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Jenny, Fabian Heinz ; Basler, Konrad

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Powerful *Drosophila* screens that paved the wingless pathway

Fabian Heinz Jenny and Konrad Basler*

University of Zurich; Institute of Molecular Life Sciences; Zurich, Switzerland

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The Wnt/Wingless (Wg) signaling cascade controls a number of biological processes in animal development and adult life; aberrant Wnt/Wg signaling can cause diseases. In the 1980s genes were discovered that encode core Wnt/Wg pathway components: their mutant phenotypes were similar and an outline of a signaling cascade emerged. Over the years our knowledge of this important signaling system increased and more components were uncovered that are instrumental for Wnt/Wg secretion and transduction. Here we provide an overview of these discoveries, the technologies involved, with a particular focus on the important role *Drosophila* screens played in this process.

A Signal Essential for Development and Relevant for Disease

Wnt/Wg signaling plays important roles in animal development. In *Drosophila melanogaster* this signaling cascade is involved in the patterning of the embryo[1-3] and in the development of adult structures from imaginal disc primordia[4,5]; this includes leg, wing, genitalia, antennae, and eye imaginal discs.[6-8] In the 1990s Wnt/Wg signaling was first associated with human disease: the *adenomatous polyposis coli* (*APC*) tumor suppressor gene was isolated, which plays a key role in hereditary and spontaneous colorectal cancer, and a few years later APC became directly linked to Wnt/Wg signaling with the discovery that it bound to the core component β-catenin.[9-13]

The Wnt/Wg signal acts either via calcium signaling,[14] by triggering the planar cell polarity pathway[15,16] or canonically by regulating the stability of Armadillo (Arm, β-catenin in vertebrates). Here we focus on the canonical signaling cascade, which principally follows 3 steps: In the sending cell Wnts first get lipid modified by the acyltransferase Porcupine (Porc) and are then secreted via the endoplasmatic reticulum and Golgi apparatus involving the transmembrane protein Wntless (Wls). In the receiving cell the Wnt ligand binds to its receptor Frizzled (Fz) and the co-receptor Arrow (Arr, LRP5/6 in mammals). When no ligand is present, a destruction complex consisting of Axin, Shaggy (Sgg, glycogen synthase kinase 3 (GSK3) in vertebrates) and APC phosphorylates Arm, and marks it for degradation by the proteasome. In presence of the Wg ligand a signal is transduced via Disheveled (Dsh) resulting in the inactivation of the destruction complex. As a consequence Arm accumulates and translocates into the nucleus, where it activates target genes together with the transcription factor Pangolin (Pan, TCF/Lef in vertebrates).[13, 17–20] A model of Wnt/Wg signaling is depicted in **Figure 1A** and a list of all signaling components with abbreviations can be found in **Table 1**.

The definition of the above pathway represents more than 30 y of research. While we have a basic understanding of the mechanics and most of the core signaling components are known, there are still gaps in pathway modulation and tissue specificity, hence there are still new discoveries to be made. Here we first look back at the model systems and techniques involved in this scientific journey and how these contributed to building our comprehensive knowledge of this signaling system. We also speculate on why certain techniques and model systems were so successful and take a look into the future, to ask what technologies could contribute to an even more complete understanding of this fundamental signaling cascade.

Glazed Eyes and Absent Wings – a Chronology of Discoveries

Discovering the ligand

The discovery of the Wg signal can be attributed to Hunt Morgan and his colleagues.[21] They isolated and described a dominant mutation in *Drosophila*, which resulted in a glazed-eye phenotype and therefore was named *Glazed* (*Gla*). 40 y later Sharma described an X-irradiation derived mutant, which frequently lacked one or both wings. This phenotype was governed by a single recessive hypomorphic mutation, which he named *wg*, not knowing that it was allelic to *Gla*.[22,23] As it was found much later, neither Morgan's *Gla* nor Sharma's *wg* alleles changed the coding sequence. *Gla* is a gain-of function allele caused by the insertion of a *roo* retrotransposon,[24] and Sharma's *wg* allele is the result of a 2.5kb deletion, downstream of the locus, which

