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RESEARCH ARTICLE

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Porcupine/Wntless-dependent trafficking of the conserved WntA ligand in butterflies

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Abstract

Wnt ligands are key signaling molecules in animals, but little is known about the evolutionary dynamics and mode of action of the WntA orthologs, which are not present in the vertebrates or in *Drosophila*. Here we show that the WntA subfamily evolved at the base of the Bilateria + Cnidaria clade, and conserved the thumb region and Ser209 acylation site present in most other Wnts, suggesting WntA requires the core Wnt secretory pathway. WntA proteins are distinguishable from other Wnts by a synapomorphic Iso/Val/Ala216 amino-acid residue that replaces the otherwise ubiquitous Thr216 position. *WntA* embryonic expression is conserved between beetles and butterflies, suggesting functionality, but the *WntA* gene was lost three times within arthropods, in podoplean copepods, in the cyclorrhaphan fly radiation, and in ensiferan crickets and katydids. Finally, CRISPR mosaic knockouts (KOs) of *porcupine* and *wntless* phenocopied the pattern-specific effects of *WntA* KOs in the wings of *Vanessa cardui* butterflies. These results highlight the molecular conservation of the WntA protein across invertebrates, and imply it functions as a typical Wnt ligand that is acylated and secreted through the Porcupine/Wntless secretory pathway.

KEYWORDS
butterfly wings, color pattern formation, porcupine, WntA, Wnt acylation, Wnt family evolution, Wnt trafficking, Wntless/Evi/Sprinter

1 | INTRODUCTION

The Wnt signaling pathway is a key component of the animal developmental genetic toolkit, but its study has been limited to a few species and pathway components, hindering a broader understanding of its mechanisms and functions (Bejsovec, 2018; Holstein, 2012; Hoppler & Moon, 2014). While the Wnt-family ligand *WntA* is widespread among bilaterian and cnidarian animals (Janssen et al., 2010; Kusserow et al., 2005; Lengfeld et al., 2009; Somorjai et al., 2018; Yuan et al., 2019), *WntA* was lost from both vertebrate and *Drosophila* genomes (Holzem et al., 2019; Murat et al., 2010; Somorjai et al., 2018) and is thus absent from the most widely used laboratory organisms. Nonetheless, there is emerging evidence for the patterning functions of *WntA* in the developing embryos and regenerating tissues of invertebrate model systems (Bolognesi et al., 2008b; Darras et al., 2018; Fritzenwanker et al., 2019; Girich et al., 2017; Hogvall et al., 2019; Janssen et al., 2010; Kraus et al., 2016; Kusserow et al., 2005; Lengfeld et al., 2009; Pruitt et al., 2014; Somorjai et al., 2018; Yuan et al., 2019), calling for a refined understanding of the conserved and divergent aspects of *WntA* secretion, reception, and transduction relative to other Wnt genes.

The secretion of Wnt ligands relies on a core endomembrane trafficking and maturation pathway involving Porcupine (Porc), an acyltransferase that acylates the Wnt glycoprotein, adding a fatty acid required for Wnt recognition and binding by Evi/Sprinter/Wntless (Wls), a cargo protein critical to its endosomal processing and release at the cell surface (Gao & Hannoush, 2014; Herr &

Basler, 2012; Nile & Hannoush, 2019; Routledge & Scholpp, 2019; Takada et al., 2006). Following intercellular transport, Wnt ligands generally bind cognate receptors of the Frizzled (Fz) family, although alternative Receptor Tyrosine Kinase receptors of the Ryk, Ror, MuSK, and PTK7 families are also known to bind and transduce Wnt signals in a variety of context-dependent processes (Hoppler & Moon, 2014; Roy et al., 2018; Wang et al., 2016). In addition to its importance for Wnt trafficking, the Porc-dependent Wnt lipid moiety is recognized by the fatty-acid binding groove in the cysteine rich domains of Fz, ROR, and MuSK receptors (Hoppler & Moon, 2014; Nile & Hannoush, 2019; Povelones & Nusse, 2005; Roy et al., 2018). Wnt acylation was originally considered essential for Wnt signaling activity (Azbazdar et al., 2019; Hosseini et al., 2019; Kakugawa et al., 2015), but a recent report depicts a more complex picture suggesting some Wnts can signal with a mutated acylation site (Speer et al., 2019).

To address the biology of WntA, one must first identify a system where WntA functions can be distinguished from other Wnts, and the butterfly wing system is ideal for this endeavor due to its two-dimensionality. Genetic and developmental studies of the family Nymphalidae have shown first, that *WntA* regulatory alleles drive adaptive pattern shape variation across populations of *Heliconius*, *Limenitis* and *Elymnias* butterflies (Gallant et al., 2014; Huber et al., 2015; Martin et al., 2012; Martin & Courtier-Orgogozo, 2017; Moest et al., 2020; Morris et al., 2019; Ruttenberg et al., 2021; Van Belleghem et al., 2017); second, that *WntA* expression in larval wing imaginal disks varies widely between species, but always prefigures the position of many color pattern elements of the Nymphalid Ground Plan (Martin & Reed, 2014; Mazo-Vargas et al., 2017; Schwanwitsch, 1956); and finally, that *WntA* is necessary for pattern induction and for the formation of color domain boundaries in all nymphalid species studied so far, as shown in clustered regularly interspaced short palindromic repeats (CRISPR) mosaic knockout (mKO) experiments (Concha et al., 2019; Mazo-Vargas et al., 2017). *WntA* is thus a key determinant of color pattern formation and diversification in butterfly wings, with both conserved and derived functions, but its molecular mode of action requires further investigation.

