Wingless Signaling: A Genetic Journey from Morphogenesis to Metastasis

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ABSTRACT This FlyBook chapter summarizes the history and the current state of our understanding of the Wingless signaling pathway. Wingless, the fly homolog of the mammalian Wnt oncoproteins, plays a central role in pattern generation during development. Much of what we know about the pathway was learned from genetic and molecular experiments in *Drosophila melanogaster*, and the core pathway works the same way in vertebrates. Like most growth factor pathways, extracellular Wingless/Wnt binds to a cell surface complex to transduce signal across the plasma membrane, triggering a series of intracellular events that lead to transcriptional changes in the nucleus. Unlike most growth factor pathways, the intracellular events regulate the protein stability of a key effector molecule, in this case Armadillo/β-catenin. A number of mysteries remain about how the "destruction complex" destabilizes β-catenin and how this process is inactivated by the ligand-bound receptor complex, so this review of the field can only serve as a snapshot of the work in progress.

KEYWORDS beta-catenin; FlyBook; signal transduction; Wingless; Wnt

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Introduction: Origin of the Wnt Name

THE story of the Wingless (Wg)/Wnt signal transduction pathway is a beautiful illustration of both the power of forward genetics and the utility of *Drosophila* as a genetic model system. The Wnt family of secreted growth factors plays a pivotal role in the embryonic development of all animal species. Wnts direct cell fate specification and morphogenesis in every tissue layer, patterning the central nervous system, the gut, the respiratory and circulatory systems, and various epidermal structures [reviewed in Nusse (2005)]. They also play a role in tumor formation; aberrant Wnt signaling is particularly associated with colorectal cancer in humans (Polakis 2007). Colorectal cancer is a leading cause of cancer deaths and second only to lung cancer, which is mostly attributable to tobacco use (Siegel *et al.* 2017). Thus, the ability to dissect the Wnt signaling pathway in *Drosophila* has broad relevance for understanding developmental processes and oncogenesis. Much of what was learned with *Drosophila* genetics inspired, and was informed by, parallel experiments on the vertebrate Wnt pathway, using mouse and *Xenopus* as model systems [reviewed in Nusse and Varmus (2012)].

Discovery of the fly gene: the wingless mutant phenotype

As the name suggests, *wingless* (*wg*) gene activity is required for generating the pattern of the adult fly wing, among its many functions during *Drosophila* development. The wingless mutant phenotype (Figure 1, A–D) was first characterized by R. P. Sharma, working at the Indian Agricultural Research Institute in New Delhi, India, who discovered this mutant in an ethyl methanesulfonate (EMS) mutagenesis (Sharma 1973). The wg^1 mutation was recessive and homozygous viable, but there was variable penetrance of winglessness: the homozygous wg^1 stock produced flies with no wings, one wing, or two normal wings, in roughly a 2:2:1 ratio. These flies also showed a variable loss of halteres, the pair of small appendages produced by the third thoracic segment, which function to counterbalance the wingbeats during flight. Mutant flies could have no halteres, one haltere, or two normal halteres, in a manner completely independent of the wing status in the second thoracic segment. The wg^1 mutation was subsequently shown to result from a small deletion 3′ to the coding region (Baker 1987), identifying an enhancer element that drives expression specifically in the wing and haltere imaginal discs, the developmental precursors to the adult structures (Schubiger *et al.* 2010). Presumably, this enhancer mutation reduces the level of *wg* expression to some critical threshold, where sometimes there is enough to pattern the appendage properly and sometimes there is not.

When the wing or haltere is absent in a wg^1 fly, the tissue is replaced by a mirror-image duplication of the dorsal thorax, a region called the notum (Figure 1, A and B). This phenotype was interpreted as a homeotic transformation of wing to notum, except that unlike other homeotic mutations, the wg^1

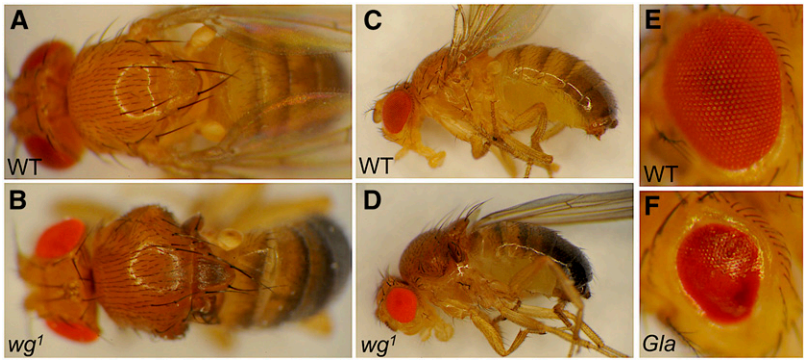

Figure 1 Viable *wg* mutant phenotypes. (A) Normal bristle pattern on the notum, the back of a fly's thorax, with both halteres visible out of focus at the posterior edge (in this and all images, posterior is to the right). (B) Notum of a wg^1 homozygous mutant showing disrupted pattern and absence of both wings and one haltere. (C) Side view of wild-type (WT) fly. (D) Side view of wg^1 homozygous mutant showing duplicated notum in place of one missing wing, and misshapen eye (*cinnabar* eye color is not part of the *wg* phenotype). (E) WT eye shows a regular pattern of ommatidia, the units of the compound eye. (F) The eye of a fly heterozygous for the *Gla* mutation shows a smooth "glazed" surface.

mutation behaves in a noncell-autonomous manner in genetic mosaics (Morata and Lawrence 1977). Mosaic flies contain patches of tissue bearing a genotype different from the rest of the fly (Figure 2A). When clones of homozygous mutant tissue were induced by mitotic recombination in a heterozygous $wg^1/+$ animal, small mutant clones were consistently found in completely normal wings. This effect showed that the normal gene product, produced in wild-type tissue, was able to rescue neighboring mutant cells, and thus showed that *wg* acts nonautonomously.

True loss-of-function alleles for *wingless* were recovered in the large-scale genetic screens for epidermal patterning defects, conducted at the European Molecular Biology Laboratory in Heidelberg (Nüsslein-Volhard and Wieschaus 1980). These screens used the cuticle pattern secreted by the embryonic epidermis as an assay to identify EMS-induced mutations that disrupt embryonic development. Among the many important mutations isolated in this effort were null mutations at the *wg* locus (Nüsslein-Volhard *et al.* 1984). The complete absence of Wg activity results in death of the embryo, with severe defects in the anterior–posterior pattern within each segment of the larval cuticle. Thus, *wg* was classified as a "segment polarity" mutant. The pattern disruption, like the wg^1 notum, involves mirror-image duplications. Late in embryogenesis, the ventral epidermal cells produce arrays of hook-like projections, called denticle belts, which are separated by expanses of bare, or naked, cuticle in each segment (Figure 3A). In *wg* null mutant embryos, these expanses of naked cuticle are replaced by denticles with a reversed polarity (Figure 3B). The behavior of the *wg* null and wg^1 mutant alleles indicated that normal activity of the *wg* gene promotes the segmental pattern of naked cuticle in the embryo, and plays a noncell-autonomous role in the development of the adult wing.

These findings inspired N. Baker, a graduate student in the Lawrence laboratory, to pursue a molecular analysis of the *wingless* locus. In these early days of cloning, the best way to find the gene sequence was to generate a transposable element (*P* element) insertion allele, and then use the *P* element sequence as a hybridization probe to recover recombinant clones that carry both the insertion and chromosomal DNA flanking the element (Rubin *et al.* 1982; Spradling and Rubin 1982). The non-*P* element sequence from these clones represents wild-type genomic DNA from the region adjacent to the insertion. This strategy is still relevant today, but has been greatly accelerated by community resource projects with the goal of isolating insertional alleles for every gene in the genome (Spradling *et al.* 1999; Bellen *et al.* 2011). Rather than mobilizing *P* elements and screening for new insertions that fail to complement a mutation of interest, we can now search for an existing insertional allele among the collection generated by the Gene Disruption Project.

The cloned *wg* gene sequence was used to make RNA *in situ* hybridization probes, which revealed that *wg* is expressed in segmental stripes (Figure 3C) in the zone of epidermal cells predicted to produce naked cuticle (Baker 1987). The stripes of *wg* expression were immediately anterior to the expression stripe of another recently cloned segment polarity gene, *engrailed* (DiNardo *et al.* 1985). Expression of *engrailed* requires *wg* gene activity (DiNardo *et al.* 1988; Martinez Arias *et al.* 1988); *wg* expression in a nonoverlapping set of cells adjacent to the *en* stripe was consistent with previous observations that the *wg* gene acts nonautonomously.

Discovery of wg homology to the mammalian oncogene int-1

The connection between Wingless signaling and cancer was discovered early on, through efforts in the Varmus laboratory to identify cellular oncogenes by insertional mutagenesis (Varmus 1984). Retroviruses, such as Mouse Mammary Tumor Virus (MMTV), carry strong promoters in their long terminal repeats. When the viral cDNA integrates into chromosomal DNA, the promoter in the downstream repeat is positioned next to host genes. If a retrovirus integrates next to a proto-oncogene, the gene is turned on at high levels and drives tumor formation. Proto-oncogenes identified as MMTV integration sites, where the proviral insertion caused breast tumors in mice, were initially named *int* genes. Characterization of the first of these, *int-1*, revealed a sequence predicted to encode a secreted, cysteine-rich molecule that was otherwise novel (Nusse and Varmus 1982; Nusse *et al.* 1984). To understand how the molecule might function to promote tumor formation, the Nusse laboratory searched for sequences homologous to *int-1* in *Drosophila*. They discovered a gene that had 54% amino acid identity with the mouse *int-1*, and found that it matched the sequence of Baker's *wg* clone (Rijsewijk *et al.* 1987). The Nusse laboratory went on to construct a wild-type *wg* transgene and introduce it into flies under the control of the heat shock

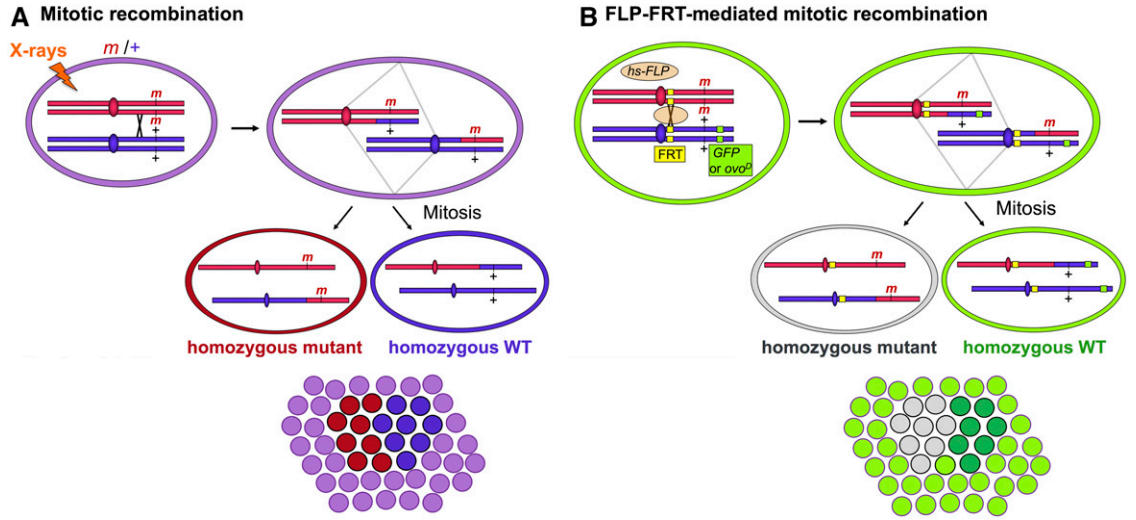

Figure 2 Generation of genetic mosaics. (A) Clones of homozygous mutant cells are generated in heterozygous flies when mitotic recombination between the homologs occurs. This rare event can be induced by exposing flies to X-rays, which cause double-stranded DNA breaks that often lead to crossing-over in the process of being repaired. If both mutant chromatids are pulled to the same mitotic spindle pole, the resulting two daughter cells will have different genotypes. Subsequent cell divisions generate mitotic clones from each daughter, producing a "twin spot" of homozygous mutant (red) and homozygous wild-type (WT) (blue) cells within a field of heterozygous cells. (B) The yeast flippase (FLP) and its target sequence (FRT) can be used instead of X-rays to induce mitotic recombination. Heat shock-induced flippase catalyzes site-specific recombination at the FRT target. The presence of a dominant marker, such as green fluorescent protein (GFP), on the WT homolog allows easy detection of mutant clones in somatic tissue. Inclusion of the ovo^D dominant female-sterile mutation blocks egg formation in heterozygous ovarian tissue. During the production of germ line clones, the only eggs recovered are derived from tissue homozygous for the non-ovo^D-bearing chromosome.

promoter, using the *P* element-mediated germ line transformation technique (Rubin and Spradling 1982; Spradling and Rubin 1982). Ectopic expression of *wg*, induced uniformly by heat shock on top of the endogenous stripes of expression, produced embryos with ventral surfaces composed entirely of naked cuticle (Noordermeer *et al.* 1992). Thus, all of the denticle-secreting cells in every segment are converted to the naked cuticle cell fate when they express high levels of *wg*, confirming that *wg* activity is both necessary and sufficient for naked cuticle specification in the embryonic epidermis.

