dCORL, y w; P[w^+; UAST.mCORL2]35.1; Df(4)dCORL. Flip-out clones were set up as follows. Virgins of y w hs.flp; AY.Gal4, UAS.GFP/ CyO.wg were mated to male UAS.dCORL7A, UAS.mCORL1, and UAS.mCORL2 lines for overexpression. Virgins of y w hs-flp; AY-Gal4, UAS.GFP/ CyO.wg; Df(4)dCORL/ CiD

were mated to male UAS.dCORL7A; Df(4)dCORL, UAS.mCORL1; Df(4)dCORL and UAS.mCORL2; Df(4)dCORL for mutant rescue. Vials were passed every 24 hours. 40 hours after the flies were passed, the vials were heat shocked in a 37°C water bath for 1 hour resulting in larvae being heat shocked between 40-64 hours after egg lay (AEL). When the larva is warmed, flipase is transcribed. The longer the larvae remains heat shocked, the more flipase is created. The flipase is capable of removing the stop cassette within the promoter region of AY.Gal4 causing it to become active. Once the stop cassette is removed, AY.Gal4 will be expressed in every cell that the stop cassette is removed. Every daughter cell of the flipped out cell will express AY.Gal4. 3rd instar wandering larvae were picked from the vials 6 days AEL and checked for GFP expression before dissection.

Recombinant lines of P[w+; UAS.cyt.GFP] (O'Connor) with P[w+; UAS.CG11093]7A and P[w+; UAST.mCORL1]3.1 on the II chromosome and P[w+; UAS.GFP] with P[w+; UAST.mCORL2]35.5 were generated for preparation of rescue and ectopic expression experiments.

Immunofluorescence

Tissues were fixed in a 4% formaldehyde solution, rinsed, and stored in methanol -20C until staining. Primary antibodies used were mouse α-EcR-B1 1:75 (Developmental Studies Hybridoma Bank), guinea pig α-Tailless 1:500 and rabbit α-GFP 1:500. Secondary antibodies used were goat α-mouse, α-rabbit, α-guinea pig Alexa Fluor 488, 546, and 633 diluted to 1:500. The secondary antibodies were obtained from Molecular

Probes. Third instar larval brains were mounted in 90% Glycerol/PBS and imaged on a Leica SP5 confocal microscope.

Imaging

Adult wings were imaged on a Leica MZ6 dissection scope at a magnification of 25x. A Spot Insight QE camera was used to capture the images. Images where cropped at a fixed size in Adobe Photoshop.

The larval brains were imaged on a Leica SP5 confocal microscope. Images were acquired every 2 μm. Lasers used for excitation were Argon (Ar) 488 nm, Krypton (Kr) 546 nm, and Helium/Neon (He/Ne) 633 nm. All third instar larval brains were imaged with sequential scanning with Ar, He/Ne, and Kr lasers exciting the fluorophores separately. Gain adjustments were made on each larval brain prior to imaging to minimize background. Images were cropped in Adobe Photoshop at a fixed size for both low magnification and high magnification figures.

Results

dCORL is More Similarly Aligned to mCORL2 in the Sno Homology Domain

Using Clustal Omega we can make sequence alignments of homologous proteins across multiple species. A schematic of the Sno homology domain shows the sequence similarity between dCORL, mCORL1 and mCORL2 (**Fig 9A**). The Sno homology domain is the region of the protein that is capable of binding Smads (Takaesu et al. 2006). In theory, a single amino acid substitution in this region can alter the affinity for a CORL protein to bind a Smad, especially if the substitution changes the electrical charge in that region of the protein (Takaesu et al. 2005). Sequence similarity between dCORL and mCORL1 is 89%, while the similarity between dCORL and mCORL2 is 90% (**Fig 9A**). The sequence similarity between Dpp and BMP2 and BMP4 is only 82% and 81% respectively, and those proteins are capable of substituting for each other in cross species studies (Padgett et al. 1993, Sampath et al. 1993).

The CORL alignment shows that there are 14 amino acid substitutions within the Sno homology domain, marked * between mCORL1 and mCORL2 (**Fig 9B**). Six of those substitutions highlighted in a red * are still conserved between dCORL and mCORL2. Within the zinc finger region, highlighted in green, there is a Serine conserved in mCORL2/dCORL that has been substituted by a Glycine in mCORL1. This alignment suggests that 6 amino acid substitutions could be responsible for a possible change in function between mCORL1 and mCORL2/dCORL. This does not support our hypothesis that dCORL and mCORL1 are more similar.

dCORL Is More Similar in Phenotype to mCORL2 in Adult Wings

Expression of Smads in cross species analysis has given similar phenotypes to their Drosophila homologs (dSmads) in the wing (Marquez et al. 2001). Though dCORL expression is limited to the CNS and does not occur naturally in wing development, Dpp/dActivin signaling is present during wing development. The effect of ectopic expression of CORLs on Dpp/dActivin signaling can be observed in the wing. We hypothesize that if the function of CORLs is conserved across multiple species, than ectopic expression of CORLs in the wing should present similar phenotypes.

Ectopic expression of mCORL1 and mCORL2 with MS1096.Gal4 caused truncation of veins in the adult female wing (**Fig 10C-D**). The loss of vein tissue is greater in both transgenic lines than in ectopic expression of dCORL (**Fig 10B**), suggesting that both mCORL lines antagonize Dpp signaling to a greater extent. The phenotypes of UAS.mCORL2 and UAS.dCORL are most similar with wild type wing size and minor vein truncations when ectopically expressed with MS1096.Gal4. The adult wings are 70% the size of wild type. The smaller wing displays 100% penetrance in the wings examined when ectopically expressing mCORL1 (**Table 1**). The size difference was not visible in ectopic expression of mCORL2 or dCORL. Posterior Cross Vein (PCV) truncation or absence was the most common vein deformity found in expressing UAS-dCORL at 100%. Both mCORLs were close to 70% PCV deformity. This is the second result that does not support the hypothesis that dCORL is more similar in function to mCORL1.

Notably the ectopic expression of dCORL created multiple chemosensory to mechanosensory bristle transformations at a high frequency and numerous locations along the lateral veins. Both mCORLs only presented chemosensory to mechanosensory bristle transformations on lateral vein 1 below the Acosta region at a low frequency, 7% for mCORL1 and 22% for mCORL2. Bristle transformations have similarly been described in an overexpression assay of dSno in the wing (Quijano et al. 2010). dCORL, mCORL1, and mCORL2 ectopically expressed in the wing appear to antagonize Dpp signaling. mCORL1 ectopic expression causes smaller than wild type wings which shows a distinct function from dCORL/mCORL2.

dCORL is More Similar to mCORL2 When Rescuing EcR-B1 Expression in the MB

Since expression of dCORL occurs naturally in the CNS, we wanted to know if either mCORL could rescue a mutant phenotype of dCORL previously described in the MB of third instar larvae (Takaesu et al. 2012). Driving UAS.dCORL in flip-out clones was used to rescue EcR-B1 expression in a dCORL mutant background. Third instar larval brains were stained with anti-GFP (green), anti-Tailless (Tll: blue) and anti EcR-B1 (red). We know that wherever GFP expression is found, UAS.CORL is present as well. The Tll antibody stains neuroblasts in the MB. Tll expression is not effected by loss of dCORL or overexpression of dCORL (Takaesu et al. 2012). Anti-Tll is used as a control to mark the location of the MB. EcR-B1 is a nuclear protein that is turned on by the TGFB/dActivin signaling pathway. EcR-B1 expression is found in the cells of the

MB within the CNS, but expression occurs outside of the MB in the CNS also. In dCORL mutants, EcR-B1 expression is not present within the MB, yet the EcR-B1 expression outside of the MB appears unaffected.

We examined flip-out clones in a Df(4)dCORL background expressing dCORL, mCORL1 and mCORL2. First generation mutants of dCORL do not have EcR-B1 expression in the MB. Expression of dCORL in large flip-out clones was previously shown to rescue EcR-B1 expression in the MB within the neurons that are expressing UAS.dCORL (Takaesu et al. 2012). Third instar larval brain hemisphere confocal images show EcR-B1 expression is absent in a dCORL mutant background within the flip-out clones in the region of the MB only expressing UAS.GFP (**Fig 11A**). The left column is the overlay of Tll (blue), EcR-B1 (red), and UAS-GFP (green). GFP expression alone is represented in the center column and EcR-B1 expression alone is represented on the right column. We repeated the rescue experiment of EcR-B1 with expression of UAS-dCORL and UAS-GFP (**Fig 11B**). The expression of EcR-B1 overlaps the expression of GFP within the MB. EcR-B1 expression is reduced outside of the clone. Expression of UAS-mCORL1 with UAS-GFP does not rescue EcR-B1 expression within the MB (**Fig 11C**). Weak EcR-B1 expression is present inside of the clone. mCORL1 is not fully capable of rescuing EcR-B1 expression in a dCORL mutant background. Expression of mCORL2 and GFP resulted in rescue of EcR-B1 expression inside the clone similar to dCORL (**Fig 11D**). Numbers of larval brains examined for each genotype are listed in **Table 2**.

As shown in a more magnified focus on the MB (**Fig 12**), this rescue experiment suggests that mCORL2 and dCORL have conserved their function to be required for

dSmad2 mediated activation of EcR-B1 and mCORL1 has not. The rescue of EcR-B1 expression using mCORL2 and not mCORL1 fails to support the hypothesis that mCORL1 is more similar to dCORL.

dCORL is More Similar to mCORL2 When Repressing EcR-B1 in Overexpression

In a wild type background, overexpression of dCORL in large flip-out clones previously resulted in a knockdown of EcR-B1 expression within the clone but had no effect on EcR-B1 expression outside of the clone (Takaesu et al. 2012). We examined flip-out clones overexpressing dCORL, mCORL1 and mCORL2 in the MB. In a wild type dCORL background, UAS-GFP expression alone does not knock down EcR-B1 expression (**Fig. 13A**). The left column is the overlay of Tll (blue), EcR-B1 (red) and UAS-GFP (green). GFP expression alone is represented in the center and EcR-B1 expression alone is represented on the right. Knockdown of EcR-B1 expression occurs within the boundaries of the clone in overexpression of dCORL (**Fig. 13B**). There is a large gap of EcR-B1 expression where the large clone expressing UAS-dCORL is present. In both clones within the MB for ectopic expression of mCORL1 (**Fig. 13C**), EcR-B1 expression appears to be unaffected. Ectopic expression of mCORL2 resulted in a knockdown of EcR-B1 expression in the regions within the clone (**Fig. 13D**). Both dCORL and mCORL2 appear to have inhibitory effects on TGF-β/dActivin signaling as dosage is increased.