In this study, we clarify the origin of WntA in the metazoan tree, assess the conservation of its acylated region, and refine its phylogenetic distribution in arthropods. Then, we generate *porc* and *wls* mKO crispants in butterflies and compare their effect with the wing pattern defects observed in *WntA* knock-outs. We conclude that WntA, widely distributed in animals, is likely secreted as a standard lipid-modified Wnt ligand.

2 | MATERIALS AND METHODS

2.1 | Phylogenetic analysis of Wnt proteins

A dataset of 119 full-length Wnt protein sequences from bilaterian and cnidarian representative species was compiled from NCBI Genbank (Supporting Information File S1), aligned with MAFFT, and curated with GUIDANCE2, leading to an alignment of 384 phylogenetically informative sites (Katoh & Standley, 2013; Sela et al., 2015). W-IQ-TREE was used for Maximum Likelihood tree reconstruction with SH-aLRT scores for node support (Anisimova et al., 2011; Trifinopoulos et al., 2016). Additional Gastropoda WntA sequences (Supporting Information File S2) were obtained by translation of messenger RNA sequences obtained by taxon-limited TBLASTN against the NCBI TSA database (*Organism* = Gastropoda [taxid:6448]). To probe for WntA presence-absence across arthropods, taxon-limited TBLASTN searches were performed against the NCBI WGS and TSA databases for 120 families, as accessed before November 2020, and hits on putative WntA orthologs were additionally screened for the presence of a non-Threonine residue at mouse Wnt3a position 216 (Table S1). A tree of 120 arthropod families was obtained using the Phylotastic web server and represents the consensus topology from those lineages in the Open Tree of Life project (Nguyen et al., 2020). Amino-acid frequency profiles of the Wnt thumb region were generated in the Geneious software.

2.2 | In situ hybridizations (ISH)

Vanessa cardui embryos were collected at the 35% stage (26 h after egg laying [AEL] at 25°C), dechorionated in 5% bleach diluted in phosphate buffer saline, and fixed overnight in 3.7% formaldehyde at 4°C. ISH were performed following a previously described wing protocol with the same *WntA* and *wg* riboprobes (Martin & Reed, 2014).

2.3 | CRISPR/Cas9 mutagenesis

All rearing, design, reagent preparation and injection procedures for *Vanessa cardui* butterflies were performed as described in a previous publication (Martin et al., 2020). Short synthetic guide RNAs (Synthego) targeting coding exons of *porc* and *wls* were designed as follows: *Vc_porc_single guide RNA* [*sgRNA*] 5′-AAACUUGGCAGCAAAUCAG-3′; *Vc_wls_sgRNA* 5′-CGUAGGUUUCGCCCUCACG-3′.

Purified *S. pyogenes* Cas9-2xNLS protein was provided by QB3/Macrolabs at UC Berkeley. Duplexes of Cas9:sgRNA were injected in butterfly syncytial embryos at 250:125 or 125:75 ng/μl at various stages (Table 1). Embryos were laid, collected, and injected at 23°C, immediately transferred to a wet chamber at 25°C. Larvae were reared on artificial diet at 25°C, transferred to room temperature as pupae, and then sprayed with water every 2 days until emergence. Adults were frozen at least 36 h after emergence to allow proper wing drying, spread-mounted, and imaged on a Nikon D5300 digital camera mounted with an AF-S VR MicroNikkor 105 mm f/2.8G lens. High resolution images of butterflies were taken on a Keyence VHX-5000 digital microscope fitted with a VH-Z100T lens.

TABLE 1 Summary of *Vanessa cardui* CRISPR injection experiments

sgRNA name	Injection time (hours) AEL	Final concentration (ng/µl) [Cas9:sgRNA]	Total injected (*N*)	L1 larvae	Egg hatching rate (%)	Pupae (total)	Pupae missing one wing or more	Emerged adults (total)	Adults with pattern defects
Vc_porc	2.5–5	250:125	675	58	9%	25	5	16	3
	4–5	250:125	169	37	22%	14	9	8	5
	6–7	250:125	324	90	28%	46	6	40	5
Vc_wls	4–6	250:125	136	52	38%	23	4	16	5
	6–8	125:75	161	64	40%	31	0	28	5

Note: We measured that *V. cardui* embryos hatch after 96 h at 23°C, or after 76 h at 25°C. As we collect and inject eggs at a room temperature of 23°C, a 4–8 h AEL window corresponds to about 4%–8% of embryonic development, an interval that spans the formation of the germ disc and precedes blastoderm differentiation (Ferguson et al., 2014; Holzem et al., 2019). Thus, a 4–8 h AEL interval is expected to be a good trade-off for maximizing mosaicism, which allows escapers to survive deleterious effects of the knockout while also allowing injected Cas9/sgRNA duplexes to reach a subset of nuclei.

Abbreviation: sgRNA, single guide RNA.

3 | RESULTS

3.1 | Evolution of the WntA proteins in Bilateria + Cnidaria

We reconstructed a phylogeny of Wnt protein sequences to remove ambiguities in Wnt nomenclature and orthology relationships (Figure 1a). Our sampling recovered 13 Wnt subfamilies across Bilateria and Cnidaria (Supporting Information File S1), in agreement with previous analyses of Wnt ligand diversification (Holstein, 2012; Murat et al., 2010; Somorjai et al., 2018). No WntA orthologs were recovered from Porifera or Ctenophora datasets (Pang et al., 2010; Reid et al., 2018), suggesting that the WntA subfamily evolved after the divergence of these two metazoan early-diverging clades (Holstein, 2012; Lengfeld et al., 2009; Nichols et al., 2006). A planarian Wnt homolog that has been variably annotated as Smed_Wnt4, Smed_Wnt11-6, or Smed_WntA (Gurley et al., 2008, 2010; Reddien, 2018), was placed near the Wnt4 and Wnt11 subfamilies, and should not be confused with a *bona fide* WntA ortholog.