Normal expression of the mouse *int-1* was found to be mostly restricted to the developing nervous system in embryos (Shackleford and Varmus 1987; Wilkinson *et al.* 1987). Knockout mutations engineered into mice produced severe defects in patterning of the brain, virtually eliminating the cerebellum (Thomas and Capecchi 1990). Indeed, the Capecchi laboratory discovered that an old neurological mutant in the mouse, called *swaying*, was caused by a lesion at the *int-1* locus, with similar effects on cerebellar development (Thomas *et al.* 1991). Overexpressing the mouse *int-1* in *Xenopus*, by injecting the mRNA into eggs, produced dramatic duplication of the frog embryo's body axis (McMahon and Moon 1989). Thus loss-of-function mutations for both *wg* and *int-1* severely disrupt the embryonic development of ectodermally derived tissues, and gain-of-function for both molecules transforms cell fates.

Wnts are a conserved gene family found throughout the animal kingdom

Homologs of *wg* and *int-1* were subsequently identified both within the mammalian and *Drosophila* genomes [reviewed in Nusse and Varmus (1992)], and more broadly in a wide variety of animal species, from leeches and starfish to humans (van 't Veer *et al.* 1984; Kostriken and Weisblat 1992; Sidow 1992). There are even some homologs in the sponge genome, but none have yet been found in single-celled organisms, suggesting that this gene family may be as old as multicellularity (Nichols *et al.* 2006; King *et al.* 2008; Loh *et al.* 2016).

The name Wnt, a combination of *wg* and *int-1*, was chosen to describe this family of growth factors (Nusse *et al.* 1991). There are now known to be 19 Wnt genes in most mammalian genomes. A comprehensive list of known Wnt family members, as well as an overview of the pathway and a database of resources for Wnt researchers, are curated by the Nusse laboratory on "The Wnt Homepage" at http://wnt.stanford.edu. Wnt proteins undergo a post-translational lipid modification that is essential for their function (Willert *et al.* 2003; Takada *et al.* 2006), as well as N-linked glycosylation as they transit through the secretory pathway (Brown *et al.* 1987; Papkoff *et al.* 1987). The covalent attachment of a lipid group, palmitoleic acid, has complicated the study of Wnts because (unlike many other growth factors) they are not freely soluble. Other secreted signals could be purified and crystallized to solve their structures, or used in assays on cultured cells to identify their cell surface receptors and intracellular components. For many years, Wnts were a signal without a receptor. The *Drosophila* model system proved to be critical in finding the core components of the Wnt pathway (Table 1) and in understanding how the pathway works.

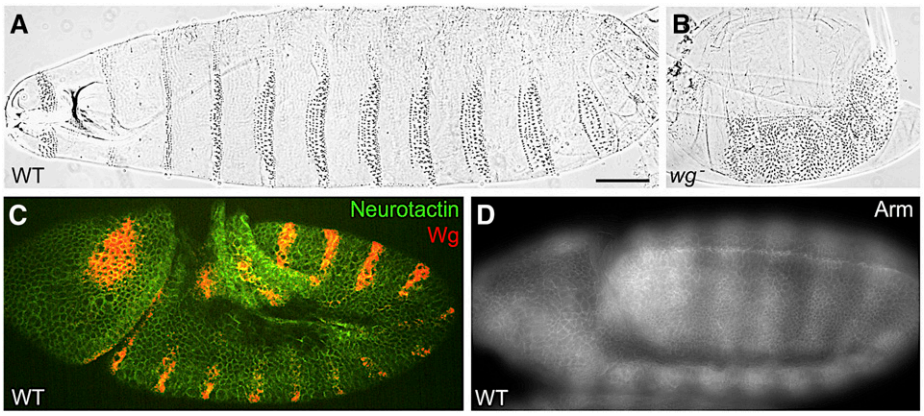

Figure 3 Embryonic *wg* phenotypes. (A) Wild-type (WT) embryos secrete a segmental pattern of denticle belts separated by naked cuticle on their ventral surface. Bar, 50 μm. (B) *wg* null mutant embryos produce a cuticle pattern with no naked cuticle, only denticles, on the ventral surface. (C) Wg antibody staining (red) shows that the protein is expressed in stripes in WT embryos, and the protein is detected over several cell diameters on either side of the stripe (cell outlines visualized with Neurotactin antibody staining, green). The stripes of *wg* expression are located within a subset of the epidermal cells that will secrete naked cuticle. (D) Arm antibody staining (white) in WT embryos shows higher levels of Arm in broad stripes that are roughly centered over the Wg-producing cells.

Identifying the Components of the Wingless/Wnt Pathway

Forward genetic screens for embryonic pattern disruption

"Heidelberg" screens for zygotic patterning phenotypes: The long history of *Drosophila* genetics was critical to gene discovery in the Wnt pathway. Starting at the turn of the last century, T. H. Morgan's laboratory, first at Columbia University and then at Caltech, generated and mapped hundreds of fly mutations to produce rough maps of the four fly chromosomes. The fly community was blessed with many generous individuals who created and shared fly stocks and information, and laid the groundwork for the large-scale screens that were to follow. Two features were particularly important for these screens: a comprehensive catalog of existing mutations and the availability of balancer chromosomes (Lindsley and Grell 1968). Balancer chromosomes are versions of the three largest fly chromosomes—the X, second, and third chromosomes—which contain multiple inversions that suppress recombination. In addition, they carry at least one dominant marker mutation, and at least one homozygous lethal mutation (or in the case of the X chromosome, a female-sterile mutation). This prevents the chromosome from surviving (or contributing to the next generation) in the homozygous state (Figure 4, A and B). For lethal mutations on the fourth chromosome, only a dominant marker mutation is needed because the fourth chromosome is mostly heterochromatic and does not undergo recombination (Ashburner 1989).

The presence of a dominant visible marker mutation also makes balancer chromosomes useful for recovering newly induced mutations that might be homozygous lethal (Figure 4C). In a mutagenesis, each chromosome exposed to the mutagen would have a unique set of mutations. To find mutations that produce autosomal recessive phenotypes requires a breeding program that produces two flies of the opposite sex that carry exactly the same mutagenized chromosome. Balancer chromosomes facilitate such breeding programs. The basic strategy used by the Heidelberg group was to cross mutagenized flies to flies carrying a balancer for a particular chromosome, so that each individual fly in the next generation carried a uniquely mutagenized chromosome balanced with an unmutagenized balancer chromosome. These flies were then bred through two generations (see Figure 4C) to produce embryos homozygous for each new mutation, which could then be examined for patterning defects.

This strategy, however tedious, was enormously successful in generating a collection of EMS-induced embryonic-lethal mutations for each of the four chromosomes (Jürgens *et al.* 1984; Nüsslein-Volhard *et al.* 1984; Wieschaus *et al.* 1984). Some embryonic-lethal mutations showed cuticle pattern disruptions that fell into one of three classes: gap, pair-rule, and segment polarity; these mutations revealed the basic mechanics of early embryonic development in the fly. The segment polarity class was particularly important to the Wnt story, because not only were *wg* loss-of-function mutations recovered in this screen, but so too were two other segment polarity mutations that produced all-denticle phenotypes similar to *wg* mutants. The two genes disrupted by these mutations, *armadillo* (*arm*) and *arrow* (*arr*), encode core components required to activate the Wnt pathway; thus, their loss-of-function produced embryonic pattern disruptions similar to loss of the signal itself. Conversely, other mutations isolated in the Heidelberg screens, such as *naked cuticle* (*nkd*) and *shaven-baby* (*svb*), produced the opposite effect on patterning: the secretion of all-naked cuticle. This mimics the effects of overexpressing *wg*, and suggested that the wild-type gene products play a role in opposing Wg pathway activity.

The *arm* gene encodes the fly β-catenin protein (Peifer and Wieschaus 1990), an intracellular effector that drives target gene expression in response to the Wnt signal. Activity of the Wg pathway hinges on the stabilization of Arm protein: cytosolic Arm is continually turned over by a set of proteins dedicated to its destruction, which is inhibited when Wg binds and activates its receptor complex (Peifer *et al.* 1994b), described in *Function of the Wingless/Wnt Pathway.* The *arrow* gene encodes part of the receptor complex, but this was not recognized until much later, mostly because the mutant

Table 1 Known components of the Wingless pathway and their human counterparts

	Drosophila gene	Human homolog	Activity
Production	*wingless*	*WNT1, 2, 2B(13), 3, 3A, 4, 5A, 5B, 6, 7A, 7B, 8A, 8B, 9A, 9B, 10A, 10B, 11, 16*	Secreted signal (ligand)
	porcupine	*PORCN*	O-acyltransferase
	Wntless/evenness interrupted/sprinter	*WLS*	Multipass transmembrane protein, chaperone?
	Swim	*TINAG, TINAGL1*	Polysaccharide binding
Activation	*arrow*	*LRP5,6*	Receptor
	frizzled, frizzled2	*FZD1, 2, 3, 4, 5, 6, 7, 8, 9, 10*	Receptor
	dishevelled	*DVL1, 2, 3, L1*	?
	armadillo	*CTNNB1*	β-catenin (effector)
	Tcf/pangolin	*TCF1, 3, 4, LEF-1*	Transcription factor
	legless	*BCL9*	Transcriptional cofactor
	pygopus	*PYGO1, 2*	Transcriptional cofactor
Inhibition	*naked cuticle*	*NKD1, 2*	?
	discs overgrown/CK1e	*CK1e*	Casein kinase
	zeste-white3/shaggy	*GSK3A, B*	Glycogen synthase kinase
	Apc, Apc2	*APC1, 2*	Destruction complex
	Axin	*AXIN1, 2*	Destruction complex
	groucho	*TLE1, 2, 3, 4, 6*	Transcriptional corepressor
	slimb	*BTRC, FBXW7, 11*	F-box of ubiquitin ligase
Modulation	*sugarless*	*UGDH*	UDP-glucose 6-dehydrogenase
	sulfateless	*NDST1, 2, 3, 4*	N-deacetylase and N-sulfotransferase
	Dally, dally-like	*GPC1, 2, 3, 4, 5, 6*	Glypican
	Notum	*NOTUM*	Palmitoleoyl-protein carboxylesterase
	CtBP	*CTBP1, 2*	Transcriptional cofactor

Based on information from FlyBase (http://flybase.org/) and the Human Genome Organisation Gene Nomenclature Committee (https://www.genenames.org/).

phenotype was not as severe as the *wg* mutant phenotype. This brings us to one of the limitations of forward genetic screens. The Heidelberg screens were designed to identify zygotic phenotypes, that is, phenotypes that result exclusively from the embryo's own genotype. However, many gene activities important for fly development are prepackaged in the egg by the mother. The maternal contribution of a gene product may allow homozygous mutant embryos to develop normally, and thus a role for that gene product in patterning would not be detected.