A magnified image of the MB is shown where EcR-B1 expression is knocked down in dCORL/mCORL2 overexpression but appears unaffected by overexpression of

mCORL1 (**Fig. 14**). The results of the overexpression assay suggest dCORL and mCORL2 have conserved their antagonistic effects on EcR-B1 when overexpressed while mCORL1 has lost that function. This is another result that does not support the hypothesis that mCORL1 is more similar in function to dCORL.

mCORL2 Is Biochemically Different from mCORL1

Biochemical analysis has been performed on mCORL1 and mCORL2 but not dCORL. In an immuno-blot assay, mCORL1 has been shown to bind phosphorylated Smad3, the dSmad2 homolog (Takaesu et al. 2012). mCORL1 has no affinity for receptor Smad 1, a homolog of Mad in the dpp signaling cascade. There is no affinity for mCORL1 to bind co-Smad4 either, which interacts with both Mad and dSmad2. We do not expect dCORL/mCORL2 to have the same affinity to smads as mCORL1 because of the 6 amino acid substitution difference between the proteins. A biochemical analysis of mCORL2 shows that it has no affinity to bind phosphorylated Smad3 (**Fig. 15A**). FLAG-tagged mCORL2 and FLAG-tagged c-Ski along with 6Myc-tagged Smad3 was examined by immunoprecipitation followed by immunoblotting in 293T cells. FLAG-tagged proteins will bind to a column and any protein that binds the FLAG-tagged protein will remain on the column also. The proteins bound to the column are eluted into a solution that only contains the FLAG-tagged proteins and any other protein that binds to it. The solution is then run on a denaturing gel and the proteins will separate based on size. Antibody staining for the tagged proteins will result in bands on the gel if a bound protein is present.

The positive control, FLAG-tagged c-Ski showed a strong affinity to bind both phosphorylated and unphosphorylated Smad3 in lanes 5-6 of the immunoblot. The bound proteins of FLAG-tagged c-Ski and 6Myc-tagged Smad3 can be observed in the top row of the blot. Lanes 3-4 are the negative controls with neither FLAG-tagged mCORL2 nor FLAG-tagged c-Ski. 6Myc-tagged Smad3 is not present in lanes 3-4 either. Lanes 1-2 shows FLAG-tagged mCORL2 but phosphorylated and unphosphorylated 6Myc-tagged Smad3 bound to mCORL2 is no stronger than the negative control observable in the top row of the immunoblot. If Smad3 bound mCORL2, lanes 1 or 2 of the immunoblot would have shown a stronger signal than the basal levels of the control. If dCORL is evolutionarily more similar in function to mCORL2 than it is likely that dCORL does not bind to dSmad2.

A luciferase assay was done to determine if mCORL1 and mCORL2 had different effects on TGF-β and BMP signaling in cell culture. Based on ectopic expression in wing data, we hypothesized that mCORL1 would antagonize TGF-β signaling. TGF-β signaling is antagonized by both mCORL1 (**Fig. 15B top**) and mCORL2 (**Fig. 15B bottom**) in a 12xCAGA-Luc reporter stimulated with caALK-5 in HepG2 cells with increased dosage. However, they do not antagonize TGF-β signaling as well as c-Ski (**Fig. 15B top and bottom**). This assay shows a functional similarity between mCORL1 and mCORL2, they both maintain the ability to antagonize TGF-β signaling.

The luciferase experiment testing for BMP signaling employed a BRE-Luc reporter stimulated with caALK-3. Given the ectopic expression phenotypes. Loaa of vein tissue in adult wings, we hypothesize that mCORL1 and mCORL2 would

antagonize BMP signaling. Increasing dosage of mCORL1 does not antagonize BMP signaling (**Fig. 15C top**). BMP signaling was antagonized with increased dosage of mCORL2 (**Fig. 15C bottom**) though not as well as c-Ski (**Fig. 5C top and bottom**). This assay does not connect a function of dCORL to either mCORL1 or mCORL2. This assay demonstrates another difference between mCORL1 and mCORL2, the ability of mCORL2 to antagonize BMP signaling in cell culture. This result is neutral to the hypothesis that mCORL1 is more similar to dCORL, however it shows a functional difference from mCORL2 which is consistent with the previous results. The hypothesis that mCORL1 is more similar in function to dCORL is not supported by any experiment we performed.

Discussion

Data Supporting Alternate Hypothesis

The hypothesis that mCORL1 is more similar to dCORL based on common function in the TGF-β/dActivin pathway has been refuted by three transgene assays. These are ectopic wing expression, third instar larval clone rescue and third instar larval clone overexpression. An updated amino acid sequence alignment with mCORL1, mCORL2 and dCORL also shows that there is more sequence similarity between mCORL2 and dCORL within the Sno homology domain. I conclude that mCORL1 is not more similar to dCORL in function. My new hypothesis is that mCORL1 is functionally different from mCORL2 and dCORL. Our three transgene assays plus biochemical analyses of mCORL1 and mCORL2 support the new hypothesis. I speculate that based on the alignment there are 6 amino acid substitutions between mCORL1 and mCORL2/dCORL that are the reason for the functional difference (**Fig. 9**).

Relevant to our understanding of multi gene families, this data illustrates how proteins with a highly conserved functional domain can have distinct activities. Once the duplication of mCORL genes occurred, the small number of advantageous amino acid substitutions created the divergence of mCORL1 and mCORL2. Overexpression studies in the fly of hSmad2 and hSmad3 showed a distinct functional difference. The reason for the functional difference was narrowed down to 5 amino acid substitutions within the MH1 domain, the portion of the protein that binds DNA. hSmad2/dSmad2, having conserved the 5 amino acids in the MH1 region, showed similar phenotypes in overexpression causing the wing to be increased in size. hSmad3 had an opposite

phenotype, inducing apoptosis in the wing, resulting in a smaller wing size (Marquez et al. 2001). If 5 amino acid substitutions alter the affinity for Smads to bind DNA, then it is possible for 6 amino acid substitutions, in the Sno homology domain to alter the affinity of CORL proteins to bind to Smads.

Ectopic expression of mCORL1 caused smaller than average wing size suggesting that cell proliferation was inhibited during development. None of the wings examined were noticeably smaller in mCORL2 and dCORL assays. This suggests that mCORL1 is functionally different from mCORL2 and dCORL in ectopic expression within the wing. Vein defects are found among all three ectopically expressed lines. mCORL2 has already been shown to antagonize BMP signaling in cell culture (Wang et al. 2011) but effects of mCORL1 on BMP signaling have not been published. The effect mCORL1 is having on wing size appears to be different from mCORL2/dCORL. Ectopic mCORL2 wing expression antagonizes vein pattern formation reinforcing the finding from Wang et al. (2011). mCORL2 antagonism of dpp signaling shows it has remained functionally conserved with dCORL. The connection of mCORL2 and dCORL's ability to antagonize Dpp signaling shows functional conservation with dSno. It is possible that the conservation goes back to the ability to bind Smad4/Medea and act as a switch between Dpp/Activin signaling.

dCORL and mCORL2 flip out clones rescue EcR-B1 expression in dCORL mutants, mCORL1 does not. This assay does not explain how dCORL or mCORL2 rescues EcR-B1 expression since wild type dCORL expression is found outside the MB (Tran et al. 2018), but the experiment shows that expression of dCORL and mCORL2 is

sufficient to activate EcR-B1 expression within the clones of the MB. mCORL2 has conserved the ability for activation of EcR-B1, while mCORL1 has lost that function.

Flip-out clone overexpression assays show that dCORL and mCORL2 repress Activin sub-family signaling by knocking down EcR-B1 expression within the clones of wild type dCORL MBs. mCORL1 expression within the clones did not effect EcR-B1 expression. This assay shows that mCORL2 has a similar dosage dependent effect on activation of EcR-B1 as dCORL.

mCORL2 differs from mCORL1 in two biochemical assays. In an immune-blot assay, mCORL2 does not bind to Smad3 unlike previously published data that shows mCORL1 binding to Smad3 (Takaesu et al. 2012). If the 6 amino acid substitutions that differentiate mCORL1 from dCORL/mCORL2 are the reason for mCORL1's affinity to bind Smad3, then it is reasonable to hypothesize that dCORL does not bind to dSmad2. The luciferase assays show that mCORL2 antagonizes both TGF-β and BMP signaling in cell culture with increased dosage. mCORL1 only antagonizes TGF-β signaling with increased dosage. Since the biochemical data shows that mCORL2 does not bind Smad3, and antagonizes BMP signaling, I speculate that mCORL2 might bind Smad4 and act as a switch between BMP and TGF-β signaling pathways similarly to the Sno proteins. If mCORL2's function is conserved with dCORL, than it is a possibility that dCORL acts as a switch between Dpp and Activin signaling similarly to dSno.

Evolutionary Genetics of CORL Proteins

CORL is a member of the family of Sno/Ski proteins (**Fig. 5**). A functional similarity of Sno/Ski proteins is the ability to bind co-Smads. This similarity can be traced back to before the nematode/arthropod divergence. This takes us further back in evolutionary time from the arthropod/vertebrate split. Dach is a Sno/Ski family member that is present in nematodes, arthropods and vertebrates. The Dach subfamily evolved before the nematode/arthropod split (**Fig. 5)**. C. elegans Dac-1 and D. melanogaster Dac have not been associated with Dpp/Activin signaling. Overexpression analysis shows that human Dach1 antagonizes BMP/Dpp signaling via binding Smad4 (Wu et al. 2003).

Ce Daf-5 is member of the Sno/Ski family in C elegans. Ce Daf-5 is located between the Dach and Sno/CORL subfamilies as shown in **Fig. 5**. It might share common ancestry with Sno/CORL subfamilies because it impacts TGF-β signaling. Ce Daf-5 binds Ce Daf-3, a co-Smad protein (Da Graca et al. 2004). Ce Daf-5 acts as a co-factor for Ce Daf-3 rather than an antagonist. Ce Daf-3 and Ce Daf-8, a receptor Smad, directly antagonize the expression of each other via a negative feedback loop in the nervous system of the C elegans (Park et al. 2010). Unlike arthropod and vertebrates, C. elegans have multiple co-Smads including Sma's 2-4 and Daf-3. The Sma co-Smads are similar in function to Medea by facilitating TGF-β signaling. The other co-Smad, Ce Daf-3, functions unlike Medea by antagonizing TGF-β signaling (Patterson et al. 1997). I speculate that Ce Daf-3 could be a part of a signaling pathway that evolved in parallel to TGF-β/Activin signaling. In this scenario Ce Daf-5 does not act as a pathway switch. Daf-5 functions in a separate signaling pathway that antagonizes signaling similar to Dpp

(Da Graca et al. 2004). Without evidence of a switch in nematodes, I conclude that the switch between Dpp/Activin signaling evolved after the divergence of nematodes and arthropods.

The ability to bind co-Smads appears to be a common function of the proteins within the Sno/Ski family (Wu et al. 2003, Da Graca et al. 2004, Takaesu et al. 2006). The Sno homology domain is the region that binds Smads and is the most highly conserved region of the proteins across multiple species. mCORL1 is currently the first known member of the Sno/CORL subfamily that can not bind to a co-Smad, rather it interacts directly with Smad3 (Takaesu et al. 2012). Since mCORL2 and dCORL are shown to have a different function from mCORL1, I speculate that mCORL2 and dCORL have conserved their original function to bind co-Smads and maintained the ability to act as a switch between the Dpp/Activin signaling pathway.

In conclusion, my thesis has shown that functional conservation between mCORL2 and dCORL has been maintained since the arthropod/vertebrate split. CORL expression is not redundant to Sno expression because CORL proteins are restricted to a small portion of the CNS in which Sno is not expressed. dCORL and mCORL2 may still function as a switch between the Dpp/Activin signaling pathway in the CNS.