From this classification, we derived a reference set of 16 eumetazoan WntA protein sequences with a GenBank entry (Supporting Information File S1). All curated *WntA* orthologs have a start codon shortly followed by a predicted signal cleavage peptide at their N-terminal end (EMBOSS/sigcleave [Rice et al., 2000] score >5). Analysis of the Wnt thumb region involved in receptor binding showed that all WntA proteins have conserved cysteines known to form disulfide bonds (Nile & Hannoush, 2016), as well as a conserved Serine (Ser) site (Figure 1b) at the aligned position 209 of *Mus musculus* Wnt3a, the site of Porc-mediated Wnt acylation (Asciolla et al., 2017; Lee et al., 2019; Rios-Esteves et al., 2014). An exception to this is that *Ciona robusta* has a Threonine at this site, an amino-acid that is also subject to acylation (Miranda et al., 2014). A noticeable change in the Wnt thumb was observed in Euthyneura, the crown group of Gastropoda, where WntA underwent a Ser211Asp change (Figure 1b). Wnt3a Ser211Ala mutants maintain acylation of Ser209 (Nile & Hannoush, 2016), so it is unclear if the euthyneuran WntA Asp211 position has any functional impact. Finally, 97% of the non-WntA ligands in our alignment show a conserved Threonine residue at reference position 216, while all 17 WntA sequences are characterized by an Isoleucine or Valine at this site (Figure 1c). This Thr216Iso/Val substitution in the Wnt thumb region is a WntA synapomorphy, and can be used as a first indicator of *WntA* orthology when analyzing metazoan Wnt sequences. As exceptions, Thr216 was substituted in three sequences outside of WntA in our dataset: Wnt8 in *Nematostella vectensis* (Ser), Wnt10 from *Ciona robusta* (Iso), and Wnt16 from *Saccoglossus kowaleski* (Ser).

3.2 | Conservation and sporadic losses of *WntA* in arthropoda

Next we performed TBLASTN searches across genome and transcriptome assemblies spanning 120 arthropod families represented in the NCBI WGS and TSA databases, using WntA protein sequences as queries. We detected WntA exons and transcripts in 100 (83%) of the 120 searched families, and all WntA sequences showed a conserved Ser209 acylation site. We can conservatively infer WntA was sporadically lost at least three times in Arthropoda (Figure 2a and Table S1): in podoplean copepods, in Cyclorrhapha, a large radiation of flies that includes *Drosophila*; and in Ensifera (crickets and katydids). Two lineages of hemipteran insects independently evolved a derived Ala216 residue, in replacement of the Iso/Val216 synapomorphic position observed in all the other hemipteran WntA orthologs, including aphids and *Oncopeltus* milkweed bugs (Table S1).

3.3 | *Tribolium*-like expression of *WntA* in lepidopteran embryos

WntA expression has been profiled in the embryos of two insects so far (Bolognesi et al., 2008a; Holzem et al., 2019; Janssen