In some cases, the maternal product is used up during embryogenesis and the homozygous mutant may survive to later stages, where defects in imaginal disc patterning can be detected. This was the case for mutations in casein kinase 1 (CK1), which were first identified in flies as alleles of *lethal(3)discs overgrown* (*dco*) (Jursnich *et al.* 1990). Homozygous *dco* mutant larvae survive but remain in the larval stage for extended periods, with dramatic overgrowth of the imaginal discs and eventual death. The *dco* gene was shown to encode the *Drosophila* CK1ε, a Ser/Thr kinase (Zilian *et al.* 1999), which had also been identified as the gene disrupted by *doubletime* mutations that affect circadian rhythm (Kloss *et al.* 1998). Overexpression of *dco* in *Drosophila* S2 cells, a cultured cell line, showed that CK1 phosphorylates Arm and that this correlates with increased Arm degradation (Yanagawa *et al.* 2002). However, subsequent experiments *in vivo* indicated that CK1ε can play a positive role in promoting Wg signaling (Klein *et al.* 2006). This discrepancy was resolved when other casein kinases in the fly genome were tested for roles in Wg signaling. Reducing the function of the closely related CK1α produced strong hyperactivity of the Wg pathway *in vivo*, indicating that CK1α has a profound negative regulatory role (Zhang *et al.* 2006). These observations suggested complex relationships between CK1 family members and the Wg pathway, but hinted at involvement in the destruction complex, which adds phosphate tags to Arm/β-catenin, making it a target for degradation by the proteasome (Figure 5).

Maternal-effect screens for embryonic patterning mutants: Specific techniques can be employed to hunt for gene products that are maternally contributed but are also required zygotically. These techniques were employed very effectively to identify other critical components of the Wg pathway. Maternal-effect mutations, where homozygous mutant female flies produce embryos with disrupted pattern, identified genes that are required during oogenesis (Perrimon *et al.* 1986; Schupbach and Wieschaus 1986). However, mutations in genes that are required both maternally and zygotically were not recovered, because the zygotic requirement prevents survival of homozygous mutant females to egg-laying adulthood. Finding mutations in this class of gene required the generation of a clone of homozygous mutant tissue in the gonad of a heterozygous female fly. Eggs produced from the mutant cells would then lack the maternally loaded gene product. N. Perrimon pioneered the use of an X-linked dominant female-sterile mutation, ovo^D, to produce germ line clones of homozygous mutant tissue (Perrimon 1984). The ovo^D mutation blocks oogenesis in heterozygous ovarian tissue, so the only eggs produced are derived from mitotic clones homozygous for the non-ovo^D-bearing X chromosome.

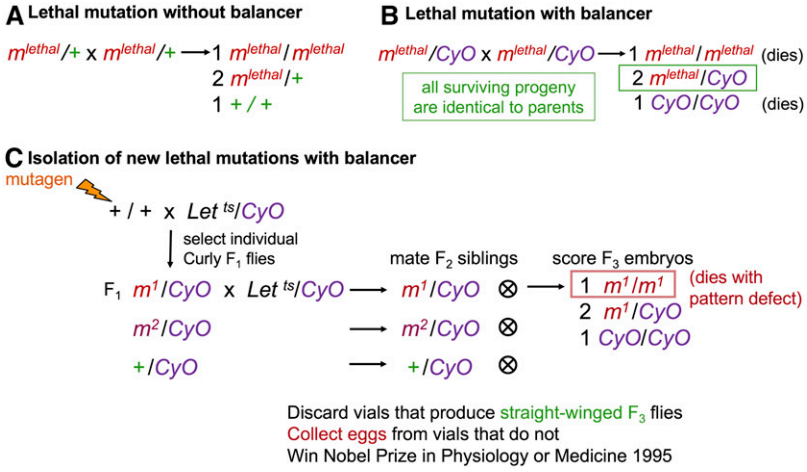

Figure 4 The magic of balancer chromosomes. (A) A lethal mutation must be maintained in the heterozygous state, with a wild-type allele of the gene on the other homolog. If the other homolog is entirely wild-type, this wild-type chromosome will predominate in future generations, and the lethal mutation may be lost. (B) Balancers, such as *Curly-O* (*CyO*), were designed so that homozygous lethal mutations could be maintained in heterozygous, balanced, fly stocks that are stable over many generations. Flies carrying a lethal mutation on one homolog and a balancer chromosome as the other homolog would produce progeny where one-quarter are homozygous lethal due to the first mutation, one-quarter are homozygous lethal (or are sterile, in the case of X chromosome balancers) due to homozygosity for the balancer, and the remaining half survive to adulthood as heterozygotes with a genotype identical to the parents. Multiple inversions on the balancer disrupt pairing between the homologs, preventing a recombination event between the desired lethal mutation and the recessive lethal mutation carried by the balancer, which could otherwise generate an entirely wild-type chromosome. (C) Balancer chromosomes can be used in genetic screens to isolate new lethal mutations. Flies are mutagenized and crossed to a stock carrying a balancer chromosome. The Heidelberg screens made use of a balancer stock carrying a dominant temperature-sensitive lethal mutation (here designated *Let* ^{ts}) to eliminate the nonbalancer chromosome in the next generation. However, any dominant mutation different from the marker on the balancer could be used and selected against in the next generation. Each F_1 individual is then crossed back to a fly of the opposite sex from the balancer stock. F_2 progeny from these individual crosses are then selected for the presence of the balancer dominant marker and the absence of the nonbalancer dominant marker, or incubated at high temperature to eliminate the nonbalancer progeny if using a dominant temperature-sensitive lethal stock. In this way, F_2 individuals of the opposite sex, each heterozygous for the same mutagenized chromosome, are generated and can be crossed together to assess the homozygous phenotype of the mutagenized chromosome.

The ability to remove the maternal contribution from developing embryos revealed several critical components of the Wg signaling pathway that are maternally provided and zygotically required. Several lethal alleles had been found for *dishevelled* (*dsh*), an X-linked gene originally identified by a weak mutation that is adult viable and causes tissue polarity disruption. Lethal *dsh* homozygous mutants have normal cuticle pattern and die during larval stages, but when germ line clones of these *dsh* mutations were generated, they resulted in embryonic lethality, with homozygous embryos showing an all-denticle phenotype identical to zygotic loss of *wg* function (Perrimon and Mahowald 1987). This discovery led to a large-scale screen for X-linked maternal-effect mutations that cause pattern disruption. This screen identified two important components of the Wg pathway in addition to new alleles of *dsh* (Perrimon *et al.* 1989). Germ line clones of *porcupine* (*porc*) mutations, like *dsh*, produced embryos with *wg*-like pattern defects. The *porc* gene product was later shown to control proper secretion of the Wg protein (van den Heuvel *et al.* 1993). Germ line clones of *zeste-white3* mutations (*zw3*, also known as *shaggy*) produced the opposite effect—all-naked cuticle—suggesting that its wild-type function was to inhibit Wg signaling activity. Indeed, *zw3/shaggy* was found to encode the fly homolog of glycogen synthase kinase 3 β (GSK), which phosphorylates Arm protein and targets it for degradation (Siegfried *et al.* 1990, 1992). The action of GSK requires prior Arm phosphorylation by CK1. Experiments in human cells (Amit *et al.* 2002) and in the *Xenopus* model system (Liu *et al.* 2002) showed that CK1 phosphorylates Ser45 in β-catenin, and that this is required to "prime" Arm/β-catenin for the GSK phosphorylations that target it for degradation.

The germ line clone technique was later improved (Chou and Perrimon 1992) by combining it with the FLP-FRT system (Figure 2B). The yeast site-specific recombinase FLP and its target, FRT, were transferred into flies (Golic and Lindquist 1989) to produce a more reliable and precise means of generating mitotic crossovers than X-irradiation. The Perrimon laboratory engineered fly strains with an FRT recombination target site inserted at the base of each chromosome arm and an inducible source of FLP recombinase, allowing easy generation of mitotic clones. They also cloned the *ovo*^{D} mutant gene, and used *P* element-mediated transformation to create fly lines with the *ovo*^{D} transgene inserted on each of the autosomal chromosome arms (Chou *et al.* 1993). Autosomal *ovo*^{D} transgenes extended the efficient production of germ line clones beyond X-linked mutations, allowing germ line clone production for mutations on the second and third chromosomes of *Drosophila*. These experiments yielded more mutations that produced *wg*-like all-denticle patterns when removed both maternally and zygotically (Perrimon *et al.* 1996). Among the autosomal genes identified were *sugarless* and *sulfateless*, which encode enzymes that function in the biosynthesis of heparan sulfate glycosaminoglycans. This implicated cell surface/extracellular matrix proteoglycans in modulating reception of the Wg signal (Häcker *et al.* 1997; Lin and Perrimon 1999). Another gene identified using this strategy was *sprinter*, since renamed *Wntless*, which encodes a multiple transmembrane domain protein that acts to chaperone Wg during its journey through the secretory pathway (Goodman *et al.* 2006).

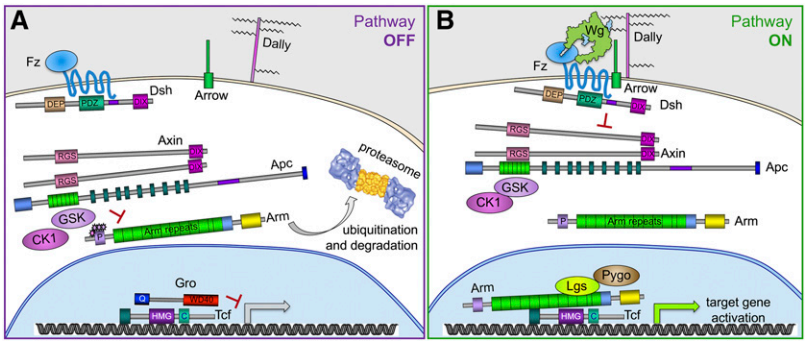

Figure 5 Diagram of core components in the Wg pathway. (A) In the absence of Wg signaling, Arm is presented by Apc and Axin to CK1 and Zw3/GSK for phosphorylation, targeting it for ubiquitination and degradation by the proteasome. Tcf binds target genes and, with the Gro transcriptional corepressor, keeps expression repressed. (B) Wg, concentrated at the cell surface by glycosaminoglycans on the glypican Dally, binds the Fz and Arrow receptors and causes them to cluster. This allows polymerization of Dsh and Axin at the plasma membrane, inactivating the kinase complex so that it cannot target Arm for destruction. Stabilized Arm translocates into the nucleus, binds to Tcf, and recruits the transcriptional activation complex, which includes Lgs and Pygo. Structural features of proteins depicted here are based on data from Wodarz and Nusse (1998), Janda *et al.* (2012).

Other genetic tricks to identify pathway components

Suppressor screens for mutations that modify loss-of-function wg phenotypes: One strategy for finding mutations that compromise a particular genetic pathway is to mutagenize a fly line that bears a weak mutation in one pathway component and screen for mutants that show an enhanced or suppressed phenotype. This "modifier" screen strategy was used to great effect in dissecting the Ras signaling pathway, through suppressor/enhancer screens with a weak mutation in the Sevenless receptor tyrosine kinase, which controls cell fate specification during *Drosophila* eye development (Rogge *et al.* 1991; Simon *et al.* 1991). My laboratory used partially functional *wg* mutant alleles (Bejsovec and Wieschaus 1995; Dierick and Bejsovec 1998) to provide a "sensitized" background for identifying modifier mutations that affect Wg signaling. We isolated a number of mutations that partly suppressed the pattern defects of the *wg* mutants, including mutations in the genes encoding the transcription factor Tcf, also known as Pangolin, and the negative Wg/Wnt regulator, Apc2 (van de Wetering *et al.* 1997; McCartney *et al.* 1999). Tcf, and its close relative LEF-1, had been characterized as HMG-box-containing transcription factors important for vertebrate T cell development. Tcf/LEF had been found to bind β-catenin in yeast two-hybrid protein–protein interaction screens, and to form a transcriptional activation complex with β-catenin (Behrens *et al.* 1996; Molenaar *et al.* 1996). Our recovery of *Tcf/pan* mutations as suppressors of *wg* loss-of-function phenotypes revealed that Tcf is not simply required for transcriptional activation of Wnt target genes, but also acts to repress those genes in the absence of Wg signaling (Cavallo *et al.* 1998). A connection between the fly Wg pathway and Tcf/LEF-1 was also found in the Bienz laboratory, who worked backward from a Wg response element they had defined upstream of *Ultrabithorax*, a target gene regulated by embryonic Wg signaling in the *Drosophila* intestine (Riese *et al.* 1997). They had noted that the DNA sequence of this element was similar to the consensus binding site for LEF-1, tested for binding of the mouse LEF-1 to this *Drosophila* sequence, and concluded that flies must have a LEF-1 homolog.