Table 1. Wing Phenotypes of MS1096.GAL4 x dCORL, mCORL1 or mCORL2

UAS dCORL	Phenotype	# phenotype	% phenotype
Total wings	Missing PCV	21	34%
62	Truncated PCV	41	66%
	1 ectopic hair L1 dorsal	10	16%
	2 ectopic hair L1 dorsal	10	16%
	3 ectopic hair L1 dorsal	2	03%
	1 ectopic hair L1 ventral	20	32%
	2 ectopic hair L1 ventral	2	03%
	Truncated L2	3	05%
	1 ectopic hair L2 dorsal	3	05%
	1 ectopic hair L2 ventral	1	02%
	1 ectopic hair L3 ventral	6	10%
	1 ectopic hair L5 dorsal	14	23%
	2 ectopic hair L5 dorsal	16	26%
	3 ectopic hair L5 dorsal	3	05%
	Truncated L5	13	21%
UAS.mCORL1	Small wing	90	100%
(III)	Missing ACV	13	14%
Total Wings	Truncated ACV	33	37%
90	Delta PCV	12	13%
	Truncated PCV	51	57%
	1 ectopic hair L1 ventral	6	07%
	Break in L1	3	03%
	Costal vein merged with L1	18	20%
	Bifurcation L2	2	02%
	Break in L2	2	02%
	Delta at margin L2	6	07%
	Ectopic vein off L2	26	29%
	Truncated L2	7	08%
	Ectopic vein connecting L2 to L3	4	04%
	Break in L3	2	02%
	Ectopic vein off L3	1	01%
	Truncated L5	90	100%

mCORL1	Phenotype	# Phenotype	% Phenotype
(II)	L5 truncated	80	100%
Total wings	L2 truncated	42	53%
80	Delta PCV	3	04%
	ACV truncated	0	0%
	Flimsy wing	80	100%
	Ectopic vein tissue connecting L2 L3	1	01%
	1 ectopic hair L1 ventral	0	0%
	ACV missing	28	35%
	Bifurcation L2	0	0%
	Break in L1	1	01%
	Delta L2 margin	0	0%
	Acosta merged with L1	0	0%
	Break in L2	23	29%
	Ectopic vein tissue L3	0	0%
	PCV missing	10	13%
	L4 truncated	8	10%
	Break in L4	14	18%
	L3 truncated	1	01%

UAS.mCORL2	Phenotype	# Phenotype	% Phenotype
(X)	Missing ACV	17	28%
Total Wings	Truncated ACV	26	43%
60	Truncated PCV	41	68%
	1 ectopic hair L1 ventral	13	22%
	Break in L1	59	98%
	Costal vein merged with L1	60	100%
	Delta at margin L2	5	08%
	Ectopic vein off L2	3	05%
	Truncated L2	12	20%
	Bifurcated L4	1	02%
	Truncated L5	60	100%

UAS.mCORL2 (II) Total wings 94	Phenotype	# Phenotype	% Phenotype
	L5 truncated	94	100%
	Break in L1	70	74%
	L2 truncated	44	47%
	ACV missing	53	56%
	ACV truncated	22	23%
	PCV truncated	50	53%
	1 ectopic hair L1 ventral	12	13%
	Acosta merged with L1	94	100%
	Ectopic vein tissue L2	24	26%
	Bifurcation L4	62	66%
	Delta L2 margin	5	05%
	Delta PCV	4	04%
	ectopic vein tissue L1 margin	1	01%
	Bifurcation L2	5	05%
	Ectopic vein tissue connecting L2 L3	3	03%
	Ectopic hair L3 margin ventral	1	01%

a. A percentage for each phenotype is shown to indicate relative frequency:
1) Percentages of phenotypes occurring less than 1% are excluded.
2) Percentages may exceed 100% because an individual wing can display multiple phenotypes

Table 2. Numbers of Third Instar Larval Brains Examined by Genotypes

Genotypes	#Brains	#Brains with clones in the MB
yw	9	6
UAS.dCORL	8	8
UAS.mCORL1	16	12
UAS.mCORL2	15	8
Df(4)dCORL	7	6
UAS.dCorl; Df(4)dCORL	7	4
UAS.mCORL1; Df(4)dCORL	15	8
UAS.mCORL2; Df(4)dCORL	18	15

Figures

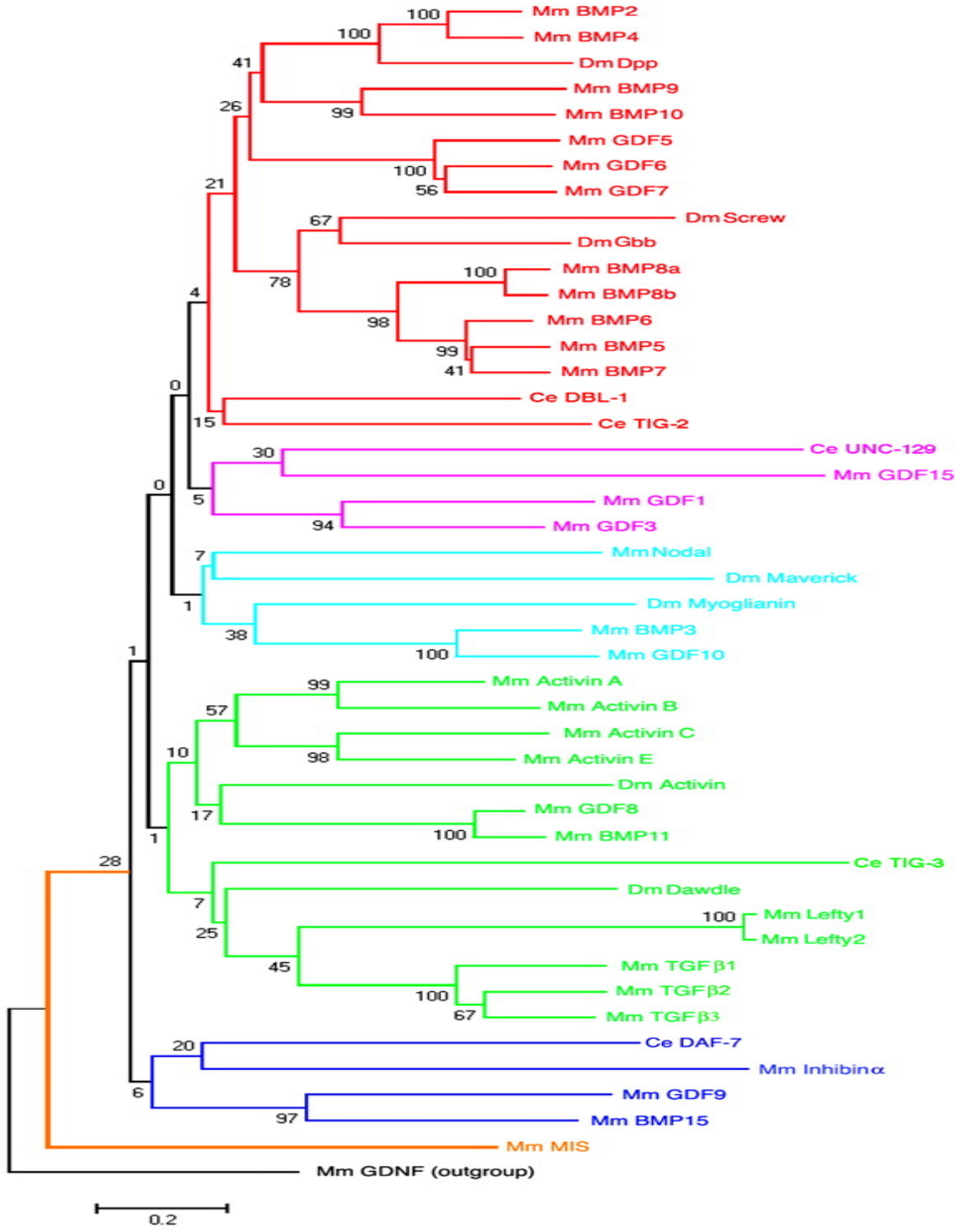
Mm BMP2
Mm BMP4
Dm Dpp
Mm BMP9
Mm BMP10
Mm GDF5
Mm GDF6
Mm GDF7
Dm Screw
Dm Gbb
Mm BMP8a
Mm BMP8b
Mm BMP6
Mm BMP5
Mm BMP7
Ce DBL-1
Ce TIG-2
Ce UNC-129
Mm GDF15
Mm GDF1
Mm GDF3
Mm Nodal
Dm Maverick
Dm Myoglianin
Mm BMP3
Mm GDF10
Mm Activin A
Mm Activin B
Mm Activin C
Mm Activin E
Dm Activin
Mm GDF8
Mm BMP11
Ce TIG-3
Dm Dawdle
Mm Lefty1
Mm Lefty2
Mm TGFβ1
Mm TGFβ2
Mm TGFβ3
Ce DAF-7
Mm Inhibinα
Mm GDF9
Mm BMP15
Mm MIS
Mm GDNF (outgroup)
0.2

Fig. 1. TGF-β ligand tree. This phylogenetic tree shows the sequence similarity of TGF-β ligands in mouse, fly, and worm. The branch at the top of the tree with BMP2, BMP4 and Dpp indicates a close relationship between the three proteins via sequence similarity. Though Dpp is a Drosophila protein, transgenic studies have shown that BMP2 and BMP4 are capable of rescuing mutant Dpp phenotypes in the fly. The values at the nodes denote statistical bootstrap values and the scale bar shows the number of amino acid substitutions between sequences.

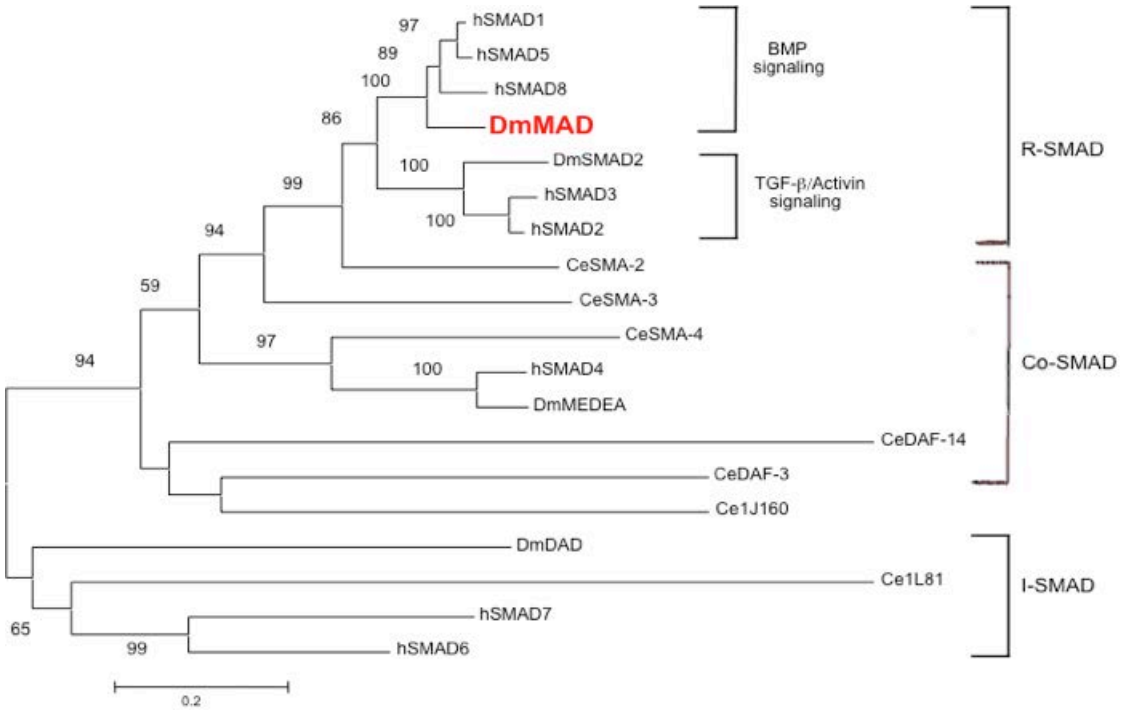

Fig. 2. The Smad signal transducer tree. This phylogenetic tree shows sequence similarity for Smad signal transducers. Receptor Smads hSmad1 and Mad are located within the BMP signaling group of the Smad phylogenetic tree. Receptor Smads dSmad2 and hSmad2, involved in TGF-β/dActivin signaling have shown similar phenotypes when ectopically expressed in wing discs (Marquez et al. 2002). hSmad4 is capable of rescuing a mutant phenotype of Medea (Hudson et al. 1998). CeSma-2, CeSma-3, and Ce-Sma-4 work together to form a co-Smad required for signaling similar to Dpp in the worm (Savage et al. 1996). CeDaf-3 is a co-Smad in the worm that antagonizes signaling similar to Dpp (da Graca et al. 2004).