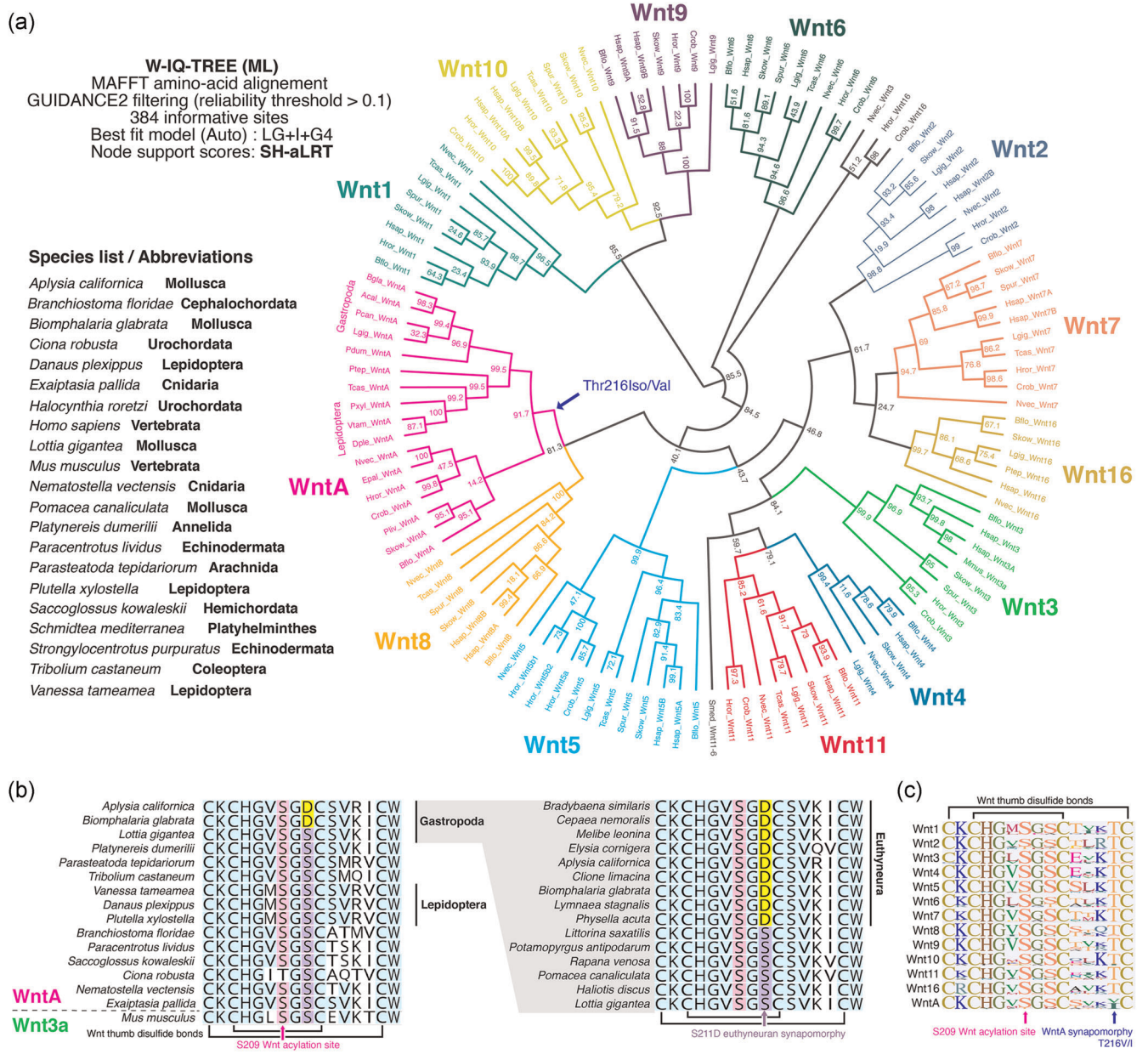

FIGURE 1 Molecular evolution of the WntA thumb region. Amino-acid positions use the murine Wnt3a reference for numbering (Nile & Hannoush, 2016). (a) Maximum likelihood phylogeny of reference Wnt proteins in Cnidaria + Bilateria, showing monophyly of the WntA orthology group. Node support is indicated as percentages by SH-aLRT scores, which are usually considered to indicate robust node support above 80% (Anisimova et al., 2011). (b) Conservation of the WntA thumb region with the mouse Wnt3a reference, including the Ser209 acylation site, and synapomorphic Ser211Asp in euthyneuran gastropods. Disulfide bonds between conserved Cysteines are indicated. (c) Amino-acid frequency profile of the Wnt thumb region for the 13 Wnt orthology groups, showing the Thr216V/I change specific to WntA [Color figure can be viewed at wileyonlinelibrary.com]

et al., 2010), the butterfly *Bicyclus anynana* (Nymphalidae, Lepidoptera) and the flour beetle *Tribolium castaneum* (Tenebrionidae, Coleoptera). Both species reveal a conserved *WntA* expression pattern in the head lobes, telson, and posterior segment compartments up to 30% of embryonic development. This pattern largely resembles the expression of *wg/Wnt6/Wnt10* up to 30% of development (Holzem et al., 2019; Janssen et al., 2010). Past that stage, *WntA* expression diverges from these other Wnt genes in *Tribolium* (Bolognesi et al., 2008a). Here we assessed if this remains true in butterflies, and found that indeed, *WntA* expression is distinguishable from *wg* in *Vanessa cardui* 35% embryos, as shown by more extensive expression domains in the head lobes and gnathal appendages, by a larger expression domain in legs, including a ring-like area in the median leg region, and by a broadening of the segmental expression domains that span from median ventral clusters to lateral edges