Our isolation of an *Apc2* mutation in the suppressor screen was particularly serendipitous because loss-of-function *Apc2* mutations are homozygous viable in the first generation. Maternally and zygotically mutant embryos derived from these *Apc2* homozygous mothers produced the all-naked cuticle phenotype that indicates deregulation of the Wg pathway (McCartney *et al.* 1999). Our mutation, $Apc2^{\Delta S}$, was temperature sensitive, allowing us to maintain a homozygous mutant fly stock at low temperature, then shift these flies to the higher temperature to characterize the effects of the mutation. Thus, we could demonstrate, without having to make germ line clones, that *Apc2* was a maternal-effect gene, and that the mutant phenotype implicates *Apc2* gene activity in the Arm destruction process. Other groups had identified the first *Apc* homolog in flies, based on similarity to the human tumor suppressor gene responsible for Adenomatous polyposis coli, a familial form of colon cancer (Polakis 1997). This first gene, when mutated, showed defects in the nervous system, such as degeneration of photoreceptor cells in the retina, but had no effect on embryonic Wg signaling (Hayashi *et al.* 1997; Ahmed *et al.* 1998). Thus flies, like humans, have two *Apc* genes that differ in their levels of expression in different tissues, which accounts for their distinct phenotypes when mutated (Hamada *et al.* 1999a; McCartney *et al.* 1999). However, both Apc molecules contribute to Wg regulation because doubly mutant *Apc Apc2* embryos or mitotic clones in various tissues show more profound hyperactivation of Wg signaling (Ahmed *et al.* 2002; Akong *et al.* 2002). This appears to be the case also with the two mammalian Apc molecules; disruption of both *Apc* and *Apc2* drives tumorigenesis in mouse mammary tissue and leads to higher Wnt target gene expression in human breast tumor samples, compared with singly mutant tissue (Daly *et al.* 2017).

Suppressor screens for mutations that modify gain-of-function wg phenotypes: An alternative strategy to identify pathway components was to screen for mutations that suppress an artificial phenotype produced by overexpressing *wg*. The Basler laboratory took advantage of a construct that fused the *sevenless* promoter to a wild-type *wg* transgene, driving *wg* overexpression exclusively in the developing fly eye. This construct disrupted the very precise organization of the adult compound eye, producing a rough-eye phenotype

that is easily observed but does not otherwise affect the health of adult flies (Cadigan and Nusse 1996). Dominant suppression of this rough-eye phenotype could then be assayed by crossing EMS-mutagenized flies to the *sev-wg* fly line. This screen identified mutations in *Tcf/pan* (Brunner *et al.* 1997), as well as mutations in a new gene, named *legless* (*lgs*), which encodes a protein essential for transcriptional activation of the Tcf-Arm complex (Kramps *et al.* 2002). The Cadigan laboratory used a variation on this theme to find gene products that, when overexpressed themselves, could suppress the rough-eye phenotype of eye-specific *wg* overexpression (Parker *et al.* 2002). This gain-of-function screen used the EP lines developed by P. Rorth (Rorth 1996). These randomly inserted upstream activating sequence (*UAS*) target sequences, recognized by the yeast Gal4 transcription factor, can be activated with the Gal4-UAS binary expression system, which uses a variety of fly promoters to drive Gal4 in defined domains (Brand and Perrimon 1993). Using eye-specific Gal4 drivers, the Cadigan laboratory identified *zw3/sgg*, and a second gene called *pygopus* (*pygo*). Pygo had also been identified in the Basler laboratory as a yeast two-hybrid interactor with Lgs (Kramps *et al.* 2002), and thus it forms part of the transcriptional complex that controls Wg target gene activation. The Cadigan laboratory also identified C-terminal-binding protein (CtBP) and CREB-binding protein (CBP) in their gain-of-function screens, and showed that these transcriptional regulators have dual roles, both positive and negative, in controlling Wg target gene expression (Fang *et al.* 2006; Li *et al.* 2007). CtBP had also been identified by the Bienz laboratory as a binding partner of Apc2, and shown to act as a transcriptional corepressor of Wg target genes (Hamada and Bienz 2004).

Broader gain-of-function screens for molecules that disrupt wing pattern also yielded Wg pathway components. A screen of the Rorth EP collection using a wing-specific Gal4 driver identified *Notum*, which, when overexpressed, causes a wg^1-like duplication of the notum at the expense of the wing, suggesting that it antagonizes Wg signaling (Giraldez *et al.* 2002). *Notum* was also identified in the Basler laboratory using a different approach: constructing a collection of randomly inserted Gal4 "enhancer traps," which will express Gal4 under the control of genomic regulatory elements close to the site of insertion, and screening for insertions that mimic the *wg* pattern of expression in the wing imaginal disc (Gerlitz and Basler 2002; Gerlitz *et al.* 2002). The similarity of expression patterns between *wg* and *Notum* suggested that *Notum* is part of a negative feedback loop in the wing disc. Indeed, *Notum* was found to be a direct target of Wg signaling (Hoffmans *et al.* 2005; Parker *et al.* 2008), and encodes an extracellular enzyme that can inactivate Wg by cleaving off its lipid group (Kakugawa *et al.* 2015).

Ironically, a gain-of-function *wg* phenotype in the eye defined the very first published report of a *wg* mutation, although it took 63 years to understand this. The dominant *Glazed* (*Gla*) mutation, which narrows the eye and smoothens its surface (Figure 1, E and F), was isolated in the Morgan laboratory (Morgan *et al.* 1936). The Basler laboratory was able to revert this phenotype by X-ray mutagenesis and by *P* element insertion, indicating that the phenotype resulted from a gain-of-function (Brunner *et al.* 1999). Their realization that the reverting *P* element was inserted very close to the *wg* gene led them to discover that the *Gla* phenotype was produced by ectopic *wg* expression during pupal development of the eye. This abnormal *wg* expression was caused by insertion of a *roo* retrotransposon, with a strong promoter in its long terminal repeat, making it analogous to the MMTV insertion that defined the first mouse Wnt, *int-1*.

Mosaic screens: Mitotic recombination, induced by X-irradiation, can be used to assess adult phenotypes of genes that are essential for embryonic development. For example, when lethal *wg* loss-of-function alleles were tested by clonal analysis, the mutant clones were mostly rescued by surrounding wild-type tissue (Baker 1988a), as was found for the wg^1 allele (Morata and Lawrence 1977). However, large clones of *wg* mutant tissue resulted in notching of the adult wing margin (Baker 1988a), correlating with a stripe of *wg* mRNA expression in this region of the wing imaginal disc (Baker 1988b) and revealing a role for Wg signaling in specifying this part of the wing structure.

The FLP-FRT system provided a much more convenient and reliable means of producing mitotic clones than the X-irradiation technique (Figure 2, A and B), and enabled large-scale genetic screens to be conducted. The Xu laboratory, using this approach to identify genes involved in growth and patterning of imaginal discs, recovered mutations in *supernumerary limbs* (*slimb*), an F-box protein that forms part of the E3 ubiquitin ligase complex that targets Arm for degradation (Xu *et al.* 1995; Theodosiou *et al.* 1998). The Struhl laboratory also found alleles of *slimb* using a similar approach, but screening specifically for mutant clones that alter the pattern of adult structures, as *slimb* mutant clones produce dramatic duplications of wing and leg tissue (Jiang and Struhl 1998). The first mutant allele of the fly CK1α was identified in a similar mosaic screen for disrupted eye development, supporting a role for CK1α in regulating Wg signaling (Legent *et al.* 2012).

The FLP-FRT system can also be used in suppression screens: the Basler laboratory adapted this strategy to find recessive mutations that suppress the rough-eye phenotype of the *sev-wg* fly line. They crossed the *sev-wg* transgene into flies carrying a FLP transgene driven specifically in the eye, so that only eye tissue would produce homozygous mutant clones of EMS-induced mutations. This strategy yielded lethal mutations in a gene that they named *Wntless* (Banziger *et al.* 2006), which was allelic with the *sprinter* locus identified through germ line clone screens for embryonic *wg*-like cuticle defects (Goodman *et al.* 2006).

Reverse genetic approaches: *Wntless* was also identified in a cell-based screen. The Perrimon laboratory had generated a library of double-stranded RNA constructs to knock down mRNA levels for $> 90\%$ of the predicted genes in the fly genome (Boutros *et al.* 2004). This RNA interference (RNAi) strategy was used to screen *wg*-expressing *Drosophila* cell lines for knockdowns that altered expression of a Wg reporter

transgene (Bartscherer *et al.* 2006). In addition to some known pathway components such as *arrow*, the Boutros laboratory discovered a gene that they initially named *evenness interrupted* (*evi*), and which is now known as *Wntless*.

Large-scale RNAi screens for whole fly phenotypes are also possible, primarily through the efforts of the Vienna *Drosophila* Resource Center (Dietzl *et al.* 2007) and Harvard's Transgenic RNAi Project (Perkins *et al.* 2015), which have produced fly lines carrying double-stranded RNA transgenes for most of the protein-coding genes in the genome. Screens using these RNAi transgenes have also detected Wg pathway modulators. For example, the Basler laboratory screened RNAi transgene lines using wing-specific Gal4 drivers, and identified the *armless* gene by its ability to disrupt wing margin pattern when knocked down (Reim *et al.* 2014). This gene encodes a ubiquitin regulatory molecule that appears to protect Arm from degradation by the proteasome. The Verheyen laboratory used a similar approach to identify phosphatases and kinases that modulate Wg signaling during wing imaginal disc patterning (Swarup *et al.* 2015).

Protein–protein interaction with known components: A key component of the Arm destruction complex, Axin, was identified in a yeast two-hybrid screen for Arm-binding proteins, and was subsequently shown to bind to both Zw3/GSK and Apc (Nakamura *et al.* 1998; Hamada *et al.* 1999b). The Akiyama laboratory identified a loss-of-function *Axin* mutation in a collection of lethal *P* element insertion alleles: maternal/zygotic mutants produced an all-naked cuticle phenotype, similar to *zw3/sgg* and *Apc2* maternal/zygotic loss-of-function. Likewise, mutant clones in imaginal disc tissues showed ectopic wing margin bristles and leg duplications, which are symptomatic of Wg pathway hyperactivity. The Nusse laboratory had also identified the *Drosophila* homolog of Axin, and used RNAi knockdown and overexpression of Axin in *Drosophila* embryos to demonstrate that Axin is required for Arm degradation (Willert *et al.* 1999).

Yeast two-hybrid screens conducted in the Bienz laboratory had found Apc as an Arm-interacting protein that can also bind to Zw3/GSK (Yu *et al.* 1999), and they identified CBP as a Tcf-interacting protein (Waltzer and Bienz 1998), thus establishing direct connections between these core pathway components. Yeast two-hybrid screens and GST pull-down assays also demonstrated contact between Arm and Lgs, and between Lgs and Pygo (Kramps *et al.* 2002), to establish direct connections among components of the transcriptional activation complex. Yeast two-hybrid screens for Tcf-interacting proteins in the *Xenopus* model system identified the vertebrate homolog of Groucho, a transcriptional corepressor in the fly (Roose *et al.* 1998). This physical interaction and corepressor function was verified in cultured cells; *Drosophila* Tcf was able to move Groucho into the nucleus, where it showed dose-dependent inhibition of a Tcf-Arm activated reporter gene (Cavallo *et al.* 1998). The Weis laboratory was able to recapitulate interactions of Tcf with β-catenin and with Groucho/TLE using purified proteins *in vitro* (Daniels and Weis 2005; Chodaparambil *et al.* 2014). How the Tcf transcriptional machinery switches from a repressor complex with Groucho to an activator complex with Arm/β-catenin is still unclear, but may involve post-translational modifications of Tcf and/or Groucho (Hikasa *et al.* 2010; Hanson *et al.* 2012), or rearrangement of the transcriptional complex proteins at Wg target gene promoters (van Tienen *et al.* 2017).