*Correspondence to: Konrad Basler; Email: konrad.basler@imls.uzh.ch
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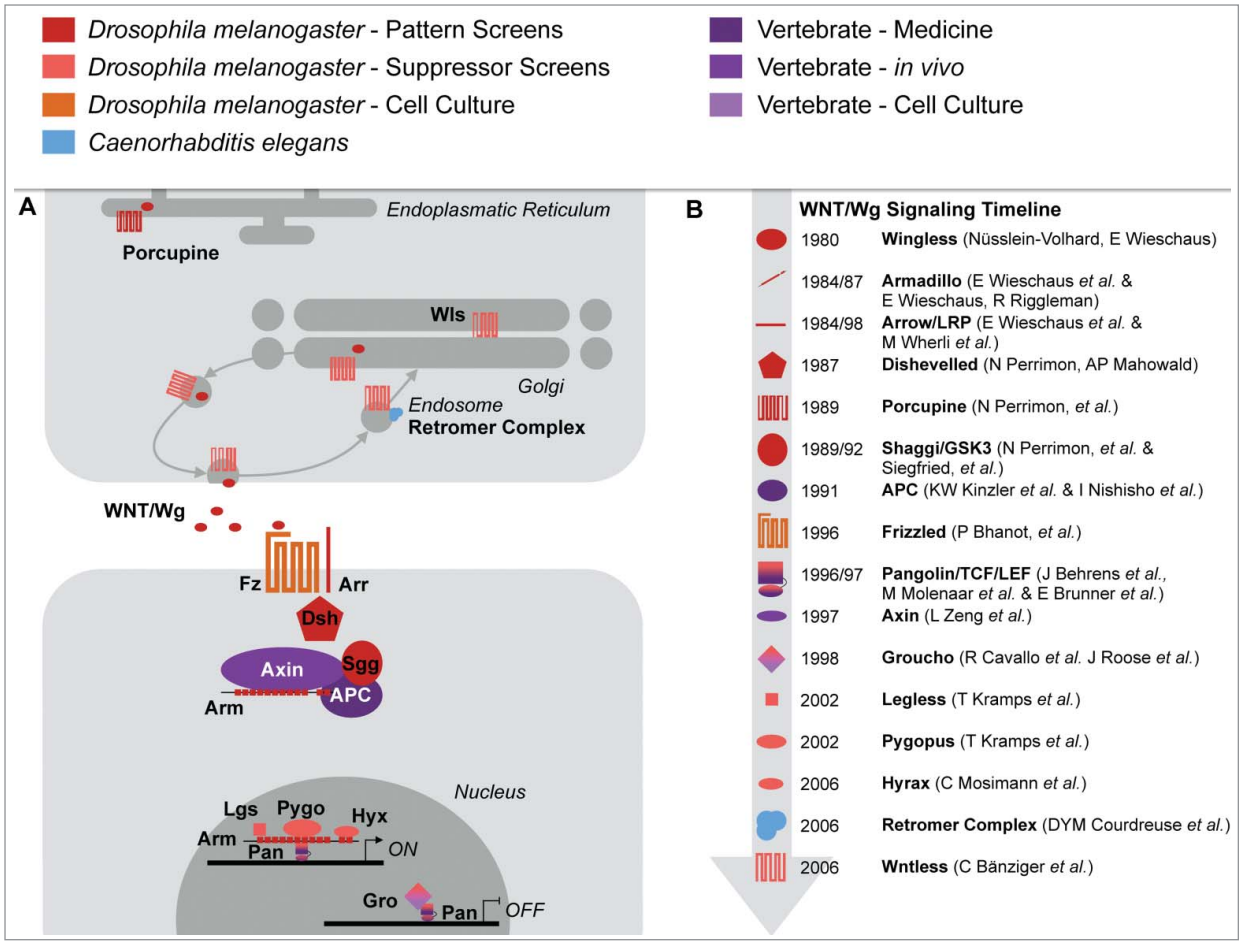

Figure 1. The mechanics and history of Wnt/Wg signaling. (**A**) The current Wnt/Wg signaling model with its core components and (**B**) a historic timeline overview regarding the discovery of these signaling components. The color code indicates whether the individual components were discovered in *Drosophila, Caenorhabditis elegans* or in vertebrates.