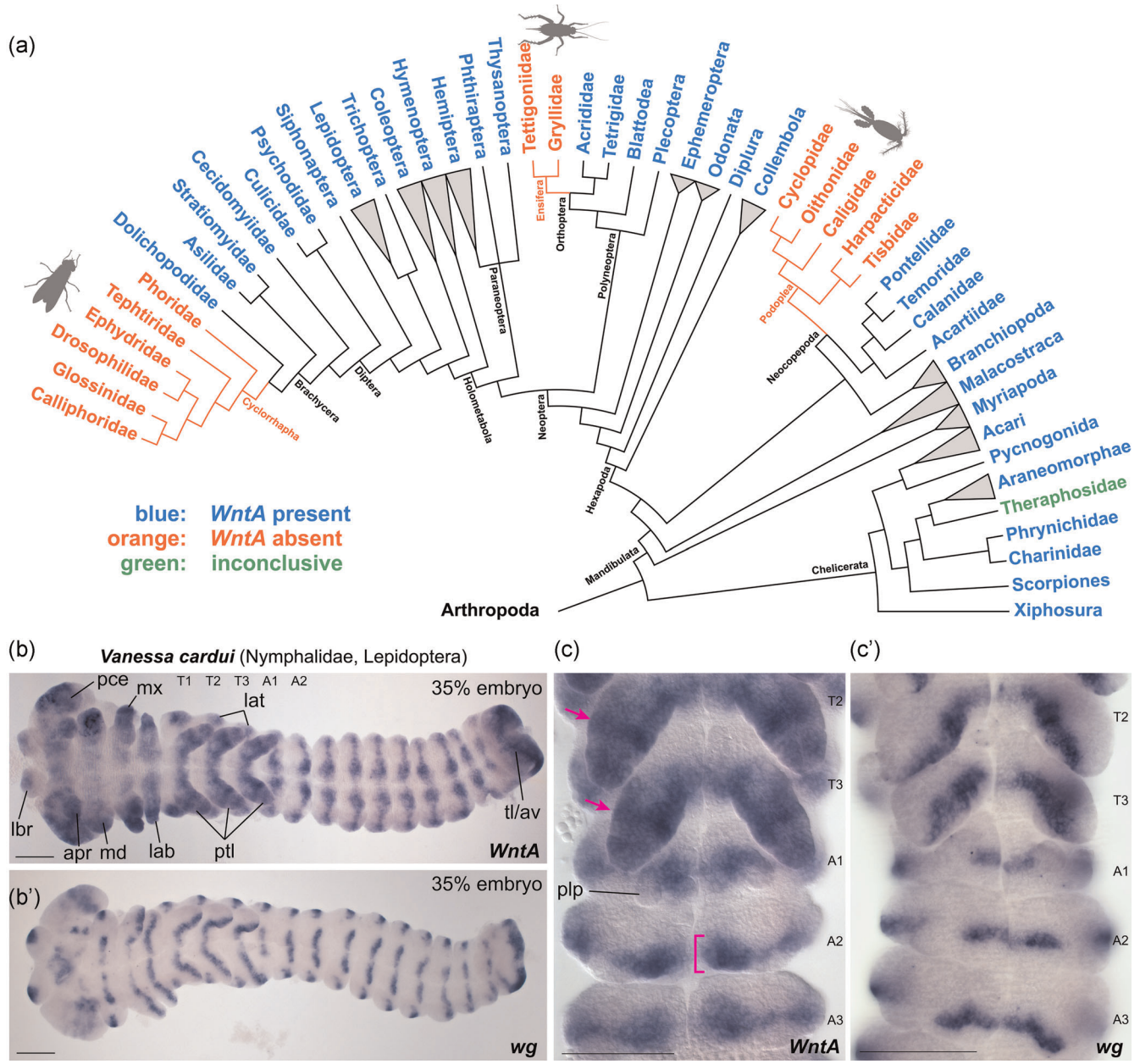

FIGURE 2 Three independent losses of *WntA* in arthropods in spite of a complex expression pattern at mid-embryogenesis. (a) Summary of the phylogenetic distribution of *WntA* in arthropods as inferred from similarity searches over available genomes and transcriptomes from 120 families (Table S1), indicating three loss events (orange). (b, b') (insets, c, c'). In situ hybridization for *WntA* and *wg* in 35% embryos. Arrows: ring-like expression of *WntA* in midlegs; bracket: segmental domain of ventro-posterior *WntA* expression. Scale bars: 100 μm. apr, antennal primodium; lab, labium; lat, lateral edges; lbr, labrum; md, mandible; mx, maxilla; pce, protocephalon/head lobes; plp, pleuropodium; ptl, prothoracic legs; tl/av, telson/anal valves [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 2b,c), forming an expression pattern similar to the one observed in *Tribolium* at an equivalent stage (Bolognesi et al., 2008a). *WntA* RNAi knockdowns yield viable embryos in *Tribolium* (Bolognesi et al., 2008b)—the embryonic functions of *WntA* will require further investigation in butterflies and other arthropods to understand if it is truly dispensable (e.g., due to redundancy), or if its repeated loss implicates a functional turnover from other *Wnt* genes.

3.4 | WntA wing patterning requires the Porc/Wls secretory pathway

Overall, our phylogenetic screen showed that WntA ligands conserved position Ser209, a target for Wnt-lipidation by Porc (Herr & Basler, 2012; Hosseini et al., 2019; Routledge & Scholpp, 2019). In addition, Wls showed enriched expression in the anterior hindwings of *Agraulis* butterflies, a region where WntA functions as a pattern

instructive signal (Hanly et al., 2019; Mazo-Vargas et al., 2017). These suggest WntA may require the core Wnt secretory factors Porc and Wls for acylation and intracellular transport. We tested this hypothesis in *V. cardui* butterflies by carrying out CRISPR-mediated mutagenesis, which yields high-frequencies of null mutations in butterfly embryos injected at a syncytial stage (Connahs et al., 2019; Lewis et al., 2019; Martin et al., 2020; Perry et al., 2016; Zhang et al., 2017a, 2017b). This method generates a fraction of mosaic G_0 "crispants" that can survive to the adult stage, due to mutations occuring in a subset of the soma following postzygotic injections (Livraghi et al., 2017). We expected *porc* and *wls* KOs to be lethal because their RNAi knockdowns yield severe embryonic defects in *Tribolium* (Bolognesi et al., 2008b), and we thus focused injection efforts in a time window 4–8 h AEL to maximize mosaicism and the number of survivors (Table 1).

mKOs of *porc* and *wls* both produced pupae with one or several missing wings (Figure 3); such morphologies are akin to the morphologies of *Bombyx* mutants for the *Fringe* gene, which completely fail to develop wing imaginal disks (Sato et al., 2008), and to *Bombyx* mutants for the *Approximated* gene, which have reduced wing imaginal disk growth (Yu et al., 2020). Lepidopteran wing imaginal disks secrete pupal cuticle (Švácha, 1992), and it is believed that insect wing tissues likely require Wingless (*syn. Wnt1*) signaling, or possibly one of the coexpressed Wnt6 and Wnt10 ligands (Koshikawa et al., 2015; Martin & Reed, 2014), for wing disk extension and establishment of the dorso-ventral wing boundary (Abouheif & Wray, 2002; Macdonald et al., 2010; Yu et al., 2014). The pupal cuticular defects from *porc* and *wls* mKOs are thus consistent with a requirement for the proper trafficking of Wingless and other Wnt ligands involved in wing growth, and thus validate the effectiveness of those KOs in depleting secreted Wnts.

Importantly, a fraction of the surviving adults showed mosaic mutant clones on their wing epithelia that replicated color pattern effects previously obtained in *WntA* KOs (Mazo-Vargas et al., 2017). In *V. cardui* and several nymphalid butterflies, *WntA* induces the central symmetry system (CSS), an antero-posterior stripe with a symmetric color composition, and modulates the size and shape of elements of the distal parafocal elements (dPf) and of the Marginal Band Symmetry system near the wing periphery. Mosaic KOs of *porc* and *wls* induced effects similar to *WntA* KOs in those three domains, demonstrating that WntA signaling requires the core Wnt secretory pathway to instruct pattern formation (Figure 4a–h and Figures S1–S2). *V. cardui* butterflies also evolved a novel *WntA* function limited to their forewing border ocelli, with loss-of-function resulting in a size reduction in white patterns that show spot-like expression of *WntA* in the fifth-instar larval wing imaginal disk (Martin & Reed, 2010; Mazo-Vargas et al., 2017). This effect was reproduced in *porc* and *wls* crispants (Figures 4f and 5a,b), consistently with a requirement of these genes for WntA trafficking.