Noncanonical Wnt signaling and its connection to the canonical pathway

Tissue polarization in the epidermis and adult eye: Some components of the Wnt pathway were hiding in plain sight. A weak mutation in *dsh* had long been known to disrupt the orientation of bristles on the back of the fly's thorax and on the legs, as well as the organization of ommatidia in the compound eye (Lindsley and Grell 1968; Held *et al.* 1986). Thus, *dsh* gene activity was first associated with polarizing the actin cytoskeleton in epidermal cells for bristle and hair production [reviewed in Adler (1992)], and then subsequently was found to mediate the response to Wg signal in a cell-autonomous fashion (Perrimon *et al.* 1989; Klingensmith *et al.* 1994). Dsh is a core component in the establishment of planar cell polarity (PCP), a process that is conserved across the animal kingdom and is important in such diverse morphogenetic events as gastrulation and hair follicle orientation [reviewed in Goodrich and Strutt (2011)].

As with *dsh*, historic mutations in *frizzled* (*fz*) disrupted ommatidial organization, bristle polarity on the back of the fly, and hair polarity on the wing blade (Bridges and Brehme 1944; Lindsley and Grell 1968). However, unlike *dsh*, *fz* mutations were found to have noncell-autonomous effects on PCP as well as cell-autonomous effects: clones of mutant cells could influence the polarity of neighboring wild-type cells (Gubb and Garcia-Bellido 1982). The Adler laboratory showed that there is a clear directionality to this nonautonomy, where only the wild-type cells distal to *fz* mutant clones in the wing showed disruption (Vinson and Adler 1987). This observation suggested the presence of graded information along the proximal–distal axis of the developing wing. The Adler laboratory went on to clone and characterize the gene, finding that *fz* encodes a predicted cell surface molecule with seven transmembrane-spanning regions (Vinson *et al.* 1989). This structure suggested similarity to G protein-coupled receptors, but it was not clear what the ligand might be. The connection to the Wg pathway was made serendipitously when the Nathans laboratory found a mammalian Fz homolog among their collection of human retina cDNA clones, and searched for other mammalian Fz homologs using degenerate PCR primers (Wang *et al.* 1996). This led to their identification and characterization of many members of this large gene family in a variety of species, including a second *Drosophila fz* gene, which they named *fz2* (also known as *Dfz2*). In collaboration with the Andrew and Nusse laboratories, they showed that this gene is expressed in segmental stripes in the embryo in a pattern similar to the *wg* expression pattern, and that when transfected into *Drosophila* S2 cells, it confers on them both the ability to

bind to Wg and to respond to it by stabilizing Arm protein (Bhanot *et al.* 1996). Finally, the Wg signal had an excellent candidate for a receptor.

Genetic redundancy hindered discovery of the Wg receptor Fz2: Why was the Wg receptor not identified earlier? The dirty little secret of genetics is that mutant phenotypes can be obscured by overlapping activity from a related gene. That is exactly what happens with *fz* and *fz2*. When mutations were made in the *fz2* gene, by mobilizing a P transposable element inserted in the promoter region and screening for an imprecise excision event that deleted part of the coding sequence, no significant cuticle pattern disruption was observed in the homozygous mutant embryos (Bhanot *et al.* 1999). However, a perfect *wg*-like lawn of denticles phenotype could be generated by combining the *fz2* mutation with both maternal and zygotic loss of *fz* function. Other signs of Wg signaling activity, such as stabilization of Arm protein and maintenance of *en* expression within each segment, were likewise affected in maternal/zygotic *fz* combined with zygotic *fz2* mutation (Bhanot *et al.* 1999; Chen and Struhl 1999; Muller *et al.* 1999). Thus, the maternally contributed *fz* product is able to compensate for zygotic loss of *fz2*, even though it appears to be *fz2* that is best able to bind to the Wg protein (Rulifson *et al.* 2000). Indeed, this capacity may be part of the reason that *fz* is able to compensate for loss of *fz2*: in *fz2* single-mutant embryos, Wg protein accumulates to levels much higher than those seen in wild-type embryos, and may therefore drive a suboptimal interaction with Fz receptor to produce normal Wg signaling (Moline *et al.* 2000).

fz and fz2 transgenic flies clarified distinctions between polarity and Wg signaling: The ease with which transgenes can be introduced into flies allowed structural comparison of the two Fz proteins (Boutros *et al.* 2000; Strapps and Tomlinson 2001). Frizzled structure can be subdivided into an extracellular domain, including a cysteine-rich domain known to bind Wg (Rulifson *et al.* 2000), a seven transmembrane-spanning region with both extracellular and intracellular loops, and an intracellular C-terminal domain that is not strongly conserved between the two *Drosophila* Frizzleds (Bhanot *et al.* 1996). These domains were swapped to create chimeric molecules that were then tested for their ability to rescue PCP *vs.* Wg signaling defects. The C-terminal domain of Fz correlated with polarity phenotypes, whereas the C-terminal domain of Fz2 correlated with Wg signaling activity (Boutros *et al.* 2000), consistent with earlier work showing that deletion of the Fz2 C-terminus produced a dominant negative effect on Wg signaling in the wing imaginal disc (Cadigan *et al.* 1998). Chimeras between Fz and Fz2 also demonstrated that C-terminal domains control apical–basal membrane targeting within epithelial cells, with basally localized Fz2 being essential for its Wg signaling function and apical Fz essential for polarization (Wu *et al.* 2004). Some of the phenotypic effects of Fz *vs.* Fz2 overexpression can be modulated by changing the dosage of Dsh, suggesting that the two Frizzleds compete for the cellular pool of Dsh molecules (Wu *et al.* 2004). Although both Fz and Fz2 can bring Dsh to the membrane when overexpressed (Boutros *et al.* 2000), it is Fz's asymmetric placement of Dsh on distal cell membranes that correlates with tissue polarization (Axelrod *et al.* 1998; Axelrod 2001, 2009). Fz and Fz2 share a small conserved domain within their C-terminal regions that directly binds the PDZ domain of Dsh (Wong *et al.* 2003).

The extracellular cysteine-rich domain (CRD) of Fz correlated with rescue of *fz* mutant effects on PCP (Strapps and Tomlinson 2001). The CRD, even though it binds Wg, appears to be dispensable for Wg signaling. *fz* and *fz2* transgenes that lack the CRD are still able to rescue Wg signaling (Chen *et al.* 2004; Povelones and Nusse 2005). Furthermore, molecular characterization of *fz* mutations found missense mutations in the extra- and intracellular loops and transmembrane domains, but none within the CRD (Povelones *et al.* 2005). Thus, the Wg-binding domain relevant to signaling may reside elsewhere, in the Fz2 extracellular N-terminus or in the extracellular loops of the transmembrane-spanning regions. However, Wg binding to the CRD of Fz may affect PCP. The Mlodzik laboratory showed that the Fz CRD binds to Van Gogh (Vang), a cell surface protein that localizes to proximal membranes, and that this interaction is essential for PCP (Wu and Mlodzik 2008). They went on to discover that Wg and one of its homologs in *Drosophila*, Wnt4, have redundant functions in modulating the interaction between Fz and Vang (Wu *et al.* 2013). Loss-of-function *wg* mutant clones were created with a temperature-sensitive allele of *wg* (Baker 1988a; Bejsovec and Martinez Arias 1991), which provides normal Wg activity to pattern the wing disc and can then be removed by shifting to higher temperature after patterning is complete. The wg^{ts} mutant clones have normal PCP, but when the wg^{ts} allele is combined with a mutation in the *Wnt4* gene, the doubly mutant clones show strong PCP defects. Thus, these two secreted Wnt molecules, both expressed along the distal margin of developing wings, could provide a redundant source of graded information that might account for the nonautonomy observed in *fz* PCP phenotypes.

Other components of the canonical Wg pathway were identified through their PCP phenotypes in the fly eye, where photoreceptor cells are precisely arranged in each ommatidium with mirror symmetry across the dorsal–ventral midline [reviewed in Axelrod (2009)]. For example, *nemo*, which encodes a Ser/Thr kinase, was identified in the Benzer laboratory through its mutant effects disrupting polarity in the eye (Choi and Benzer 1994). *nemo* mutations were also found in a genetic screen for modifiers of signaling pathways (Verheyen *et al.* 1996), and the Verheyen laboratory showed that Nemo kinase acts as a negative regulator of Wg signaling, as well as of other signaling pathways (Verheyen *et al.* 2001).

Function of the Wingless/Wnt Pathway

Fly genetics showed how the pathway works

Stabilization of Arm protein as the key effector: Although the *arm* gene product is contributed maternally (Wieschaus

and Noell 1986), there is not enough to rescue embryonic patterning, so zygotic loss of *arm* function produces strong segment polarity mutant phenotypes. The Wieschaus laboratory set out to clone the *arm* gene with the same strategy used to isolate the *wg* gene sequence, by mobilizing *P* elements to generate new insertional alleles of the gene and then using the *P* element sequence as a hybridization probe for clone recovery (Riggleman *et al.* 1989). Cloning the *arm* gene allowed the Wieschaus laboratory to perform mRNA *in situ* hybridization in embryos, which revealed that the gene is broadly expressed in a uniform pattern during development (Riggleman *et al.* 1989). The breakthrough came when antibodies were made against the Arm protein. By contrast to the uniform mRNA distribution in embryos, Arm antibody staining showed a dramatic striped pattern (Figure 3D), which mirrored the striped pattern of *wg* expression and depended on wild-type Wg activity (Riggleman *et al.* 1990). Similar responsiveness to Wg was demonstrated in wing imaginal disc tissue (Peifer *et al.* 1991), confirming that *wg* gene activity caused a post-transcriptional stabilization of the *arm* gene product. The status of Arm protein stability correlated with cell fates: *wg* mutant embryos that produce no naked cuticle show no striping (Riggleman *et al.* 1990), whereas *wg* overexpression produces entirely naked cuticle and causes high uniform accumulation of Arm protein (Noordermeer *et al.* 1992). Other mutants that produce excess naked cuticle, such as *naked* and *zw3/sgg*, show corresponding increases in Arm protein accumulation as well (Noordermeer *et al.* 1992; Peifer *et al.* 1994b).

The Wieschaus laboratory then discovered that the Arm protein was homologous to human plakoglobin and β-catenin (Peifer and Wieschaus 1990; Peifer *et al.* 1992). These molecules are critically important for cell–cell adhesion, and indeed, so is Arm when maternal production is disrupted (Peifer *et al.* 1993). Germ line clones removing *arm* gene activity in the ovaries produce defective eggs, because of disrupted cell–cell adhesion and defects in the actin cytoskeleton, which is essential for transfer of maternal components into the developing oocyte (Cooley *et al.* 1992). Characterization of the *arm* sequence and the position of mutations within that sequence showed that Arm is a modular protein, with conserved N- and C-terminal domains and a more highly conserved repetitive middle region (Figure 5). This middle region contains 12 copies of a 42-amino acid motif, called Arm repeats, which forms a "superhelix" of helices with an extended positively charged groove (Huber *et al.* 1997). Within the groove are binding sites for the Arm/β-catenin-binding domains of various interacting molecules, such as cadherins, Tcf/LEF, and Apc [reviewed in Valenta *et al.* (2012)]. The C-terminal Arm domain appears essential for Arm's role in Wg signaling activity, as many *arm* mutant alleles encode truncated proteins lacking this domain (Peifer and Wieschaus 1990). The Peifer laboratory corroborated this with structure/function analyses of Arm. Using *arm* transgenes, they showed that at least some Wg signaling function resides in the C-terminus, and found that this region can interact with the Arm repeat region in a yeast two-hybrid assay (Orsulic and Peifer 1996; Cox *et al.* 1999). The vertebrate β-catenin shows the same intramolecular interaction, with the C-terminus folding back to contact the repeat region, where it may regulate access of potential binding partners to favor adhesion *vs.* signaling function (Gottardi and Gumbiner 2004).