presumably contains a regulatory element involved in controlling *wg* expression during wing development.[25,26] Other known mutations, like *Sternopleural* (*Sp*),[27] *spade* and *flag*,[28] were later shown to be regulatory alleles of *wg* as well. The first *wg* null allele was isolated in 1980 as a segment polarity gene influencing embryonic patterning, in the famous screen for embryonic lethal mutations in *Drosophila* conducted by Nüsslein-Volhard and Wieschaus,[29] which laid the foundation for their Nobel Prize

Table 1. List of Wnt/Wg signaling core components. All components are shown with their full *Drosophila* name, abbreviation and the names of their vertebrate homologs. The information is based on Flybase (http://www.flybase.org) and the Wnt Homepage (http://wnt.stanford.edu).

	Drosophila	**Vertebrate**
Signal-transducing components	Wingless (Wg) (6 other WNTs)	WNT1 (18 other WNTs)
	Arrow(Arr)	LRP 5 and 6
	Frizzled (Fz) and Frizzled2 (Fz2)	Fzd 1 to 9
	Disheveled (Dsh)	Dvl-1 to 3
	Armadillo (Arm)	β-Catenin
	Pangolin (Pan)	TCF1 to 4 and LEF-1
	Legless (Lgs)	BCL9
	Pygopus (Pygo)	PYGO 1 and 2
	Porcupine (Porc)	Porc
Signal-repressing components	Adenomatous polyposis coli (APC) and APC2	APC1 and APC2
	Axin	Axin 1 and 2
	Groucho (Gro)	Grg/TLE 1 to 4
	Shaggy (sgg)	GSK3β

winning experiments. Two years later, the mouse *Int-1 g*ene was described, as a locus activated by the integration of MMTV proviral DNA in virally induced mammary tumors (note the analogy of the retrovirus-induced *Int-1* and the retrotransposon-induced *Gla* lesions).[30] *Int-1* was later found to encode a homolog of Wg,[31] and the entire protein family was therefore called Wnt - a blend of the names Wg and Int.[32] The discovery of *wg* in *Drosophila* showed a relevance of this pathway in fly development, whereas the characterization of the murine *Int-1 g*ene implied a role in oncogenesis. A role in vertebrate development became evident when ectopic expression of Wnts was observed to cause axis duplications in *Xenopus* embryos.[33] These factors, their importance in development and oncogenesis, as well as the high degree of conservation between different species, were the underpinnings of the entire Wnt/Wg signaling research field.

Sketching the pathway

Drosophila continued to play an important role early on with genetic fly screens yielding the building blocks of the pathway. In the Nüsslein-Volhard and Wieschaus screen, other genes were identified which were later shown to play a role in the Wnt/Wg pathway. Alleles of *arm* and *arr* also showed segment polarity defects, similar to those of *wg* null alleles, but their relationship remained obscure.[34,35] In subsequent screens, where also the maternal gene function was removed, Perrimon and other researchers identified alleles of *dsh*, *sgg* (also known as *zeste-white 3*) and *porc*.[36-42] This clustering of phenotypes among segment polarity genes indicated already the vague outline of a signaling cascade and therefore pushed research to the next level: The discovery that Wg stabilizes *engrailed* (*en*) expression in embryonic segmentation[43] enabled researchers to add roles, functions and relationships between these genes. The Perrimon lab studied the effect of *wg* on *en* expression in *sgg* mutants.[44] They reported that Wg inactivates the Sgg-induced repression of *en* and that Sgg is a homolog of GSK3 in mammals. Several epistasis experiments helped to sharpen our view of the Wnt/Wg pathway (e.g., *porc*, *dsh*, *arm* and *sgg*)[44-46] and the Nusse lab could show that Porc provides a relevant function for Wg protein secretion.[26]