3.5 | WntA-independent color patterning effects of Wnt trafficking perturbation

As *porc* and *wls* loss-of-function should affect most Wnt ligands (see Section 4), KO experiments could highlight *WntA*-independent effects:

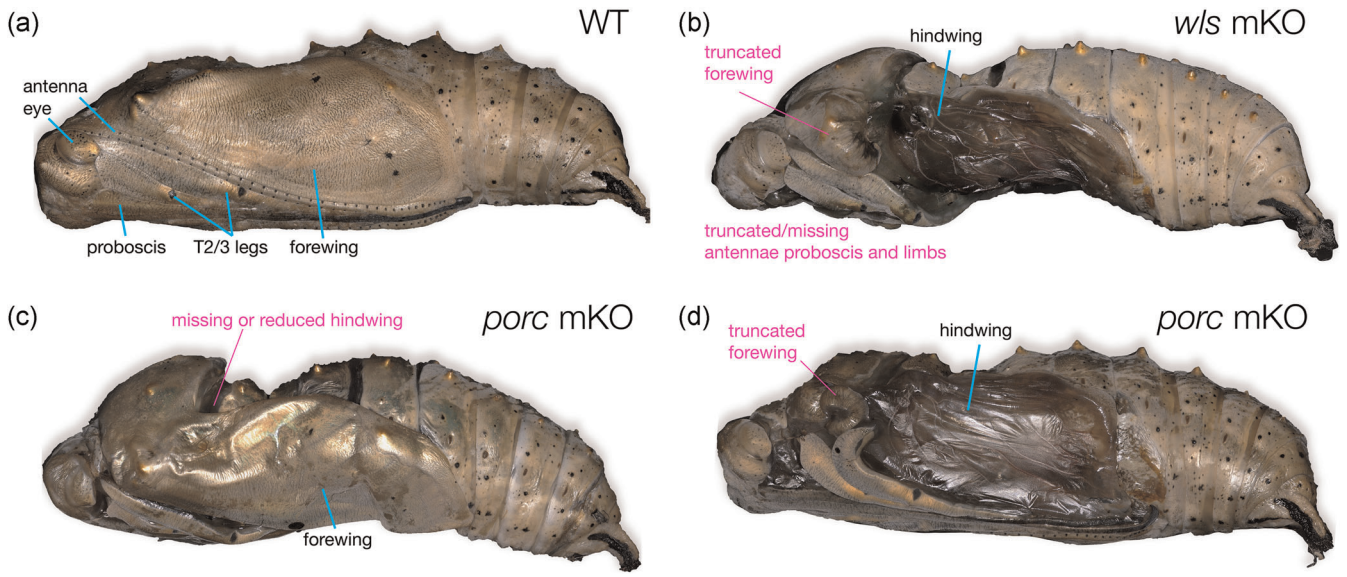

FIGURE 3 Wing and appendage growth defects in pupae following *porc* and *wls* mosaic knockout (mKO). These crispant phenotypes are representative of the variation observed for each gene, and did not survive the pupal stage. (a) Wild-type pupa, lateral view. (b) Example of a *wls* crispant pupa with a bilaterally truncated forewing as well as a severe reduction and loss of head appendages and legs. (c, d) Individual crispants obtained after *porc* mKO, with bilateral loss of the hindwing pouch (c), and an unilateral loss of the forewing pouch and cuticle (d). See Table 1 for phenotype frequencies. porc, Porcupine; wls, Wntless [Color figure can be viewed at wileyonlinelibrary.com]