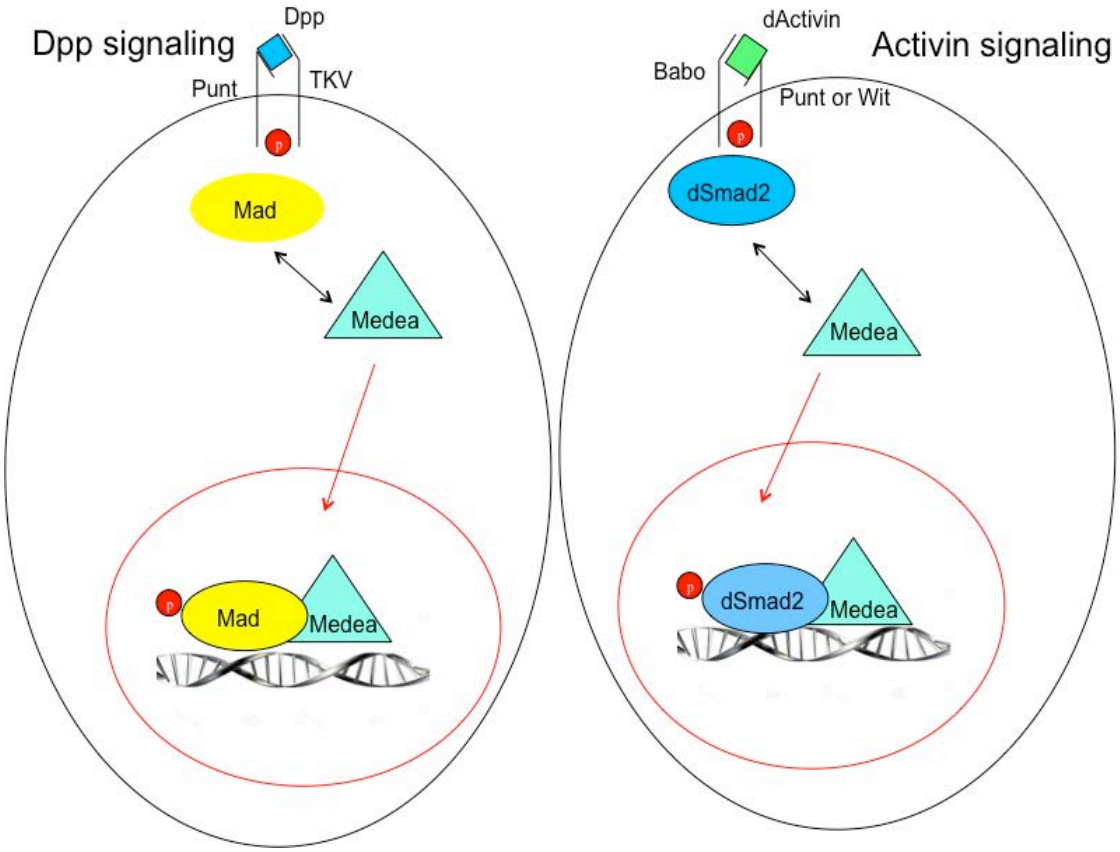

Fig. 3. Dpp and dActivin signaling in parallel. Dpp intracellular signaling and related proteins are shown on the left. dActivin intracellular signaling and related proteins are shown on the right. The ligand Dpp binds the type II receptor punt. The type I receptor, Thickveins can be recruited and the receptor complex becomes activated. The receptor complex phosphorylates Mad, which then binds the Co-Smad Medea. The Mad/Medea complex translocates to the nucleus and becomes a transcription factor that sets off a cascade of target gene expression. The ligand dActivin binds to the type II receptor Punt or Wit, the type I receptor Baboon can then be recruited and the receptor complex becomes activated. The receptor complex phosphorylates dSmad2 which then binds the Medea upon phosphorylation. The dSmad2/Medea complex becomes a transcription factor that and sets off a different cascade of gene expression.

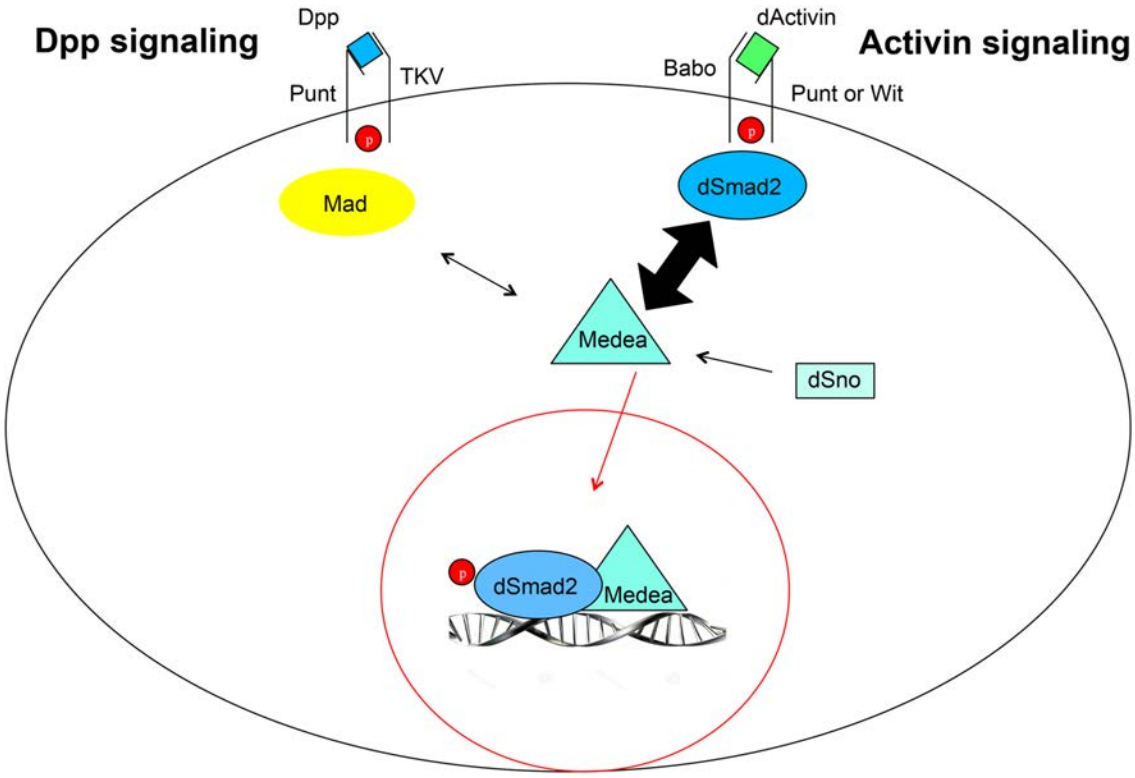

Fig. 4. Impact of dSno on Dpp/dActivin signaling. When dSno is present in the cell, it switches Medea's affinity from binding Mad to binding dSmad2. dSno acts as a switch between Dpp and dActivin signaling.

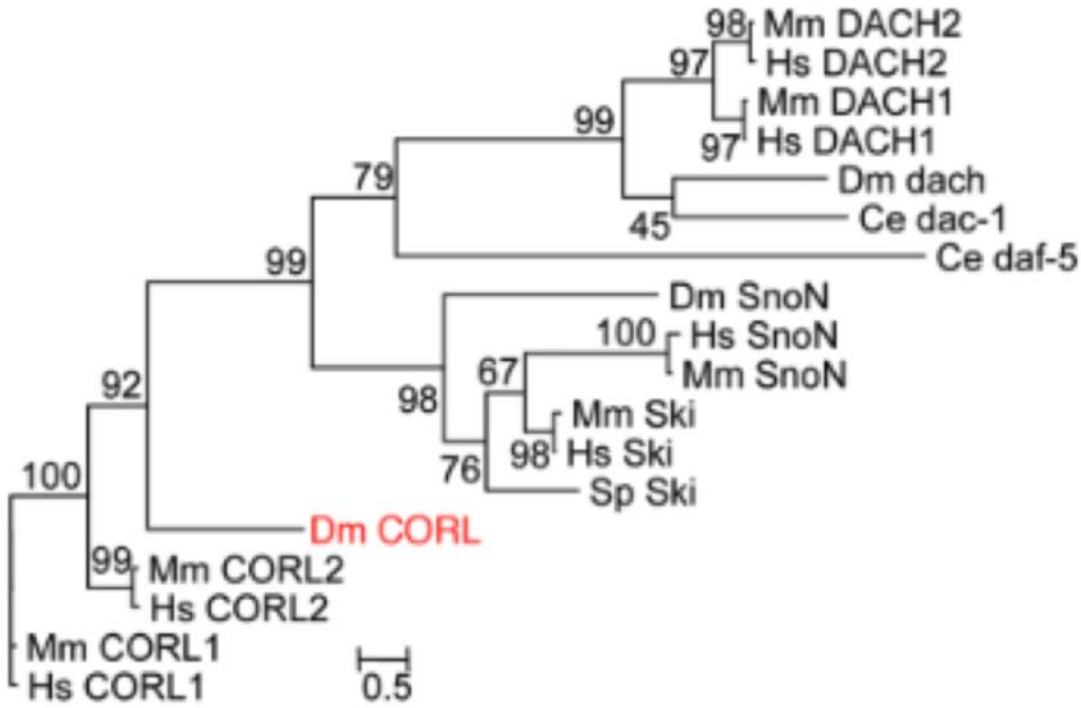

Fig. 5. Sno/Ski Smad binding proteins Tree. A phylogenetic tree that places CORL proteins within the Sno/Ski family. Proteins from worm, mouse, human, and fly are represented. Ce daf-5 is not placed with confidence in any subfamily of the Sno/Ski family tree.