A signaling system related to cancer: the Wnt/Wg field takes off

In the early 1990s, studies in patients identified a genomic region on chromosome *5q21* and associated mutations at this locus (termed APC) with familial adenomatous polyposis.[9,10] A few years later, immunoprecipitation experiments showed that the APC protein interacts with β-catenin,[11] which reinforced the hypothesis that the Wnt/Wg signaling cascade is involved in cancer. Driven by this finding and its clinical relevance, the Wnt/Wg field started a quest for additional pathway components, in particular the receptor(s). Attempts to biochemically isolate Wg and its receptors failed and research focused on genetic approaches. Initially Notch was postulated to transduce the Wg signal,[47] but Rulifson and Blair presented evidence that while the cross-talk between Notch and Wg functions is substantial, Notch is not the long-sought Wg receptor.[48] It was established that 2 *Drosophila* Fz-family genes encode the Wg receptors. The Nusse and Nathan labs could identify these membrane proteins by a cell culture assays in *Drosophila*: Wg insensitive Schneider 2 cells, transfected with a *fz2* expression construct, were able to respond to the Wg signal and stabilize Arm.[49,50] Pathway specific screens in *Drosophila* using an ectopically active *wg* transgene were started in our laboratory and yielded nuclear signaling components. Brunner et al. described the in vivo role of the *Drosophila pan* gene in a suppressor screen.[51] It encodes a homolog of vertebrate LEF-1, which can, as a transgene, substitute the Pan function.[52] It was found to interact with the Wnt/Wg signaling component β-catenin in a yeast 2-hybrid assay[53] and of *Xenopus* XTcf-3, which forms a complex with β-catenin in the nucleus. This illustrates that this family of transcription factors enables Arm and β-catenin to activate specific target genes.[54]

In 1997 the Costantini lab started to characterize the *Fused* (*Fu*) locus in mice.[55] *Fu* mutations cause pleiotropic developmental effects, including axis duplications[56]; the gene was thus renamed to *Axin*. Dorsally injected *Axin* mRNA inhibited *Xenopus* axis formation and this ventralization was shown to be related to perturbed Wnt/Wg signaling. Epistasis experiments indicated that Axin acts up-stream of β-catenin. A few years later, a *Drosophila* screen identified Axin as a modifier of *dsh* over-expression in the eye.[57]

Soon after, the Wnt/Wg co-receptor *arr* was phenotypically characterized and Wehrli et al. showed that *arr* is essential in cells receiving Wg input, where it acts upstream of Dsh.[58] The low density lipoprotein receptor-related protein (LRP) Arr was essential for proper segmentation in the *Drosophila* embryo and null-mutants (upon removing also the maternal contribution) were indistinguishable from *wg* mutants.

Events in the nucleus

Functional analyses in *Drosophila*, *Caenorhabditis elegans* and *Xenopus* indicated that Pan/TCF/LEF might also act as a transcriptional repressor.[52,59-64] The protein Groucho (Gro) was already known as a co-repressor in segmentation, neurogenesis and sex determination in *Drosophila,*[65-68] however there was no direct link to Wnt/Wg signaling. In the Pan/TCF/LEF yeast 2-hybrid screen that established the link of this transcription factor to β-catenin,[54] a murine homolog of *gro* was identified and implicated in Wnt/Wg target gene repression.[69] In parallel, the co-repressor function of Gro was described in *Drosophila.*[70]

After the turn of the century 3 further genes were identified, that encode products which interact with the nuclear β-catenin-Pan/TCF/LEF complex: *legless* (*lgs*), *pygopus* (*pygo*) and *hyrax* (*hyx*). Lgs recruits Pygo to the Pan/TCF/LEF complex and together with Hyx and other factors they assist β-catenin in the activation of Wg target genes.[71-76] Whereas Lgs and Pygo also emerged from the ectopic Wg signaling screen in the *Drosophila* eye for dominant suppressors that uncovered pan,[51] Hyx was identified in a complementary overexpression screen in the wing.[76]

Secretion of the ligand

In an improved version of the Brunner et al. suppressor screen, which can also retrieve recessive suppressors (see below),

the *wls* gene was discovered, which encodes a factor involved in Wg secretion.[77,78] At the same time, Coudreuse et al. showed that the Retromer complex, previously implicated in intracellular protein trafficking, is required for signaling in Wnt/Wg producing cells.[79] The mechanism necessitating the Retromer complex was further characterized in *Drosophila*, where it is required for Wls protein recycling: In the absence of components of the Retromer complex, Wls is degraded and the Wg pathway is impaired.[80,81]**Figure 1B** illustrates the chronology of Wnt/Wg signaling component discoveries.