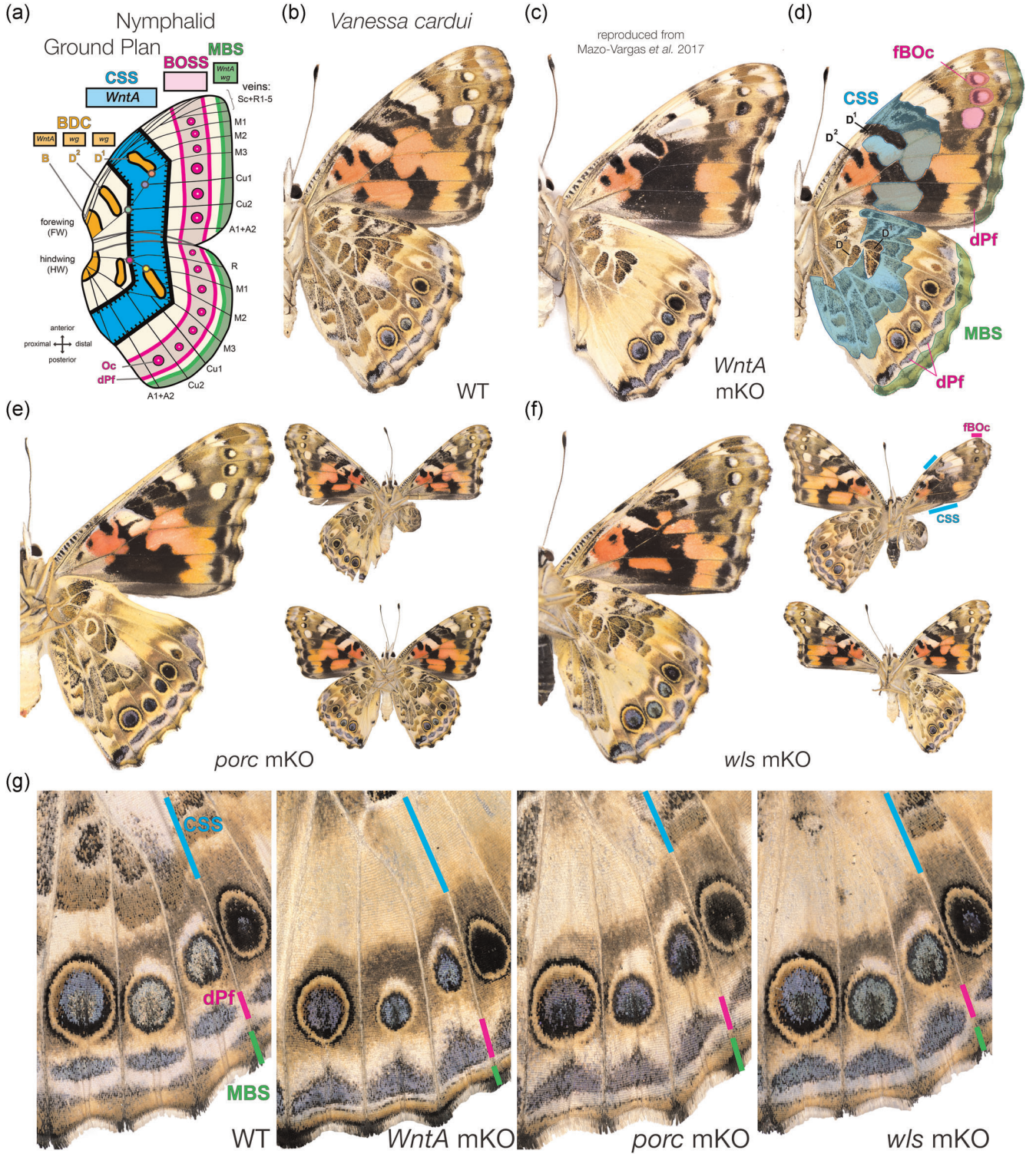

FIGURE 4 Mosaic knockouts (mKO) of *porc* and *wls* phenocopy the wing pattern phenotypes observed in *WntA* crispants. (a) Schematic representation of the Nymphalid Ground Plan and where *WntA* and *wg* genes are expressed in various nymphalids (Martin & Reed, 2014; Mazo-Vargas et al., 2017). (b–d) Summary of the effects of WntA mKO on the ventral wing surfaces of *V. cardui* (Mazo-Vargas et al., 2017). (e, f) Representative examples of phenotypes obtained after *porc* and *wls* mKO, including wing growth defects, local ablations of the CSS, shrinking of the fBOc, and distalization of the dPf and MBS. (g) *WntA* phenocopies of *porc* and *wls* mKO in the CSS, dPF shape and position, and MBS patterning in the ventral hindwing. CSS, central symmetry system; dPF, distal parafocal elements; fBOc, forewing border ocelli; MBS, marginal band symmetry system; porc, Porcupine; wls, Wntless [Color figure can be viewed at wileyonlinelibrary.com]

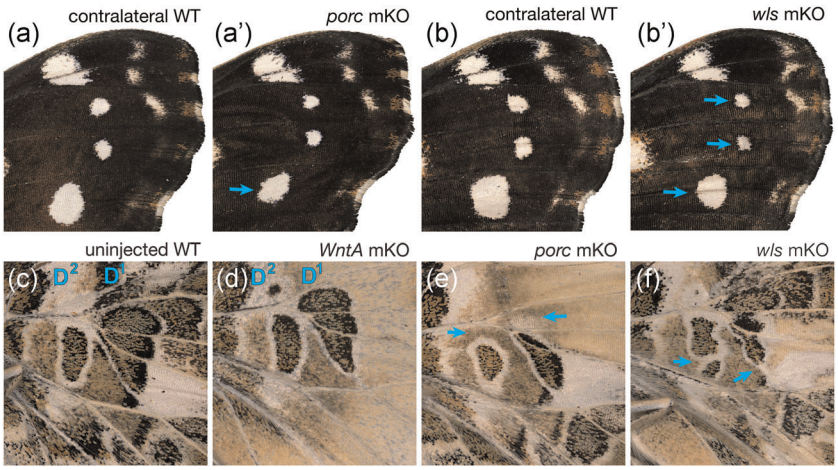

FIGURE 5 Pattern reduction effects of *porc* and *wls* mosaic knockouts (mKOs) in *WntA*-dependent forewing Border Ocelli and the WntA-independent Discalis elements. (a, b) Left-right comparisons of dorsal forewing distal regions, revealing asymmetries in eyespot sizes due to mosaic *porc* and *wls* mKOs (arrows), similar to *WntA* mKO (Mazo-Vargas et al., 2017). Contralateral wild-types are mirror-reversed and provide comparisons within the same mosaic mutant individual. (c) Wild-type ventral hindwing centered on the *wg*-expressing Discalis elements. (d) Corresponding area in a complete *WntA* crispant, where the mKO does not affect the Discalis elements but make them more prominent in the absence of a CSS. (e, f) Mosaic clones of *porc* and *wls* result in localized removal of the CSS, as well as in disruptions of the Discalis elements (arrows), indicating those knockouts affect Wnt ligands involved in adjacent wing patterning systems. CSS, central symmetry system; dPF, distal parafocal elements; fBOc, forewing border ocelli; MBS, marginal band symmetry system; porc, Porcupine; wls, Wntless [Color figure can be viewed at wileyonlinelibrary.com]