The N-terminal domain of Arm has a particularly important role in Wg signaling, as this is the portion of the protein that regulates its stability. The discovery that *zw3/sgg* encodes a Ser/Thr kinase strongly suggested that phosphorylation plays a role in activating the Wg pathway (Siegfried *et al.* 1990, 1992). Indeed, Arm protein was shown to be phosphorylated by GSK, and consensus sites for GSK phosphorylation were found in the N-terminal domain (Peifer *et al.* 1994a). The Peifer laboratory deleted the putative phosphorylation domain and found that ectopic expression of this arm^{S10} transgene showed higher-than-wild-type accumulation of the transgenic Arm protein, along with uniform naked cuticle production in the embryo (Pai *et al.* 1997). This phenotype mimicked the effect of knocking down GSK or CK1 function (Peifer *et al.* 1994b; Yanagawa *et al.* 2002). Thus, Arm is stabilized artificially when it cannot be phosphorylated by GSK, the precursor step to Slimb-mediated ubiquitination and destruction by the proteasome. Under normal conditions, Apc and Axin, which each have multiple protein–protein interaction domains, bring the kinases into close proximity with Arm (Figure 5A). The group of interacting scaffold and kinase proteins is known collectively as the destruction complex, since it is dedicated to the phosphorylation and degradation of constitutively synthesized Arm [reviewed in Stamos and Weis (2013)]. This central feature of Wnt pathway control is conserved in humans; mutations in the corresponding GSK and CK1 phosphorylation sites of β-catenin, as well as loss-of-function mutations disrupting Apc, have been found in a variety of cancers [reviewed in Oving and Clevers (2002) and Polakis (2007)].

Genetic epistasis experiments determined the order of steps in the pathway: The opposite cuticle patterns produced by loss and gain of Wg activity, all-denticle *vs.* all-naked cuticle, respectively, allowed researchers to deduce the molecular steps of pathway activation. The heat shock-driven wg^{+} transgene was used to determine that *porc* acts upstream of Wg, as the *porc* mutant phenotype was partially rescued by ectopic *wg* expression, whereas *dsh* and *arm* mutant phenotypes were not rescued (Noordermeer *et al.* 1994). This order matched expectations, as *porc* mutations disrupt Wg secretion but had no effect in Wg-responding cells (van den Heuvel *et al.* 1993; Kadowaki *et al.* 1996), whereas *dsh* and *arm* both showed cell-autonomous loss of Wg signaling in responding cells (Wieschaus and Riggleman 1987; Klingensmith *et al.* 1994). The excess naked cuticle phenotype of *zw3* mutants (Perrimon and Smouse 1989) was used to test the order of intracellular gene activities. Removing *arm* function, in *arm zw3* double-mutant embryos, resulted in the all-denticle *arm*

mutant phenotype, indicating that Arm is required for the all-naked *zw3* phenotype. By contrast, removing *dsh* function, in the *zw3 dsh* double-mutant embryos, did not eliminate the all-naked *zw3* phenotype (Siegfried *et al.* 1994). This positioned Dsh as the most upstream cytosolic component, acting between receptor activation and destruction complex inhibition.

The *nkd* gene product may also act at this upstream position. Double-mutant experiments indicated that both *dsh;nkd* and *arm;nkd* showed the all-denticle phenotype (Rousset *et al.* 2001). However, the all-denticle phenotype was also observed in *wg;nkd* double mutants, with only a slight rescue of segmentation in the denticle pattern (Bejsovec and Wieschaus 1993), suggesting that *nkd* gene activity is most important when the Wg pathway is active. Indeed, Wg signaling induces *nkd* gene expression, suggesting that Nkd is an inducible negative feedback inhibitor of the pathway (Zeng *et al.* 2000). Overexpression of *nkd* suppressed *dsh* overexpression phenotypes in the eye, and Nkd bound to Dsh in yeast two-hybrid assays, indicating a direct interaction between them (Rousset *et al.* 2001). Experiments with the vertebrate homologs of Nkd and Dsh corroborated this, showing that Nkd and Dsh colocalize at the plasma membrane, where Nkd may influence the stability of Dsh (Hu *et al.* 2010; Schneider *et al.* 2010). In addition, Nkd has two nuclear localization sequences, and its localization to the nucleus correlated with partial rescue of the *nkd* mutant phenotype by a mechanism that is still unclear (Waldrop *et al.* 2006; Chan *et al.* 2008).

The *arrow* mutation, although it was identified in the Heidelberg screens, was not connected to the Wg pathway until the DiNardo laboratory tested it for maternal contribution. Homozygous mutant embryos from germ line clones produced the all-denticle cuticle pattern typical of lost Wg signaling (Wehrli *et al.* 2000). This phenotype was not altered by overexpression of *wg*, but was substantially rescued by overexpressing *dsh*, indicating that this predicted transmembrane protein acts between the Wg signal and its intracellular effectors. The vertebrate homologs of Arrow, LDL receptor-related proteins LRP5 and 6, were found to mediate Wnt signaling in *Xenopus* (Tamai *et al.* 2000). The intracellular domain of LRP5 was also shown to bind to Axin in mouse cells (Mao *et al.* 2001), providing another link between the cell surface and cytosolic components of the Wg/Wnt pathway. These results suggested that Arrow/LRP acts as a coreceptor with Fz to transduce the Wnt signal (Figure 5B). Perhaps the most convincing evidence of this was the construction of transgenic *Drosophila* lines carrying a chimeric molecule, with the intracellular domain of Arrow fused to the C-terminus of Fz2 (Tolwinski *et al.* 2003). The chimeric transgene was able to rescue naked cuticle formation in *wg* null mutant and in *fz fz2* maternal/zygotic mutant embryos, but not in *dsh* or *arm* maternal/zygotic mutants. The behavior of the chimera suggested that Wg binding to the cell surface receptors may bring Fz2 and Arrow together, and that close proximity of the Arrow cytoplasmic domain with some or all of Fz's three intracellular loops, and/or with Fz's C-terminus, may trigger the intracellular response. This idea was corroborated in cultured *Drosophila* cells and in human cells by the Varmus laboratory, who engineered mutations into the rat Fz1 and human Fz5 genes, and demonstrated that residues in all three Fz intracellular loops and in the C-terminal tail were important for Wg-induced reporter gene expression (Cong *et al.* 2004). These Fz mutations also reduced binding to Dsh and reduced the activity of a Fz-LRP chimeric molecule, confirming that Wg/Wnt binding induced the clustering of Fz with LRP for intracellular transduction of the signal. Subsequent experiments with an artificial ligand designed to cross-link the extracellular portions of Fz and LRP also produced Wg pathway activation (Janda *et al.* 2017).

Downstream steps in the transcriptional activation of *wg* target genes were also deduced in genetic epistasis experiments. The all-naked cuticle phenotype produced by the arm^{S10} transgene, which lacks the phosphorylation sites targeting Arm for degradation, was suppressed by *Tcf/pan* loss-of-function (van de Wetering *et al.* 1997). Thus, *Tcf* gene activity is essential for the phenotypic effects of Arm stabilization, indicating that the physical interaction detected in yeast two-hybrid assays is relevant to *in vivo* target gene activation. Conversely, deleting the predicted Arm-binding domain of Tcf yields a dominant negative molecule that strongly represses target gene expression, replacing naked cuticle with denticles when expressed in otherwise wild-type embryos. Even a wild-type *Tcf* transgene can increase denticle specification when overexpressed in sensitized embryos with low levels of *wg* expression (Cavallo *et al.* 1998). In both cases, the effect was weakened when the transgene was expressed in embryos from mothers (but not fathers) that were heterozygous for *groucho* mutations. This confirmed the identity of Groucho as the transcriptional corepressor that mediates Tcf's repressive mode *in vivo* (Figure 5A), and demonstrated that it is maternally provided.

Properties of the Wg signal

Lipid modification and glycosylation of Wg: The isolation and characterization of *porc* mutations marked the beginning of our understanding of Wg protein processing. *porc* mutant embryos show retention of Wg protein within the *wg*-expressing cells, very similar to what is observed in embryos mutant for some missense alleles of *wg*, such as the wg^{ts} allele at restrictive temperature (van den Heuvel *et al.* 1993). The *porc* gene encodes a multiple transmembrane-spanning protein resident in the endoplasmic reticulum (Kadowaki *et al.* 1996), with homology to O-acyltransferases (Hofmann 2000). Heroic efforts in the Nusse laboratory pioneered the purification of active Wg (van Leeuwen *et al.* 1994) and vertebrate Wnt molecules (Willert *et al.* 2003), and demonstrated the presence of a fatty acyl group covalently attached to the protein. The mouse homolog of *porc* was associated with this lipid modification of vertebrate Wnts, where a monounsaturated palmitoleic acid is attached to a conserved serine residue (Takada *et al.* 2006). Overexpression of *porc* also increased the N-linked glycosylation of Wg protein passing through the secretory pathway (Tanaka *et al.* 2002), suggesting that lipid modification is a

required precursor for subsequent modifications. However, mutation of both reported asparagines eliminated glycosylation of Wg without affecting its secretion or signaling activity in cultured cells or in transgenic flies (Herr and Basler 2012; Tang *et al.* 2012). By contrast, mutating the lipidation site on Wg, Ser239, blocked secretion and signaling activity (Franch-Marro *et al.* 2008; Herr and Basler 2012; Tang *et al.* 2012).

The discovery of lipid attachment explained why the Wnts had been so difficult to characterize biochemically, but was puzzling in light of observations that Wg protein was detected and could act over many cell diameters both in embryos (van den Heuvel *et al.* 1989; Bejsovec and Wieschaus 1995; Lawrence *et al.* 1996; Sanson *et al.* 1999) and in imaginal discs (Struhl and Basler 1993; Zecca *et al.* 1996; Neumann and Cohen 1997; Strigini and Cohen 2000). One possible explanation is that lipid-anchored Wg molecules are sorted into lipid rafts in the membrane (Zhai *et al.* 2004), where they can then associate with extracellular lipoprotein particles (Panakova *et al.* 2005). Indeed, the Eaton laboratory showed that RNAi knockdown of the fly apo-lipophorin, which organizes lipoprotein particles, reduces the range of Wg protein movement and signaling activity within the wing imaginal disc (Panakova *et al.* 2005). A second possible explanation is that Wntless might be involved not only in chaperoning Wg through the secretory pathway, but also in packaging Wg into extracellular vesicles called exosomes. The Vincent laboratory showed that Wg and Wntless can be recovered together, along with homologs of mammalian exosome proteins, from the culture medium of Wg-expressing *Drosophila* S2 cells (Beckett *et al.* 2013). However, their RNAi perturbation of exosome production did not disrupt Wg secretion and gradient formation in imaginal discs, leading them to question whether exosomes are relevant to Wg signaling in the wing disc. A third possibility is that there are dedicated chaperone molecules that bind Wg, shielding the lipid and allowing the complex to be soluble. The Nusse laboratory realized that they were losing activity of Wg during its purification from the culture medium of Wg-expressing S2 cells. They deduced that some component in the conditioned medium was essential for Wg activity, and used mass spectrometry to identify a fly lipocalin homolog that they call Swim, for Secreted Wg-interacting molecule (Mulligan *et al.* 2012). RNAi knockdown for *Swim* restricted the movement of Wg and signaling activity in the wing imaginal disc in a manner similar to what the Eaton laboratory observed for lipophorin knockdown. Thus, it is possible that any or all three of these processes may function in delivering Wg to more distant cell populations [reviewed in Langton *et al.* (2016) and McGough and Vincent (2016)].

Interaction with receptors and proteoglycans: The lipid attached to Wg may also be shielded by interaction with Frizzled receptors. To solve the structure of a Wnt molecule, it was coexpressed and cocrystallized with the extracellular CRD of a Fz molecule. The resulting structure showed that the palmitoleic acid was inserted into a hydrophobic groove in the CRD, rendering the complex soluble for crystallization (Janda *et al.* 2012). However, as discussed earlier (in *fz and fz2 transgenic flies clarified distinctions between polarity and Wg signaling*), the Fz CRD was shown to be dispensable for Wnt signal transduction (Chen *et al.* 2004; Povelones *et al.* 2005), therefore the biological relevance of this particular interaction is not clear. Replacing the CRD with a Wg molecule fused to the N-terminus of Fz results in a constitutively active molecule (Povelones and Nusse 2005), suggesting that the CRD may help to concentrate Wg at the cell surface.