The Power of *Drosophila* Screens and the Contribution of Other Systems

It is striking that 13 out of 16 components were discovered first in *Drosophila*. This illustrates nicely the power of phenotype based screens and the associated techniques (**Fig. 1A and B**).

Traditional screens in *Drosophila*

In a first phase of Wnt/Wg research, patterning screens were the key to success. Systematic searches for mutations that affect the segmental pattern, like the one from Nüsslein-Volhard and Wieschaus, identified the first components of this signaling cascade (**Fig. 2A**) .[29,35] A number of segment polarity mutants, but not all, displayed similar phenotypes and helped to vaguely outline the scheme of the Wnt/Wg pathway. Because these experiments were conducted in embryos, where the zygotic mutant phenotype of some loci were masked by maternally provided gene product, a number of signaling components were missed. For example, maternal contribution was the reason, why mutations in the *arm* gene did not entirely mimic *wg* null alleles.[35] As the embryo only gradually runs out of maternal product, the analysis of their early zygotic phenotypes is difficult. Among Wnt/Wg signaling genes, *wg*, *arr* and *arm* alleles show a clear segment-polarity phenotype in homozygous mutant embryos whereas other genes, such as *dsh*, *porc* and *sgg* were initially missed.[36,38,39] Irradiation-based generation of homozygous germline clones allowed the analysis of zygotes that lacked the maternal contribution of the gene under investigation (**Fig. 2B**). This approach permitted Perrimon and colleagues to identify the Wnt/Wg signaling components *dsh*, *porc* and *sgg*, which were missed in earlier screens.

Suppressor screens in *Drosophila*

In a second phase, genetic pathways were further explored with dedicated screens in *Drosophila* based on sensitized phenotypes. Ectopic expression of the *wg* gene in the eye was used to induce a gain-of-Wg signaling phenotype, which allowed for a suppressor screening. The approach of using a *wg* transgene under the control of the eye-specific *sevenless* (*sev*) enhancer has proven valuable for a genome-wide screen for *dominant* suppressors, and led to the identification of *pan*, *lgs* and *pygo* (**Fig. 2E**).[51,71] In a second version, Flp-mediated mitotic recombination was included, which allowed for a chromosome arm-specific screening for *recessive* suppressors (**Fig. 2F**). On chromosome arm 3L this approach uncovered the *wls* gene.[77] A further improved, inducible version of this screen, taking advantage of a conditional *sev-wg* transgene, was recently used for all remaining chromosome arms (F. Jenny & M. Hediger Niessen et al., unpublished). A similar set-up with a sensitized background was used by the Nusse lab to carry out a screen for modifiers of a *dsh* mis-expression phenotype in the Drosophila eye. Flies expressing *UAS-dsh* driven by an eye-specific *Gal4* transgene were mutagenized (**Fig. 2D**); this resulted in the identification of the Drosophila *Axin* gene.[57]

In a suppressor screen with an Arm-depleted sensitized background that favors the discovery of signal-repressing components, the Bejsovec lab was able to describe the negative role of Pan in Wg signaling (together with the co-repressor Gro) and to isolate one of the *Drosophila APC* homologues, *APC2* (**Fig. 2C**).[70,82,83]

Other Approaches

While traditional and suppressor screens in *Drosophila* were highly successful, they also had their limitations in identifying redundant or negatively acting components.