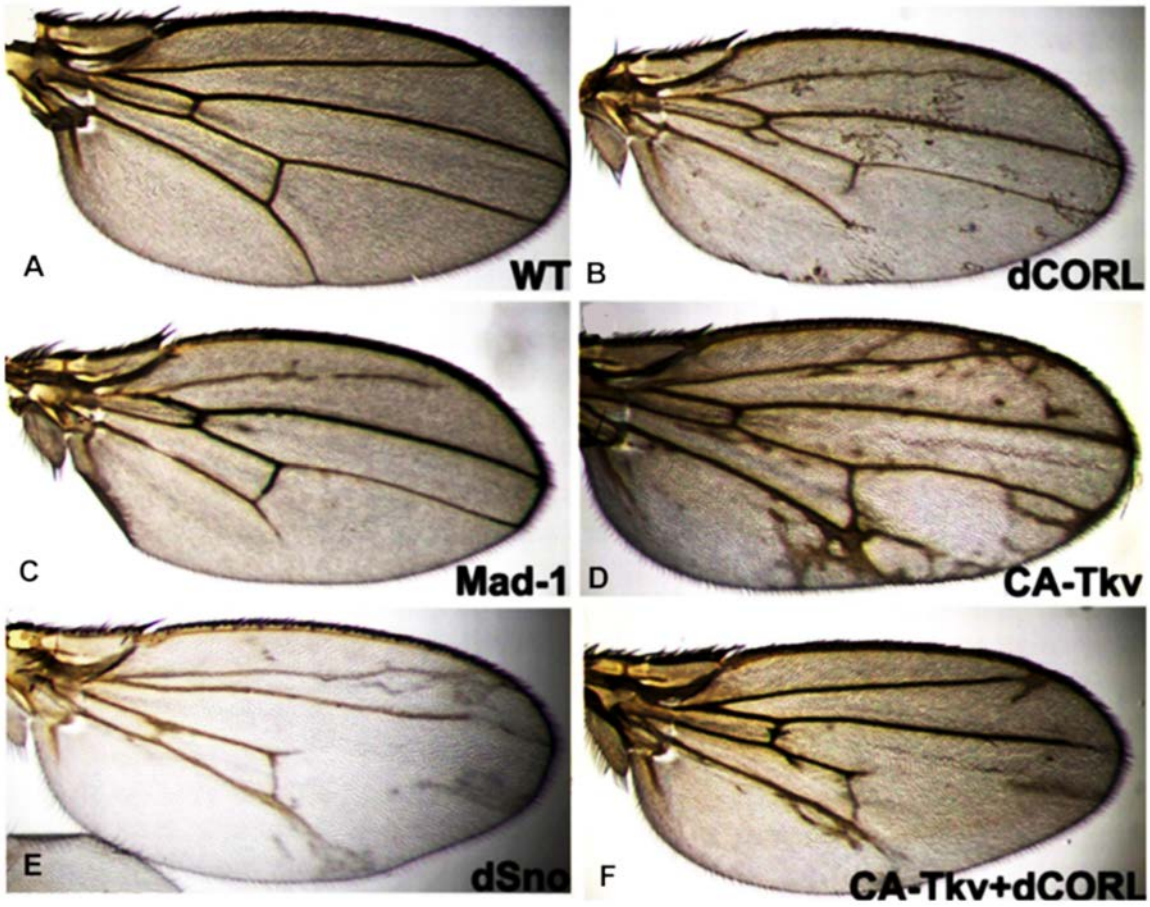

Fig. 6. dCORL overexpression antagonizes Dpp signaling. A) Wild type wing. B) Overexpression of dCORL shows vein truncations associated with suppression of Dpp signaling. C) Expression of Mad-1, a dominant negative allele that is a control for suppressed Dpp signaling, shows vein truncations. D) Constitutively activated Tkv causes overexpression of the Dpp signaling pathway downstream of the receptor causing ectopic vein tissue to form on the wing and increased wing size. E) dSno overexpression produces vein truncations that show suppression of Dpp signaling. F) In an epistasis experiment, dCORL is overexpressed with caTkv, vein truncations are visible and excess ectopic vein tissue is not found. Dpp signaling is repressed downstream of the receptor.

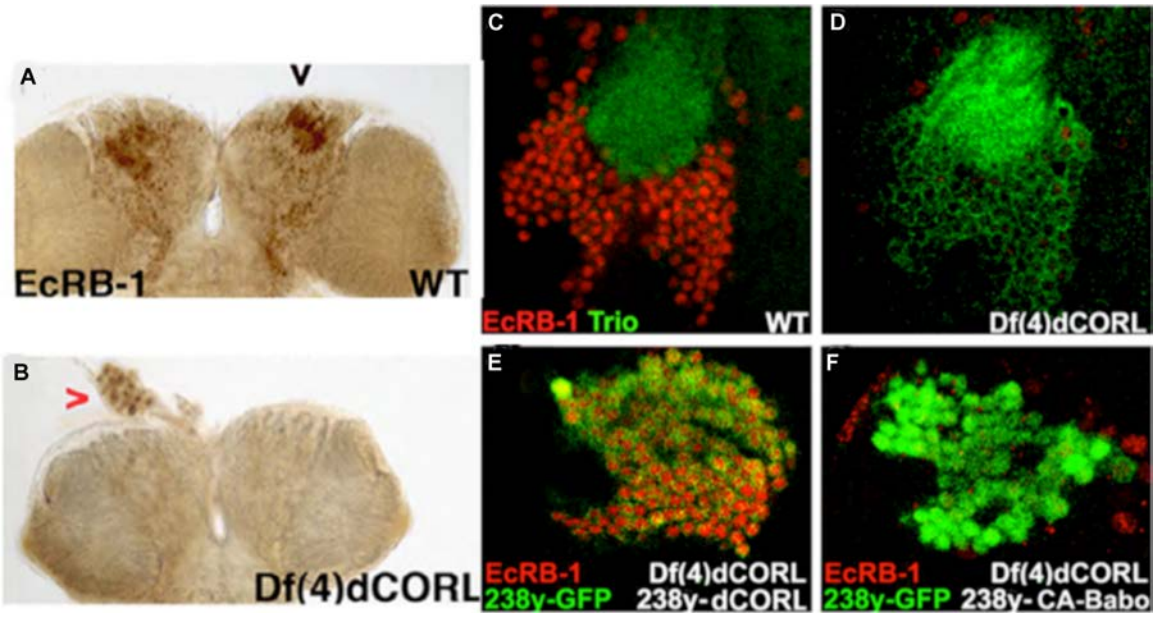

Fig. 7. dCORL is required for EcR-B1 expression in the MB. A) Wild type expression of EcR-B1 in a 3rd instar larval brain. B) EcR-B1 expression in a Df(4)dCORL 3rd instar larval brain. Note the ring gland still expresses EcR-B1 and there is absence of EcR-B1 in the MB. C) Wild type expression of EcR-B1 (red) and Trio (green), a neural membrane specific protein, in the Mushroom Body. D) EcR-B1 (red) and Trio (green) expression in a Df(4)dCORL brain, EcR-B1 expression is absent. E) UAS.dCORL expressed by 238Y.Gal4 (green) in a Df(4)dCORL brain rescues EcR-B1 (red) expression in cells with GFP. F) Constitutively activated Babo (green) causes overexpression of dActivin signaling from the receptor. caBabo does not rescue EcR-B1 (red) expression in a Df(4)dCORL brain, meaning dCORL is required for dActivin to complete its signaling cascade downstream of the receptor to activate EcR-B1 expression.

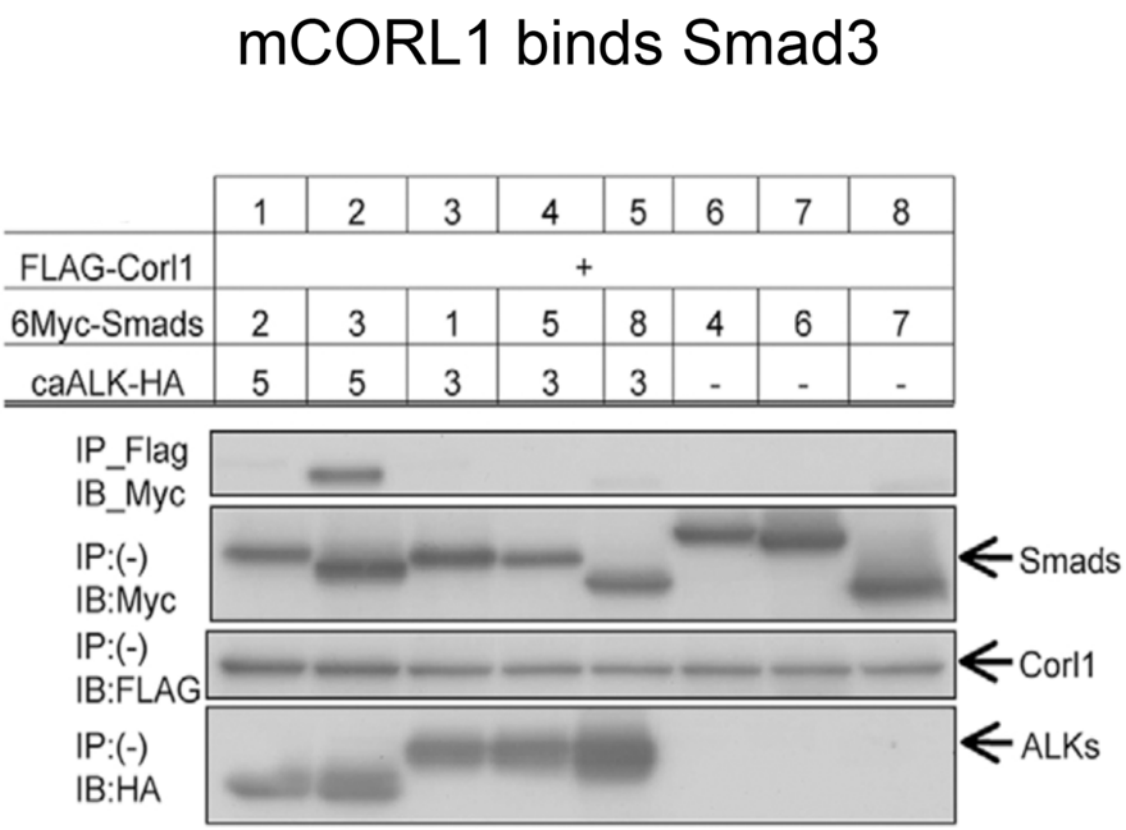

Fig. 8. mCORL1 binds Smad3. An immunoblot of FLAG-tagged mCORL1 and Myc-tagged Smads. Lanes 1-2 included caALK5 to activate TGF-β signaling. In lane 2, the top row shows that mCORL1 binds Smad3, a dSmad2 homolog. Lanes 3-5 included caALK3 to activate BMP signaling. Lanes 6-8 included Smad4 and inhibitory Smad6 and Smad7. There is no band in the top row, of lane 6 which would represent mCORL1 binding to Smad4, a homolog of Medea. The other rows are controls for protein expression.