porc/wls KO phenotypes that are not observed in *WntA* KOs would suggest other Wnt signals participate in color pattern formation. The small Discalis I and II pattern elements (D_1 and D_2) are known to express *wingless* in the larval wing imaginal disk (Macdonald et al., 2010; Martin & Reed, 2010; Martin & Reed, 2014). D_1 and D_2 patterns were reduced in both *porc* and *wls* crispants (Figure 5c–f), indicating that Wg signaling is effectively impacted by the *porc/wls* KOs. Thus, Wg is important for the proper patterning of the Discalis elements in *V. cardui*, but is not required for their induction. Wg is also thought to modulate eyespot development in *Bicyclus* butterflies (Özsu et al., 2017), but we did not find support for a secreted Wnt input in *V. cardui* hindwing eyespots, including in wing regions that had extensive *porc* or *wls* clones impacting the surrounding CSS or dPf elements (Figure 4g). Notably, border ocelli vary naturally in size and color in our wild-type colony, so we can exclude here effects of mKOs of *porc* (Figure S1), *wls* (Figure S2), and *WntA* (Mazo-Vargas et al., 2017*)* on eyespot size, as we never observed left/right asymmetries within individuals in the *Vanessa* hindwing eyespots. Further Wnt loss-of-function and expression assays will be needed to clarify if *Bicyclus* and *Vanessa* evolved distinct eyespot modulatory signals in their hindwings. Finally, we did not find evidence of wing scale growth or orientation defects that would support a role for butterfly secreted Wnts in a putative wing Frizzled/Planar Cell Polarit (Fz/PCP) pathway. This result is in agreement with recent findings showing that they Fz/PCP pathway is Wnt-independent in *Drosophila* (Ewen-Campen et al., 2020; Yu et al., 2020).

4 | DISCUSSION

4.1 | Origin of the WntA ligand family in metazoa

The *WntA* gene family was named when first identified as a monophyletic group in an early phylogeny that grouped polychaete, mollusk, spider and sea urchin orthologues (Prud'homme et al., 2002). In this study, we confirmed the monophyly of WntA sequences within Bilateria + Cnidaria. Of note, a previous article proposed WntA originated before the split between Bilateria/Cnidaria and Ctenophora (Pang et al., 2010). We were unable to replicate robust placement of ctenophoran Wnt sequences in bilaterian orthology groups with our extended dataset, a result that was also found in studies that increased sampling within sponges, ctenophorans, and placozoans (Borisenko et al., 2016; Jager et al., 2013). The current data thus indicate that the WntA orthology group evolved at the base of Bilateria + Cnidaria, and further genomic analyses will help to clarify the evolutionary history of the Wnt family in early-branching metazoans. While we identified the synapomorphic Thr216Iso/Val/Ala amino-acid replacement as a rapid diagnostic for *WntA* orthology assignment, we also observed three convergent Iso/Ser216 positions in non-WntA ligands, and reemphasize that further studies should examine other positions or perform proper phylogenetic reconstruction to name Wnt ligands.

4.2 | WntA requires the core Wnt trafficking pathway

Regardless of their subfamily assignment, orthologs of Wnt pathway components (including Wnt ligands, Fz, Porc, and Wls) are found across all Bilateria, as well as in early diverging lineages of metazoans, including Cnidaria, Ctenophora, Placozoa, and Porifera (Schenkelaars et al., 2017), suggesting the Porc/Wls secretory pathway originated in conjunction with Wnt ligands at the onset of animal multicellularity (Holstein, 2012). Investigations of Wnt secretion has shown that most Wnt ligands require Porc and Wls, indicating an essential and conserved role. As exceptions to this trend, the atypical *Drosophila WntD* lacks a Ser/Thr209 acylation site, and was shown to be secreted independently of Porc-mediated lipidation and Wls processing (Ching et al., 2008; Gao & Hannoush, 2014; Herr & Basler, 2012; MacDonald et al., 2014; Miranda et al., 2014). Wnt3A and Wnt4 were found to elicit signaling intracellularly without requirement for Porc/Wls in human cancer cell lines (Rao et al., 2019). And in *Tribolium* embryos, it was also suggested that Wnt8 requires Porc, but not Wls (Bolognesi et al., 2008b). Thus, even in the presence of a putative Ser/Thr209 acylation site, the requirement of Wnts for the Porc/Wls core trafficking pathway may have exceptions in certain subfamilies or developmental contexts. We have here determined that the conservation of the WntA thumb region is indicative of traditional acylation across Bilateria and Cnidaria. Taking advantage of the highly specific effects of WntA deficiency on butterfly wing patterning, we generated Porc and Wls deficient clones that phenocopy the effects of WntA removal. Thus WntA can reasonably be assumed to function as a lipid-modified secreted ligand across the wide range of invertebrate lineages where it is studied (Constantinou et al., 2016; Darras et al., 2018; Fenner et al., 2020; Hayden & Arthur, 2014; Hogvall et al., 2019; Janssen et al., 2010; Kraus et al., 2016; Pruitt et al., 2014; Robert et al., 2014; Somorjai et al., 2018; Yuan et al., 2019).

4.3 | Depletion of secreted Wnts in mosaic mutant clones

Both *porc* and *wls* mKOs were highly lethal, as observed for knockdowns performed in *Tribolium* embryos (Bolognesi et al., 2008b). Later in development, *porc/wls* KOs generated pupae with wing defects, consistent with a reduction in *wg* signaling (see Section 3). We must, therefore, assume survivorship bias, where the wings that are the most affected by CRISPR mutations fail to grow or emerge, while only wings with small or no mutant clones are able to complete development. This explains the observation of smaller mutant *porc* and *wls* clones relative to *WntA* KOs, which are viable and can extend to entire wing surfaces (Concha et al., 2019; Mazo-Vargas et al., 2017). That said, within surviving adults the mosaicism in *porc* and *wls* KOs likely generated clones that are entirely lacking secreted Wnts altogether. For instance, the Discalis elements of the ventral hindwing that express *wg* (Martin & Reed, 2014) were reduced in the secretory pathway KOs, implying Wg requires Porc and Wls for secretion. These mKOs will be useful for further dissecting the division of labor between Wnt ligands during lepidopteran wing development, by allowing the generation of clones depleted of all extracellular Wnt activity.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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DATA AVAILABILITY STATEMENT

Supporting information and data files are available online in the Supporting Information Material section of this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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