Redundancy was the main issue in the discovery of the Wnt/Wg signaling receptors. Both *fz*-family genes, *fz* and *fz2*, encode functional Wg receptors and were therefore missed in genetic screens. However, the availability of well characterized *Drosophila* cell lines helped. It was known that Schneider 2 cells are insensitive to the Wg signal. Transfection with *fz2* conferred pathway activity and thus demonstrated that *fz2* encodes a Wg receptor.[49] In addition, overexpression of Fz-family proteins resulted in phenotypes similar to ectopic Wg signaling.[84,85] Three years later then, firm genetic data was obtained demonstrating that only when the function of both genes, *fz* and *fz2*, are abolished, Wg is no longer transduced *in vivo*.[50,86] With the exception of Sgg, all negatively acting components were first discovered in systems other than *Drosophila*, perhaps because the setups in modifier screens favors the identification of suppressors (positively acting components) and not enhancers, which often unspecifically aggravate the initial phenotype and are thus difficult to incontestably score. Hence the negative component APC was first associated in clinical research with heritable colorectal cancers and later shown to interact with Arm by being part of the destruction complex.[9-11] APC was missed by most *Drosophila* screens, perhaps because of the special nature of the 2 Drosophila APC homologs: While Wg signaling is essential for embryonic development, APC function is confined to the central nervous system.[87] Only the above mentioned screen aimed at negative Wg components in the Bejsovec lab identified APC2 as a Wnt/Wg signaling component.[82] Axin is another component of the destruction complex, which was first described in a Wnt-unrelated context in the mouse.[56] cDNA microinjection experiments in *Xenopus*, an excellent vertebrate model for gain-of-function phenotypes, linked this protein then to Wnt/Wg signaling.[61,88,89] In large scale screens, this system led to the discovery of Wnt-7b, Wnt-10, β-catenin[90] and the Wnt-inhibitor Dickkopf.[91] Since it is ideally suited to assay for gain-of-function activities, this screening system does not suffer from the redundancy issue.