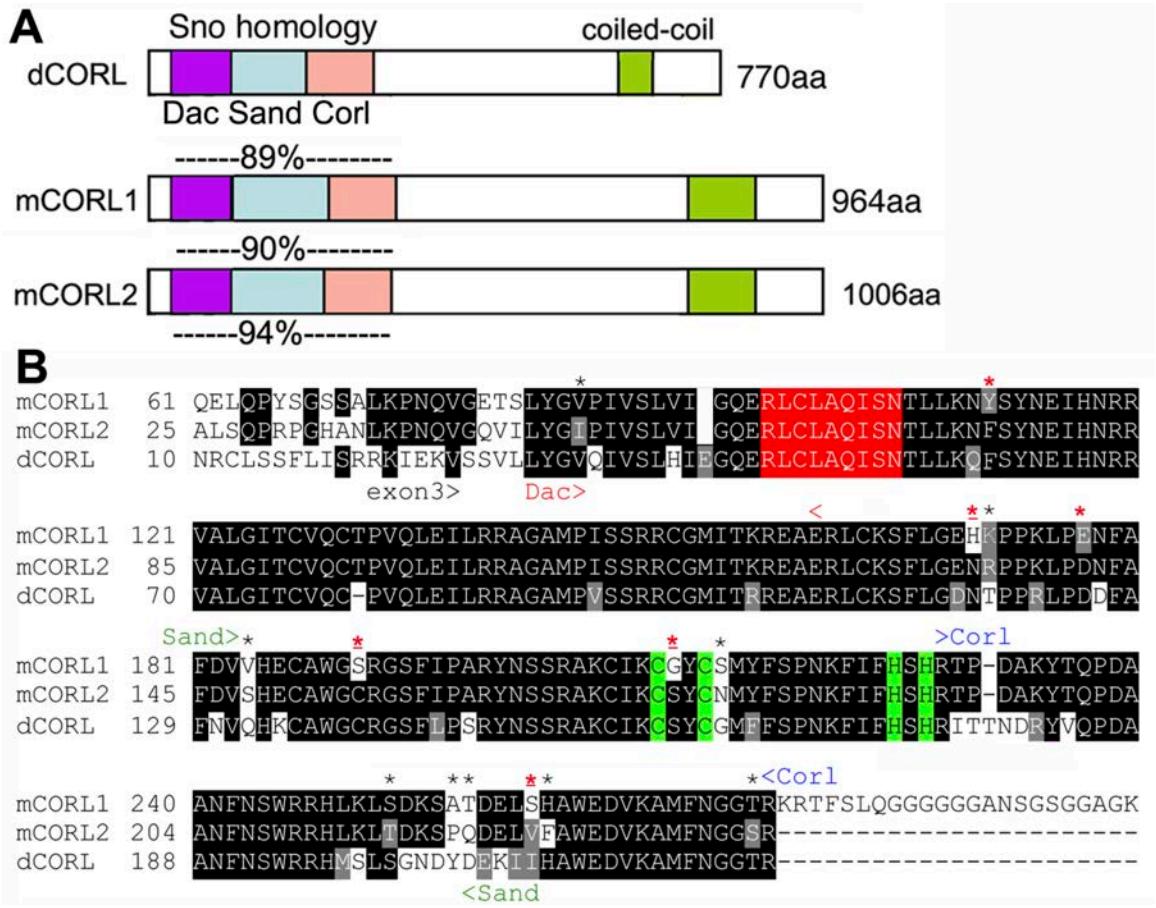

Fig. 9. mCORL1 differs from mCORL2/dCORL at six amino acids in the Sno homology domain. A) Schematic of CORL proteins from fly and mouse with the locations of five named domains shown. Amino acid similarity in the Sno homology domain (Smad binding; 195 residues) between dCORL and mCORL1 is shown above mCORL1, similarity between dCORL and mCORL2 above mCORL2 and similarity between mCORL1 and mCORL2 below mCORL2. Similarity is the sum of identical residues and conservative substitutions where both amino acids share biochemical properties: D/E, K/R/H, N/Q, S/T, I/L/V, F/W/Y, A/G (Smith and Smith 1990). B) Sno homology domain alignment. An amino acid is shaded if the residue is identical (black) or similar (gray) in two or three proteins. The APC recognition site is red and Cys2-His2

zinc finger in green. The Dac, Sand and CORL domains are shown with arrowheads. Fourteen amino acids different between mCORL1 and mCORL2 are indicated by *, a red * indicates 6 positions where dCORL and mCORL2 both differ from mCORL1 and red * indicates 4 positions where mCORL1 has a biochemically distinct amino acid from both dCORL and mCORL2.

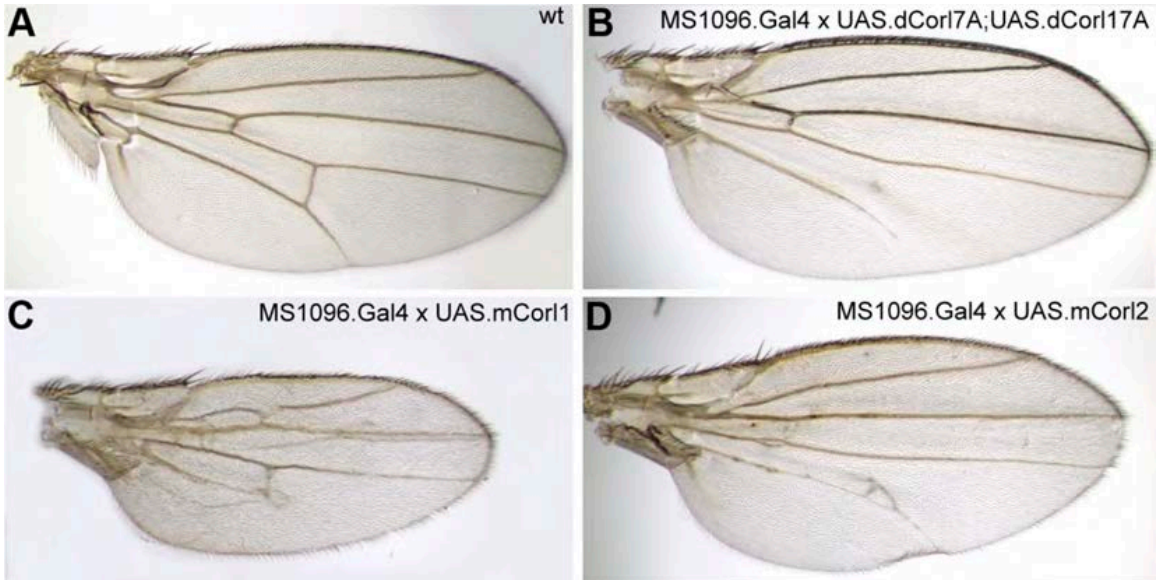

Fig. 10. mCORL1 differs from mCORL2/dCORL in ectopic wing expression assays. Ectopic expression of dCORL, mCORL1 and mCORL2 in adult wings driven by MS1096.Gal4. A) Wild type adult female wing. B) UAS.dCORL7A, UAS.dCORL17A is normal wing size with longitudinal vein 5 (L5) truncation, posterior cross vein (PCV) truncation and 4 ectopic bristles on longitudinal vein 1 (L1) below the Acosta region. C) UAS.mCORL1 wing is 25% smaller and less rigid than normal. L5 truncates prior to the PCV and there is a break in longitudinal vein 2 (L2). D) UAS.mCORL2 is normal size, and there are PCV and anterior cross vein (ACV) truncations. There is a delta of vein tissue in the PCV/L5 region. The acosta vein merges with L1 and there is a break in L1 below the Acosta region.

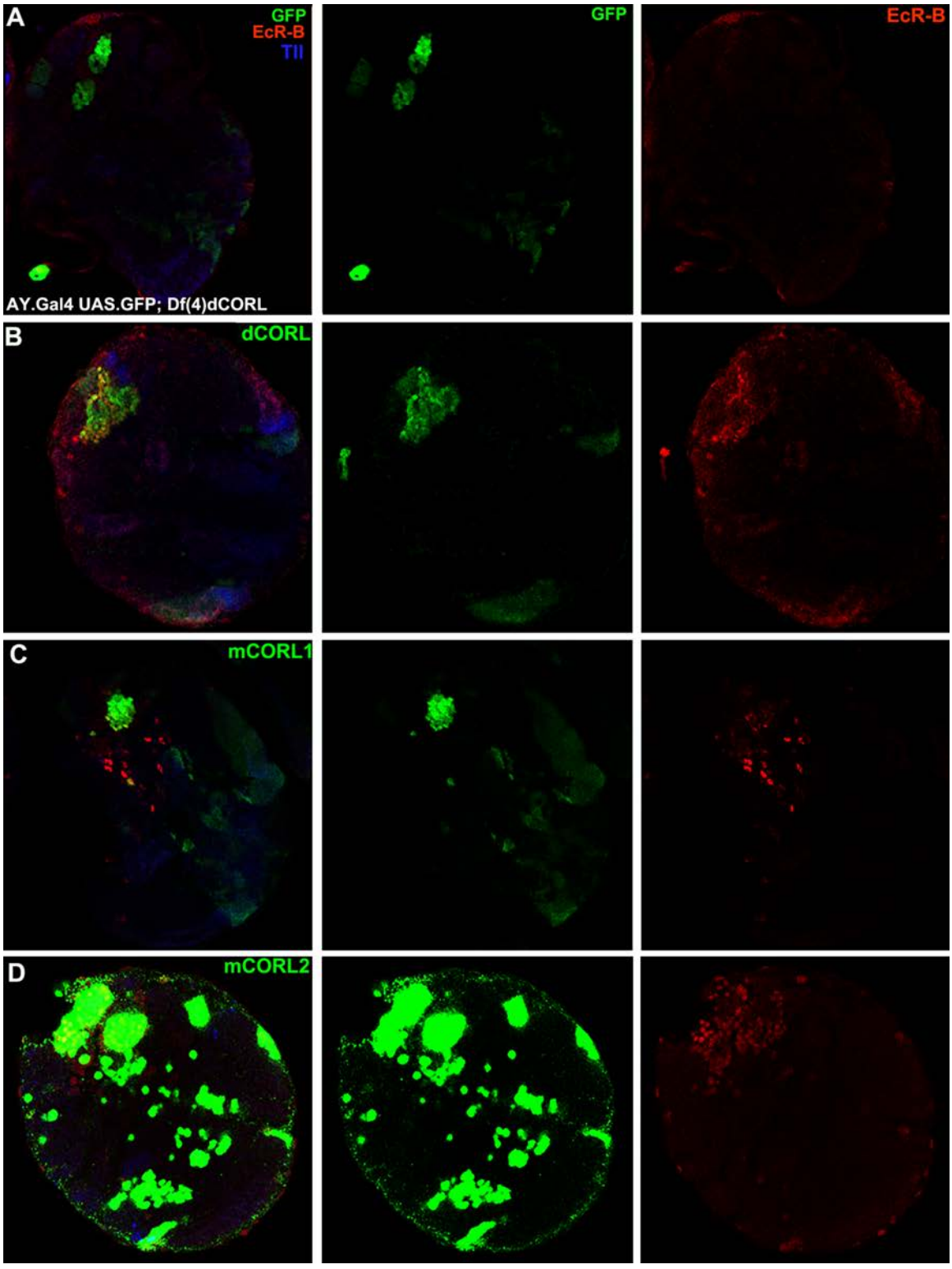
A
GFP
EcR-B
Tll
AY.Gal4 UAS.GFP; Df(4)dCORL
GFP
EcR-B
B
dCORL
C
mCORL1
D
mCORL2

Fig. 11. Rescue of Ecr-B1 in the MB of dCORL mutants by mCORL2/dCORL not mCORL1 (lobe). A single image of one slice is shown from left to right as three color (Tailless in blue, GFP in green and EcR-B1 in red), green alone (GFP), and red alone (EcR-B1). The genotype was determined by reference to the sister brain lobe. The clones location within the MB is referenced by the presence of Tll. High magnification views of each clone are displayed in figure 12 A) Single slice clone expressing only GFP. EcR-B1 expression is absent within the entire MB including the clone. B) Single slice image of a clone expressing dCORL and GFP. EcR-B1 expression is rescued within the boundaries of the clone but is still absent outside of the clone boundaries. C) Single slice image of a clone expressing mCORL1 and GFP. EcR-B1 expression is faint within the boundaries of the clone. D) Single slice image of two large clones expressing mCORL2 and GFP. EcR-B1 is rescued within the clone boundaries similar to the dCORL rescue.