A similar role for sulfated glycosaminoglycans may explain why the *sugarless* and *sulfateless* genes were recovered through their *wg*-like mutant effects in germ line clones (Häcker *et al.* 1997; Lin and Perrimon 1999). Overexpressing *wg* can rescue these effects (Häcker *et al.* 1997), suggesting that proteoglycans might act to increase the local concentration of Wg and increase signaling efficiency. Dally, the fly glypican, was examined as a candidate for the proteoglycan core protein modified by these enzymes because mutant alleles had been associated with adult wing margin notching, a *wg*-like phenotype (Nakato *et al.* 1995). Indeed, *dally* homozygous mutants showed partial loss of naked cuticle (Tsuda *et al.* 1999) and strong *wg*-like cuticle patterns when derived from germ line clones (Lin and Perrimon 1999). Mutations in *dally* also suppressed the formation of ectopic wing margin bristles caused by overexpressing *fz2* in the wing imaginal disc, indicating that Dally function is required for optimal Fz2 activity (Lin and Perrimon 1999). A second proteoglycan, Dally-like (Dlp), was also found to have effects on modulating Wg signaling in embryonic and imaginal disc patterning (Baeg *et al.* 2001). Heparan-sulfated Dlp restricted Wg protein distribution in the wing imaginal disc (Baeg *et al.* 2004; Kirkpatrick *et al.* 2004), suggesting that it can bind to extracellular Wg and modulate its interaction with Fz receptors. The *dlp* mutants showed genetic interactions with *Notum* mutations; in particular, *dlp* mutations suppressed the effects of overexpressing *Notum* (Kirkpatrick *et al.* 2004). Thus, Dlp can influence Notum enzymatic activity, perhaps because Notum's interaction with glycosaminoglycans may colocalize it with Wg at the cell surface and facilitate its cleavage of the Wg lipid group (Kakugawa *et al.* 2015).

Interactions with both the Fz receptors and cell surface proteoglycans help to shape the graded distribution of Wg protein across the epithelium, as it moves away from the Wg-producing cells (Franch-Marro *et al.* 2005; Piddini *et al.* 2005; Hufnagel *et al.* 2006). In the embryo, endocytosis also plays a role in shaping the Wg distribution (Dubois *et al.* 2001). Blocking endocytosis, by disrupting dynamin function in a stripe of cells within each embryonic segment, causes Wg-dependent pattern defects on the far side of the disrupted domain (Moline *et al.* 1999). The "shadow" cast by a spatially restricted endocytosis block suggests that, in the embryo, movement of Wg protein requires internalization and transit through cells. In the wing imaginal disc, blocking endocytosis does not disrupt the extracellular distribution of Wg protein (Strigini and Cohen 2000), but instead disrupts the apical-to-basal movement of Wg, which is required for its signaling

activity (Yamazaki *et al.* 2016). The Vincent laboratory has used new gene-editing techniques to address the functional significance of the Wg gradient, engineering the loci of several key Wg pathway components with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 to allow replacement of the wild-type gene with tagged and/or mutated forms (Baena-Lopez *et al.* 2013). A membrane-tethered form of Wg containing the Neurotactin transmembrane domain (Zecca *et al.* 1996), in addition to the normal lipidation site, was inserted into the endogenous *wg* locus; the resulting molecule was able to support normal cell proliferation and patterning of the wing (Alexandre *et al.* 2014). Studies of Wg/Wnt trafficking are complicated by high accumulation of Wg in the Wg-producing cells when export or movement is impaired; this intense signal can obscure detection of low levels of Wg elsewhere. For example, embryos that are heterozygous for the wg^{ts} allele at restrictive temperature appear to have no detectable Wg outside of the Wg-expressing cells, even though they produce half a dose of wild-type Wg, and show completely normal patterning and viability (van den Heuvel *et al.* 1993). It is also difficult to spatially restrict cell surface molecules with confidence. Bride-of-sevenless, the ligand for the Sevenless receptor, is internalized in its entirety into the adjacent responding cell, in spite of being anchored with seven transmembrane domains (Kramer *et al.* 1991; Cagan *et al.* 1992). Further work, particularly with the valuable new reagents generated by the Vincent laboratory, will build a clearer understanding of the processes that move Wg from cell to cell, and of possible roles for Wg as a graded morphogen.

Genetically separable domains within the Wg protein: Wg differs from vertebrate Wnts in having a hydrophilic 80-amino acid region that is not conserved (Rijsewijk *et al.* 1987; Willert and Nusse 2012). This appears to have been the most immunogenic epitope when antibodies were raised against Wg, as an in-frame deletion of this region yields a functional molecule that does not cross-react with polyclonal Wg antibodies (Hays *et al.* 1997). This nonconserved region divides the molecule into two regions (Figure 6), each predicted to have internal disulfide bond pairs—five in the N-terminal half and six in the C-terminal half—suggesting that the regions fold into separate domains. The Nusse laboratory showed that the halves could be separately produced and secreted in *Drosophila* S2 cells, but could transduce signal only when they were expressed together (Wu and Nusse 2002). Many new mutant alleles of *wg* have been generated, facilitated by the wg^{1} allele, which is still viable *in trans* with lethal mutations, but with a strong, fully penetrant wingless phenotype. A mutation that truncates *wg* at the end of the nonconserved region produces mutant protein that showed normal distribution and endocytosis into neighboring cells, but had no detectable signaling activity (Bejsovec and Wieschaus 1995). Missense mutations that alter highly conserved amino acids in the N-terminal half (marked by a blue box in Figure 6) produced the opposite effect: the mutant proteins could signal locally, stabilizing *en* expression in the neighboring cells, but showed a restricted distribution in the embryo (Dierick and Bejsovec 1998). These observations suggest that the cell-to-cell movement of Wg protein is independent of its ability to signal, and confirm that the C-terminal half is essential for signaling. Indeed, a missense mutation in the C-terminal domain was found to reduce the efficiency of Wg signaling, but was rescued to wild-type activity by overexpressing *fz2* or by reducing *dally* (Moline *et al.* 2000), suggesting that this portion of Wg interacts with the receptor complex.

Consequences of response to Wg

Cell fate specification in different tissues: Although much of the pathway was identified through the effects on embryonic and imaginal disc patterning, many tissues throughout the fly body require Wg signaling. The nervous system requires multiple inputs from Wg signaling throughout development, starting with neurogenesis in the early embryo, where *wg* mutations cause loss or duplication of specific neuroblasts (Patel *et al.* 1989; Chu-LaGraff and Doe 1993). To study *wg* function in the nervous system at later stages requires either mosaic analysis or use of the wg^{ts} mutation, which can be cultured at low temperatures through the early stages of development. The latter was used to show that Wg signaling across the larval neuromuscular junction is required for proper synapse formation (Packard *et al.* 2002), in a process that requires Wntless for the packaging and release of Wg in exosomes (Korkut *et al.* 2009; Koles *et al.* 2012). Wg signaling in the eye-antennal disc initially blocks neuronal fate to specify the portion of the disc that will form the head capsule [reviewed in Legent and Treisman (2008)], and then later specifies neural cell fates along the edge of the retina (Tomlinson 2003). Wg signaling even persists in the adult fly brain, where it has been associated with long-term memory formation (Tan *et al.* 2013).

In the embryo, Wg secreted by the ectoderm signals to the developing mesoderm, establishing fates in the somatic musculature (Baylies *et al.* 1995) and in the heart precursor cells (Lawrence *et al.* 1995). Embryonic Wg signaling has also been associated with the formation of a number of tubular structures: the foregut (Pankratz and Hoch 1995), midgut [reviewed in Bienz (1994)], hindgut (San Martin and Bate 2001), Malpighian tubules (Skaer and Martinez Arias 1992), trachae (Llimargas 2000), and salivary glands (Bradley and Andrew 2001). The requirement for Wg signaling in these disparate tissues hints at a very diverse array of potential target genes that might respond to Wg activation.

Interaction with other signaling pathways during development: Often, Wg-mediated cell fate specification events involve the modulation of other signaling pathway activities, particularly the Decapentaplegic (Dpp), Epidermal growth factor receptor (EGFR), Hedgehog (Hh), and Notch pathways. For example, Wg and Hh signaling from adjacent rows of cells in the embryonic epidermis mutually reinforces their striped expression patterns, as *hh* gene expression is turned on by the En transcription factor, which requires Wg signaling for its stable expression (Bejsovec and Martinez Arias 1991;

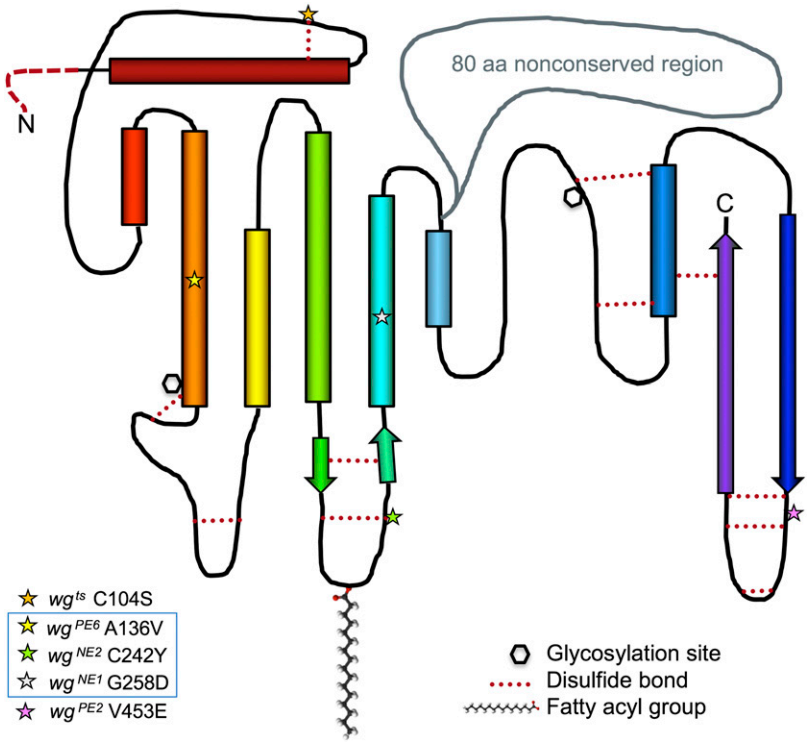

Figure 6 Diagram of putative Wg protein structure. The structure of *Xenopus* Wnt8 was solved by cocrystallizing it with the mouse Fz8 CRD, allowing determination of the positions for glycosylations, conserved cysteines involved in disulfide bonds, and the fatty acyl attachment (Janda *et al.* 2012). Cylinders depict α-helical regions and block arrows depict β-sheets predicted in the crystal structure. Approximate positions of *wg* mutations are indicated by stars (those implicated in Wg protein transport are within the blue box), and the position of the nonconserved insert region of Wg is indicated in gray (van den Heuvel *et al.* 1993; Bejsovec and Wieschaus 1995; Dierick and Bejsovec 1998).

Heemskerk *et al.* 1991; Bejsovec and Wieschaus 1993; Heemskerk and DiNardo 1994). A similar positive feedback loop between Wg and Dpp signaling, where each pathway regulates expression of the other signal, appears to function in embryonic midgut development (Yu *et al.* 1996). Both Wg and Dpp in turn activate the EGFR pathway in the midgut, with Dpp and EGFR signaling also showing an interdependence (Szuts *et al.* 1998). The EGFR pathway plays a role in shaping the segmental pattern in the embryonic epidermis as well, acting in opposition to Wg signaling to define the denticle-secreting field of cells (Szuts *et al.* 1997; Urban *et al.* 2004; Walters *et al.* 2005). The interplay of EGFR and Wg in the embryo requires input from the Hh and Notch signaling pathways, both of which activate the production of EGF ligand, whereas Wg signaling represses it (O'Keefe *et al.* 1997; Alexandre *et al.* 1999).

A combination of Wg, Dpp, and Hh signaling generates the overall pattern of wing and leg imaginal discs (Blair 2007; Estella *et al.* 2012). Spatially restricted expression of each signal organizes the anterior–posterior, dorsal–ventral, and proximal–distal axes for proper morphogenesis of the appendage (Basler and Struhl 1994; Zecca *et al.* 1995; Jiang and Struhl 1996; Penton and Hoffmann 1996). The Wg and Notch pathways also interact in wing patterning. Notch signaling is required for the expression of *wg* along the presumptive wing margin in the imaginal disc (Rulifson and Blair 1995), and both pathways are required for proper patterning of sensory neurons in the wing (Couso *et al.* 1994; Bray 1997). Indeed, the Wg and Notch pathway share several downstream components, such as Zw3/Shaggy and Groucho, leading to strong genetic interaction among mutations in the two pathways (Couso and Martinez Arias 1994).