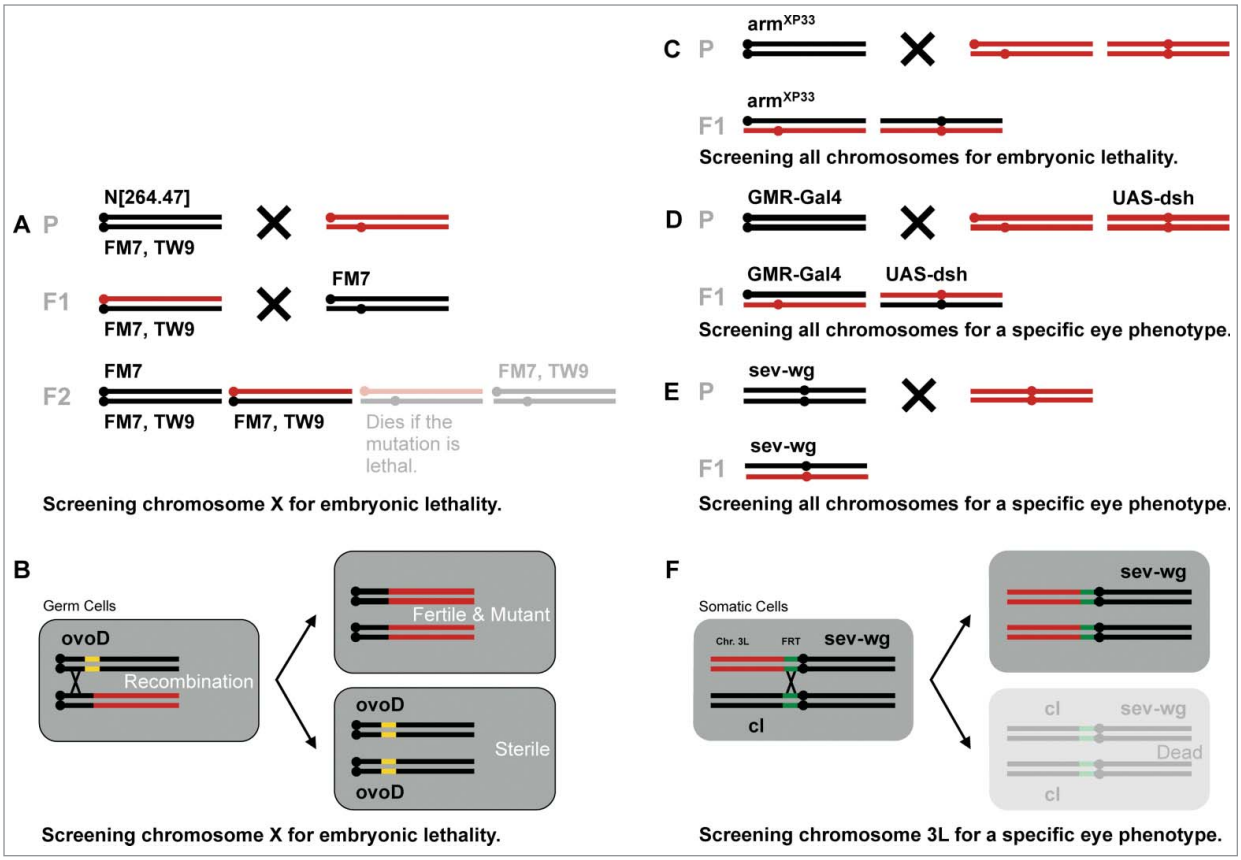

Figure 2. Genetics of Drosophila screens for Wnt/Wg signaling components. (**A**) The first patterning screens were performed by Nüsslein-Volhard and Wieschaus.[29,34] Flies were mutagenized and lines with interesting candidates were established. Here we show the crossing schemes for the isolation of X-linked lethal mutations. (**B**) Perrimon adapted these first screens for zygotic lethals and removed maternal contributions using the *ovoD* system, which relies on a female-sterile mutation and mitotic recombination by X irradiation. (**C**) The Bejsovec lab sensitized the genetic background with a hypomorphic *arm* allele, which was more susceptible for negative components, such as Pan/Gro and APC2 and (**D**) the Nusse group identified *Axin* in a *dsh* overexpression screen. (**E and F**) Our lab has carried out suppressor screens using *wg* mis-expression, induced by the activity of the *sev* enhancer. (**E**) A dominant suppressor screen for suppressors of the *sev-wg* phenotype yielded *pan*, *lgs* and *pygo*. (**F**) This setting was further developed for recessive suppressor screens based on Flp-induced recombination. In this screen *wls* was discovered. The remaining chromosome arms are screened with an improved method, where the *wg* transgene carries a flp-out cassette, which is removed in the eye by *ey-Flp* (*eyeless* promoter driven Flipase). The corresponding tester lines carry an FRT site as well as a cell lethal (*cl*) allele. Marked in red are the mutagenized chromosomes.

Perspectives and Conclusions

At present, we can assume that most of the core components of Wnt/Wg signaling are discovered, so we may ask, whether remaining questions, such as tissue specificity and pathway modulation, can be answered by conventional *Drosophila* research or whether new technologies and or different model systems are needed.

RNA interference screens are already commonly used and contributed to the identification of factors influencing Wnt/Wg signaling.[92,93] The method using clustered regularly inter-spaced short palindromic repeats (CRISPR) with the protein Cas9 could herald the start of a new age of knock-out screens in *Drosophila*. While both approaches allow systematic screens, they are limited in one aspect, compared to classical mutagenesis screens: Knockdowns by RNAi mimic hypomorphic alleles and CRISPR/Cas9 knockouts might yield most of the time null alleles (due to small deletions and frame shifts). Screens based on EMS mutagenesis, however, can lead to a wide spectrum of alleles, from hypomorphs, to dominant negatives, as well as null alleles. Missense mutations destroying functional protein domains can even identify proteins with multiple functions and help putting them into the right context. This becomes even more relevant when one of these functions is cell essential and a complete null would cause cell death. Classical mutagenesis screens have been performed since the early 1980s and millions of flies were screened ever since. So the question arises: have we reached saturation? Modifier screen setups with different sensitized backgrounds in combination with new genetic tools are still fruitfully ongoing in several labs. Hence chemical mutagenesis screens should not yet be considered experimental relics. These approaches might also be helpful, when focusing on Wnt/Wg signaling in specific tissues identifying context specific regulators.

An interesting new approach might come from population genetics: So far scientists used reductionist approaches to investigate signaling pathways. However these do not live up to the complexity of cell-to-cell communication and cross talk between

pathways. The McKay lab has created a resource of 192 inbred and sequenced lines.[94] They can be used as a resource for systems genetics and might reveal quantitative trait loci (QTL) influencing Wnt/Wg signaling. Even a directed evolution approach would be possible: these inbred lines could be crossed with one another, and in every generation one could select for Wnt/Wg specific features. A sensitized background (e.g., *sev-wg*, as in our screens) could facilitate the process of selecting for a specific trait at every generation. Isolation of QTLs, with or without prior selection, will likely also reveal components that do not have a Wnt-pathway-dedicated function.

It is foreseeable therefore, that a combination of classical and new tools in *Drosophila*, but also in other model organisms, will help us to shed further light on this intricate pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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