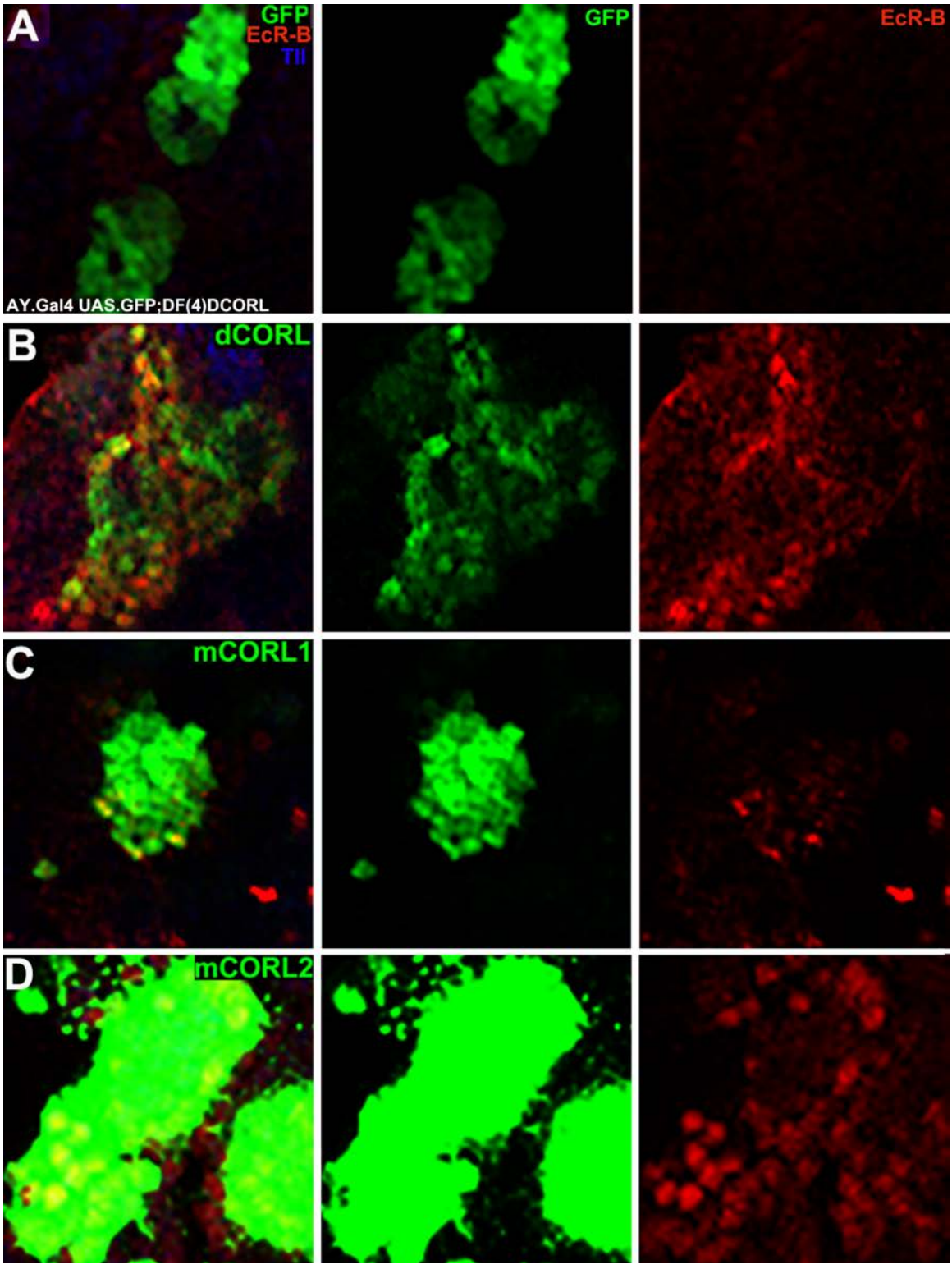
A
GFP
EcR-B
Tll
AY.Gal4 UAS.GFP;DF(4)DCORL
GFP
EcR-B
B
dCORL
C
mCORL1
D
mCORL2

Fig. 12. Rescue of Ecr-B1 in the MB of dCORL mutants by mCORL2/dCORL not mCORL1. High magnification dorsal view of the MB neurons in AY.Gal4, UAS-GFP, Df(4)dCORL brains with anterior up. These panels depict the same brains shown in **Fig. 11.** A single image of one slice is shown from left to right as three color (Tailless in blue, GFP in green and EcR-B1 in red), green alone (GFP), and red alone (EcR-B1). The genotype is determined by reference to the sister brain lobe. The clone location within the MB is referenced by the presence of Tll. A) Single slice clone expressing only GFP. EcR-B1 expression is absent within the entire MB including the clone. B) Single slice image of a clone expressing dCORL and GFP. EcR-B1 expression is rescued within the boundaries of the clone. C) Single slice image of a clone expressing mCORL1 and GFP. EcR-B1 expression is faint within the boundaries of the clone. D) Single slice image of a clone expressing mCORL2 and GFP. EcR-B1 is rescued within the clone boundaries similar to the dCORL rescue.

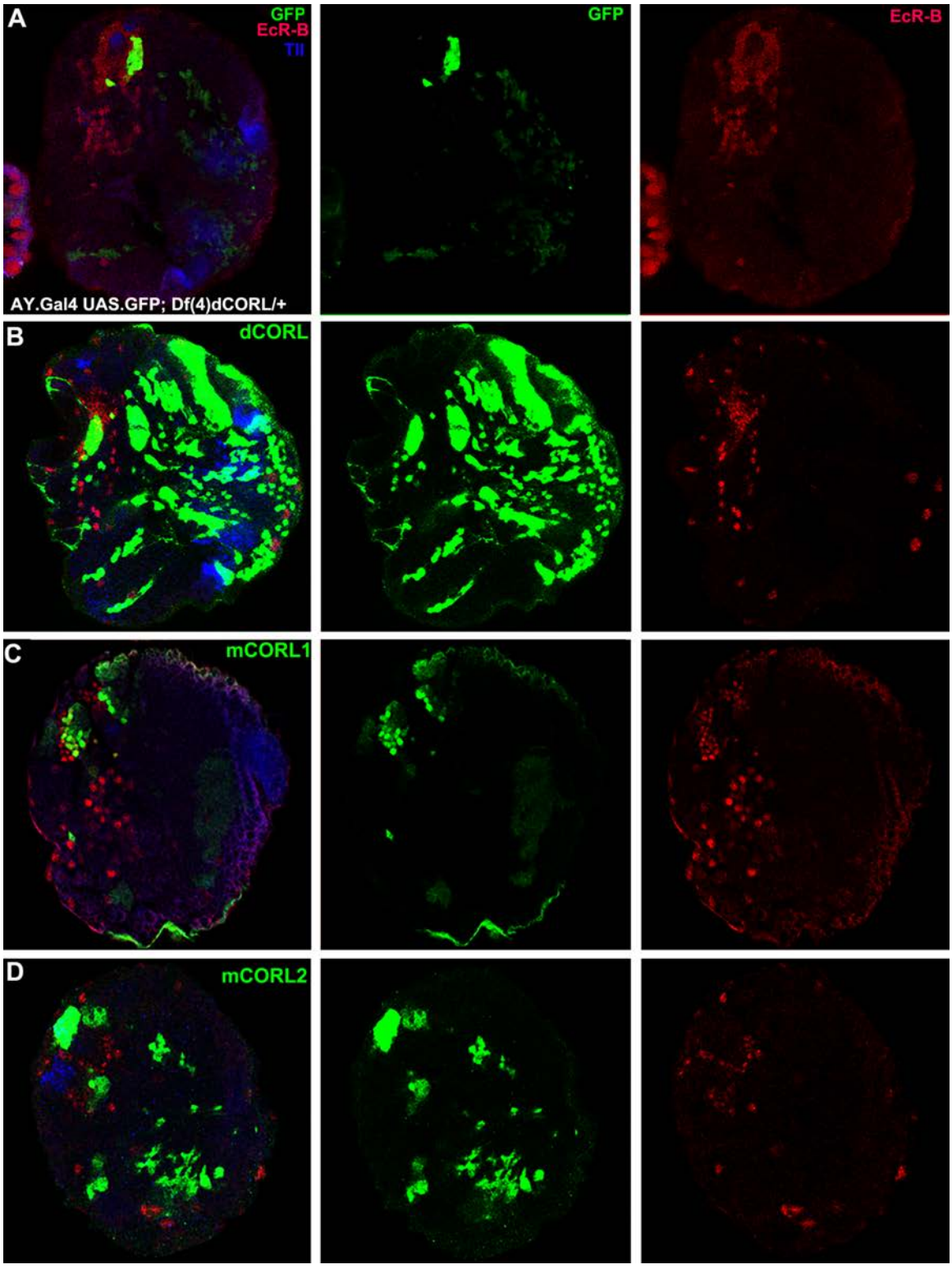
A
GFP
EcR-B
Tll
AY.Gal4 UAS.GFP; Df(4)dCORL/+
GFP
EcR-B
B
dCORL
C
mCORL1
D
mCORL2

Fig. 13. Repression of EcR-B1 in the MB of wild type by mCORL2/dCORL not mCORL1 (lobe). A single image of one slice is shown from left to right as three color (Tailess in blue, GFP in green, and EcR-B1 in red), Green alone (GFP), and red alone (EcR-B1). The genotype was determined by reference to the sister brain lobe. The clones location within the MB is referenced by the presence of Tll. High magnification views of each clone are displayed in **Fig. 14.** A) Single slice image of a lobe with a clone in the MB expressing GFP alone. EcR-B1 is unaffected. B) Single slice image of a lobe with a clone in the MB expressing dCORL and GFP. EcR-B1 expression is repressed in the clone. C) Single slice image of a lobe with a clone in the MB region expressing mCORL1 and GFP. EcR-B1 expression overlaps the clone and is unaffected. D) Single slice image of a lobe with a clone in the MB expressing mCORL2 and GFP. EcR-B1 expression is repressed in the clone, a phenocopy of dCORL.