The search for Wg target genes and their functions: The complex interaction of signaling pathways, and their various effects on promoting specific cell fates, begs the question of how they do what they do. Presumably the target genes activated in response to each signal include, or control expression of, structural gene products that are physically responsible for morphogenesis of the tissue. A few Wg target genes were identified early on: *engrailed* in the epidermis (DiNardo *et al.* 1988; Martinez Arias *et al.* 1988), and *Ultrabithorax* and *labial* in the midgut (Immergluck *et al.* 1990; Thuringer and Bienz 1993; Thuringer *et al.* 1993), all of which encode homeobox-containing transcription factors. Some of the targets required for particular cell fate specifications in other tissues have been identified as well. For example, the *sloppy-paired* and *even-skipped* pair-rule genes are induced by Wg in heart progenitor cells, and were shown to have functional Tcf-binding sites in their regulatory regions (Lee and Frasch 2000; Knirr and Frasch 2001).

Two of the segment polarity genes associated with the all-naked cuticle phenotype, *naked* (*nkd*) (Zeng *et al.* 2000) and *shaven-baby* (*svb*) (Payre *et al.* 1999), were also shown to be targets of Wg signaling but with opposite control. Expression of *nkd* is induced by Wg, whereas *svb* is repressed by it. *Notum* is another example of a Wg-activated gene that, like *nkd*, is part of a negative feedback loop (Gerlitz and Basler 2002). The Cadigan laboratory has characterized Tcf-binding sites in the *cis* regulatory modules at a number of Wg target loci, particularly *Notum* and *nkd*. They have demonstrated direct binding of the transcription complex at two distinct consensus sequences: the HMG-box-binding motif (SCTTTGWWSWW; S = G/C, W = A/T) and a second "helper" motif (GCCGCCR;

R = A/G) that is bound by a separate DNA-binding domain in Tcf, called the C-clamp (Chang *et al.* 2008; Parker *et al.* 2008; Archbold *et al.* 2014).

A number of examples of Wg-mediated repression beyond *svb* have been characterized: in the developing wing imaginal disc, Wg represses expression of its own Fz2 receptor, and this helps to shape the graded distribution of Wg protein within the disc (Cadigan *et al.* 1998); in the ventral embryonic epidermis, Wg represses the expression of *SoxN* (Overton *et al.* 2007), a gene that imposes negative control on the transcriptional response to Wg signaling (Chao *et al.* 2007). The Wg-mediated repression of *SoxN* and *svb* in the late embryonic epidermis is critical for the segmental pattern of denticle belts. Both genes encode transcription factors that control the expression of many structural genes required for denticle construction (Chanut-Delalande *et al.* 2006; Fernandes *et al.* 2010; Rizzo and Bejsovec 2017).

Wg-mediated repression of these genes may be indirect, through the induction of targets that encode transcriptional repressors, but it may be direct. The Cadigan laboratory had conducted a genome-wide microarray screen for transcriptional targets of Wg in the *Drosophila* Kc167 cell line, and characterized a number of genes that were either activated or repressed by Wg signaling. They discovered a *cis* regulatory sequence in *Ugt36Bc*, *Ugt58Fa*, *Peroxidasin* (*Pxn*), and *Tiggrin*, which is associated with direct repression of these genes by Tcf-Arm (Blauwkamp *et al.* 2008; Parker *et al.* 2008; Zhang *et al.* 2014). This sequence (AGAWAW, W = A/T) is distinct from sequences found in promoters of genes activated by Tcf-Arm, implying that interaction with this alternative sequence correlates with altered function of the Tcf-Arm complex. The Basler laboratory identified some of these Wg targets as well, in an RNA-sequencing-based screen in Kc cells (Franz *et al.* 2017). Cells exposed to Wg-conditioned medium upregulated 40 genes at least twofold, and downregulated 11 genes. These transcriptional changes were eliminated in Kc cells lines with CRISPR-generated null alleles for *arm* or *Tcf/pan*, clearly demonstrating that both activation and repression depend on Arm and Tcf function (Franz *et al.* 2017).

Conclusions

Current questions and controversies

Role of Dishevelled in linking receptor activation to Arm stabilization: Dsh remains the most upstream cytosolic component of the pathway known, but even after years of study in the fly and in vertebrate systems, it is still not clear exactly what it does. Dsh can bind to the cytosolic domain of Fz, and Axin can interact with the cytosolic portion of Arrow. Thus, it seems likely that Wg-mediated clustering of Fz and Arrow brings together Dsh and Axin, in a way that antagonizes destruction complex activity and allows accumulation of Arm inside the cell. The Bienz laboratory, who showed that Dsh can recruit Axin to the membrane (Cliffe *et al.* 2003), postulate a model where Dsh and Axin polymerize in response to Wg signaling (Schwarz-Romond *et al.* 2007a,b). Mutating the Dsh interaction (DIX) domain of Axin prevents it from rescuing the *axin* mutant phenotype in embryos, supporting the idea that sequestering Axin in these polymers blocks its destruction complex activity (Fiedler *et al.* 2011). Others have proposed a variety of possible roles for Dsh: (1) direct binding between Dsh and Axin inhibits destruction complex activity (Logan and Nusse 2004; Malbon and Wang 2006), (2) recruitment of Axin to the membrane leads to its degradation and thereby inactivates the destruction complex (Mao *et al.* 2001; Tolwinski *et al.* 2003), or (3) modifications of the Arrow/LRP cytosolic domain directly inactivate GSK and block Arm/β-catenin phosphorylation (Piao *et al.* 2008; Wu *et al.* 2009). However, data from a vertebrate system suggest that the destruction complex remains intact during response to Wnt signaling (Hernandez *et al.* 2012; Li *et al.* 2012). These experiments led the Kirschner laboratory to propose a model (Hernandez *et al.* 2012) where Wnt signaling diminishes, but does not eliminate phosphorylation of β-catenin by the destruction complex, and that a reduced degradation rate is sufficient to explain the β-catenin accumulation observed in cells responding to Wnt. The Clevers laboratory proposes an alternative model (Li *et al.* 2012), where the destruction complex becomes saturated with phosphorylated β-catenin due to Wnt-mediated blockage of the ubiquitination step. This then allows newly synthesized β-catenin to accumulate, free of destruction complex activity, and drive target gene expression. However, neither of these models explains how Dsh controls this process.

Reorganization of the destruction complex as a means of controlling Arm stability: Axin is the primary scaffolding molecule in the destruction complex, with binding sites for Arm, CK1, GSK, protein phosphatase 2A, and Apc (Ha *et al.* 2004). Although mutations in Apc are strongly correlated with colon cancer initiation in the general population (Kandoth *et al.* 2013), the role of Apc in the destruction complex has been unclear. The Bienz laboratory found that Apc competes with Dsh for Axin binding, and interferes with a default tendency for Axin polymerization at the plasma membrane (Mendoza-Topaz *et al.* 2011). They made point mutations in the Apc-binding region of Axin and found that these fail to rescue *axin* mutant phenotypes, and cause Axin to move to the plasma membrane in a Dsh-dependent fashion. The Ahmed laboratory also found that plasma membrane association of Axin in puncta was relevant to its destruction complex role (Wang *et al.* 2016a). The Peifer laboratory examined the interaction of Apc2 with Axin by expressing the fly molecules in SW80 cells, a human colorectal cancer cell line that lacks Apc function (Pronobis *et al.* 2015). They discovered that Axin forms large, complex macromolecular structures in the presence of Apc2 through two different contact points between the molecules. Both interactions are required for proper β-catenin degradation, and one is regulated by GSK phosphorylation of Apc2. They propose a model where Apc2 cross-links the Axin scaffold and forms pockets where Arm/β-catenin is presented to the kinases. Apc2 then undergoes

phosphorylation itself, causing it to swing one end away from Axin and present Arm/β-catenin to the E3-ligase for ubiquitination. This model is consistent with both the Kirschner and Clevers models for Wnt regulation of the destruction complex.

Modifications of Arm downstream of stabilization: In the simplest view of Wg pathway activation, Arm accumulation drives its interaction with the Tcf transcription factor, recruiting Legless and Pygopus to form a transcriptional activation complex. However, the high flux of Arm may be in excess of what is needed to transduce a signal. We have found that embryos mutant for weak *wg* alleles show substantial cuticle patterning and expression of target genes, but produce no change in Arm levels from the uniform low level typical of *wg* null mutants (Moline *et al.* 2000). This raises the possibility that only a subset of the stabilized Arm molecules observed in wild-type are required for signal transduction. The essential role of Arm/β-catenin in cell adhesion also creates a tension with Wg/Wnt signaling, where increased cadherin can pull Arm/β-catenin away from the signaling pool and diminish pathway activity (Hinck *et al.* 1994; Sanson *et al.* 1996; Gottardi and Gumbiner 2004; Wodarz *et al.* 2006). Thus, part of the response to Wg/Wnt signals may involve shifting the balance between these competing functions of Arm/β-catenin.

The central Arm repeat region of β-catenin provides interaction domains for a variety of proteins: α-catenin and E-cadherin for its adhesive role, and Axin, Apc, Lgs, and Tcf for its signaling role. A number of phosphorylation sites in vertebrate β-catenin have been identified that correlate with enhanced signaling activity rather than degradation [reviewed in Valenta *et al.* (2012)]. These phosphorylations appear to decrease affinity for adhesive binding partners and increase the recruitment of signaling partners; for example, phosphorylation of a tyrosine in the first Arm repeat decreases β-catenin binding to α-catenin and favors binding to BCL9 (Brembeck *et al.* 2004). However, the corresponding Tyr in *Drosophila* Arm does not have a similar function (Hoffmans and Basler 2007). Other phosphorylation sites might play as yet uncharacterized roles in activating Arm or changing its subcellular location. In particular, we do not know whether Arm translocation to the nucleus is regulated. Arm lacks classical nuclear import and export signals, but a small portion of the Arm repeat region is sufficient for nuclear localization; the conventional nuclear import chaperone, Importin-β, possesses similar Arm repeats, and both molecules interact with the same nuclear pore complex protein as they move through the pore (Sharma *et al.* 2012). It is easy to imagine that phosphorylation, or other modification, of Arm might increase or decrease its transit into the nucleus and have profound effects on the activation of target genes.

Future directions

The power of the fly system for gene discovery and functional analysis will only increase as technology evolves. Basic forward genetic screens are still important as an unbiased means to identify unexpected players in the Wg pathway, and the rich array of community resources—FlyBase, the Bloomington *Drosophila* Stock Center, the Gene Disruption Project, the Transgenic RNAi Project, the Vienna *Drosophila* Resource Center, and the *Drosophila* Genomic Resources Center, for example—has dramatically shortened the time it takes to go from a new mutation to a fully characterized gene function. Big questions still remain about how the Wg signal is transduced from the cell surface to the nucleus, and it may be because we are still missing some key components. Finding these is crucial to our understanding of how cell fates are specified during development, and how cell function goes awry in the many human diseases associated with deranged Wnt signaling [reviewed in Nusse and Clevers (2017)]. The essential role of Arm/β-catenin in both cell adhesion and Wg/Wnt signaling makes it particularly tricky to find suitable therapies for these disorders. For example, tumors that are caused by Wnt pathway hyperactivation could metastasize if β-catenin were targeted directly, as this would lead to loss of adherens junctions and epithelial-to-mesenchymal transition [reviewed in Heuberger and Birchmeier (2010)]. Thus, there is great interest in learning how the Wnt pathway might be downregulated in ways that do not affect cell–cell adhesion. Perhaps the best candidate for intervention is Axin, which is degraded in response to tankyrase-mediated ADP-ribosylation in flies (Wang *et al.* 2016b) and in human cells (Huang *et al.* 2009; Zhang *et al.* 2011). Small-molecule inhibitors of tankyrase have been shown to stabilize Axin (Chen *et al.* 2009; Huang *et al.* 2009), and to block the proliferation of colon cancer cell lines (Kulak *et al.* 2015) and of *Apc* mutant tumors in mice (Waaler *et al.* 2012; Lau *et al.* 2013). More potential targets of intervention may present themselves as we discover new components and understand the Wnt pathway in ever greater detail.

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