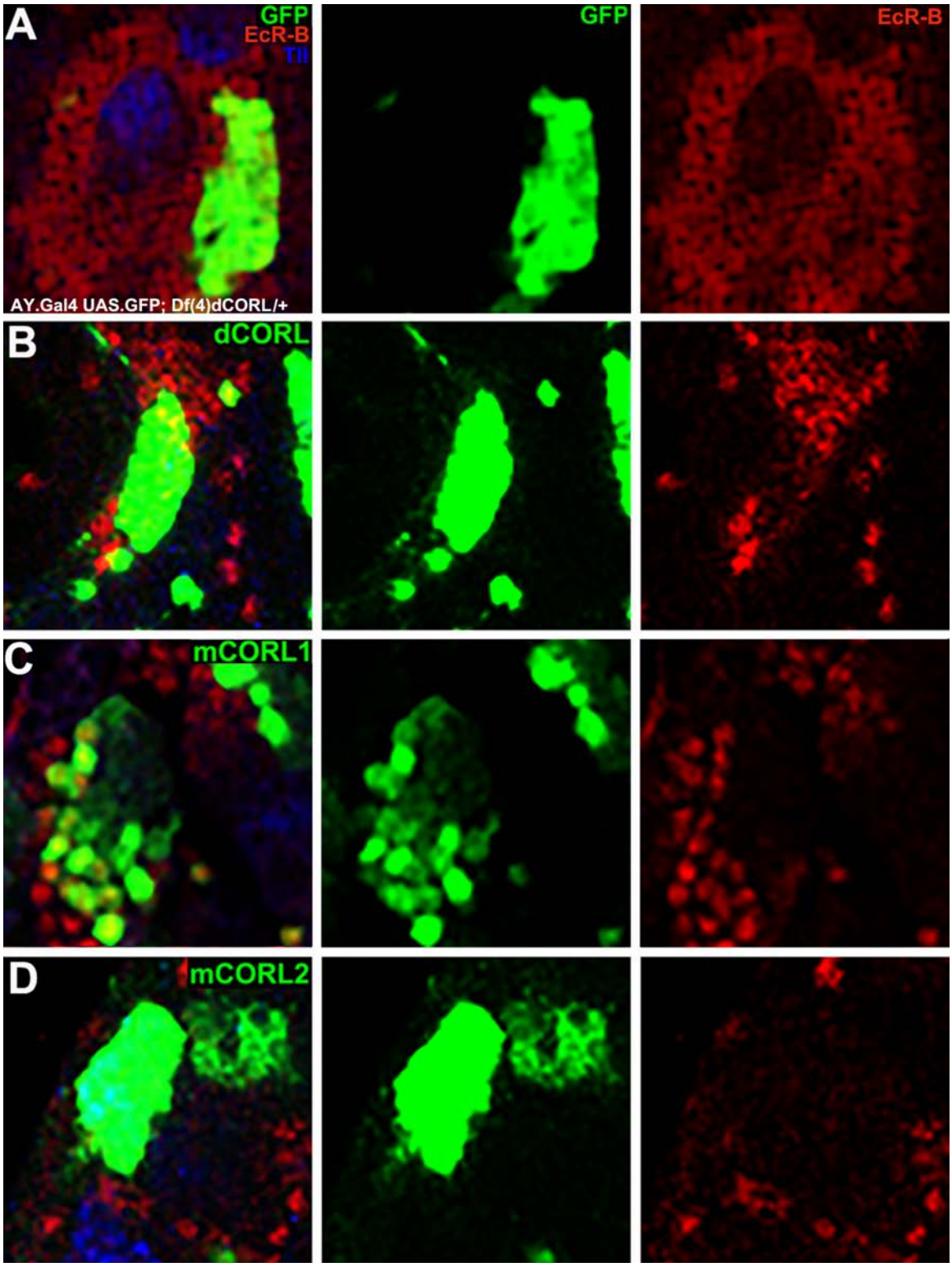
A
GFP
EcR-B
Tll
AY.Gal4 UAS.GFP; Df(4)dCORL/+
GFP
EcR-B
B
dCORL
C
mCORL1
D
mCORL2

Fig. 14. Repression of EcR-B1 in the MB of wild type by mCORL2/dCORL not mCORL1. High magnification dorsal view of mushroom body neurons in wild type siblings of Df(4)dCORL mutants with anterior up. These panels depict the same brains as displayed in **Fig.** 13. A single image of one slice is shown from left to right as three color (Tailless in blue, GFP in green and EcR-B1 in red), green alone (GFP), and red alone (EcR-B1). The genotype was determined by reference to the sister brain lobe. The clones location within the MB is reference by the presence of TLL. A) Single slice image of a clone expressing GFP alone. EcR-B1 is unaffected within the clone expressing GFP. B) Single slice image of a clone expressing dCORL and GFP. EcR-B1 is reduced within the region of the clone. C) Single slice image of a clone expressing mCORL1 and GFP. EcR-B1 expression is unaffected within the region of the clone. D) Single slice image of a clone expressing mCORL2 and GFP. EcR-B1 is reduced in the clone, like dCORL.

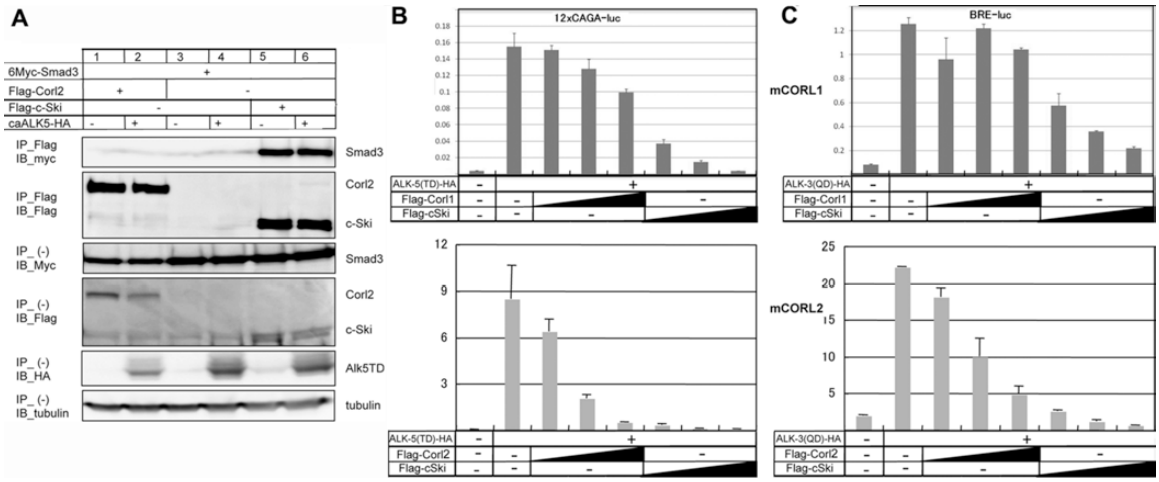

Fig. 15. mCORL2 functions are biochemically distinct from mCORL1. A) Interaction of FLAG-tagged mCORL2 and c-Ski with 6Myc-tagged Smad3 was examined by IP followed by IB in 293T cells. HA-tagged constitutively active ALK-5 (caALK-5) was utilized to activate TGF-β signaling. The top panel shows the interaction of c-Ski with Smad3, but no interaction between mCORL2 and Smad3 (lane 2). The lower five panels are controls for protein expression. B) mCORL1 (top), mCORL2 (bottom) and c-Ski (top and bottom) reduce TGF-β signaling with increasing efficacy as shown by a 12xCAGA-Luc reporter stimulated with caALK-5 in HepG2 cells (in duplicate, error bars are shown). Activity was normalized against co-transfected Renilla luciferase and reported in arbitrary units. mCORL1 and mCORL2 are effective but not as effective at antagonizing TGF-β signaling as c-Ski. C) The same experiment for BMP signaling via a BRE-Luc reporter stimulated with caALK-3. mCORL1 (top) is distinct from mCORL2 (bottom) and c-Ski (top and bottom). mCORL1 cannot antagonize BMP signaling but mCORL2 and c-Ski can, though c-Ski is a more effective antagonist than mCORL2.

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APPENDIX A

PROTOCOLS

Experiment:
Date:

Fixing 3rd Instar Brains

1. Dissect larvae in PBS at room temperature for no longer than 15 minutes
2. Place all dissected larval cuticles with brains in a 9-well dish on ice full of PBST
3. After 15 minutes, transfer all dissected larvae into a 1.5 mL Eppendorf tube, remove excess PBST
4. Fix in 4% formaldehyde/PBST (200 µl 16% formaldehyde + 600 µl PBST) for 25 minutes at room temperature
5. Remove fixative and discard into waste container
6. Wash 2x with PBST (~1 mL), 10 minutes each time
7. Remove liquid, rinse 2x with 25% MeOH quickly (~1 mL)
8. Remove liquid, wash 2x with 50% MeOH (~1 mL), 10 minutes each time
9. Remove liquid, add 100% MeOH (~1 mL)
10. Store brains at 4^{o}C between 12 hours and 5 days

Fluorescent Staining 3rd Instar Brains

1. Take out carcasses that have been stored in MeOH at 4oC
a. genotype (date): _____
_____
_____
2. Remove MeOH
3. Wash 2x with 50% MeOH 10 minutes each time
4. Remove liquid; rinse 2x with 25% MeOH quickly
5. Remove liquid; wash 2x PBST 10 minutes each time
6. Remove liquid, add Perm. Buffer (0.3% Triton-X/PBS), 1 ml/tube
7. Incubate for 30 minutes at room temperature (RT)
8. Remove liquid, rinse with PBST quickly (~1 mL)
9. Add Block (1% BSA/PBST), 1 ml/tube
10. Incubate for 30 minutes at RT
11. Remove liquid, rinse with PBST quickly (~1 mL)
12. Transfer brains to 0.5 mL Eppendorf tube
13. Add Primary Ab, µl/tube
a. Primary: _____ diluted 1: _____ in PBST
b. Primary: _____ diluted 1: _____ in PBST
c. Primary: _____ diluted 1: _____ in PBST
14. Incubate at 4oC overnight
15. Remove liquid, store diluted primary Ab in clean tube at 4oC
16. Rinse 4x in PBST, 20 minutes each time
17. Add Secondary Ab, µl/tube
a. Secondary: _____ diluted 1: _____ in PBST
b. Secondary: _____ diluted 1: _____ in PBST
c. Secondary: _____ diluted 1: _____ in PBST
18. Incubate at 4oC overnight; cover tray with tin foil
19. Remove liquid, rinse 4x in PBST, 20 minutes each time
20. Re-suspend in 300 µl 90% Glycerol/PBS, store at 4oC
21. Dissect brain off of carcass under a dissecting microscope using a needle and thin-tipped forceps
22. Mount 100 µl of 90% Glycerol/PBS with brains onto glass slide
23. Set clay on cover slip or broken glass onto glass slide
24. Put cover slip on top of mounted solution, avoiding air bubbles
25. Use nail polish to seal cover slip onto slide
a. date on slide: _____