Powerful *Drosophila* screens that paved the wingless pathway

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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 and in the development of adult structures from imaginal disc primordia; this includes leg, wing, genitalia, antennae, and eye imaginal discs. 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.

The Wnt/Wg signal acts either via calcium signaling, by triggering the planar cell polarity pathway 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).

### 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. 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*. 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 *rho* retrotransposon, and Sharma’s *wg* allele is the result of a 2.5kb deletion, downstream of the locus, which...
presumably contains a regulatory element involved in controlling $wg$ expression during wing development. Other known mutations, like Sternopleural ($Sp$), spade and flag, 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, which laid the foundation for their Nobel Prize.

**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.

### 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) | TCF 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 gene 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 
ug in Drosophila showed a relevance of this pathway in fly development,
whereas the characterization of the murine
Int-1 gene implied a role in oncogenesis. A role in vertebrate development became evi-
dent when ectopic expression of Wnts was observed to cause axis
duplications in Xenopus embryos.33 These factors, their impor-
tance in development and oncogenesis, as well as the high degree
of conservation between different species, were the underpin-
nings 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 
ug 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 dis-
covery that 
Wg stabilizes 
engrailed (en) expression in embryonic
segmentation44 enabled researchers to add roles, functions and
relationships between these genes. The Perrimon lab studied the
effect of 
ug 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 can-
cer. 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 
ug transgene were
started in our laboratory and yielded nuclear signaling compo-
ments. Brunner et al. described the in vivo role of the Drosophila

pan gene in a suppressor screen.51 It encodes a homolog of verte-
brate LEF-1, which can, as a transgene, substitute the Pan func-
tion.52 It was found to interact with the 
Wnt/Wg signaling
component 
β-catenin in a yeast 2-hybrid assay53 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 develop-
mental effects, including axis duplications56; 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 essen-
tial for proper segmentation in the Drosophila embryo and null-
mutants (upon removing also the maternal contribution) were
indistinguishable from 
ug mutants.

Events in the nucleus

Functional analyses in Drosophila, Caenorhabditis elegans and
Xenopus indicated that Pan/TCF/LEF might also act as a tran-
scriptional 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 (lg), pygopus (pyg) and
hryax (hry). Lgs recruits Pygo to the Pan/TCF/LEF complex and
together with Hry 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 Hry 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),
Wieschaus, identified the first components of this signaling cas-
the segmental pattern, like the one from N
romer complex, Wls is degraded and the Wg pathway is
Wls protein recycling: In the absence of components of the Ret-
first in
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).

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 ugg null alleles.

As the embryo only gradually runs out of maternal product, the analysis of their early zygotic phenotypes is difficult. Among Wnt/Wg signaling genes, tgg, 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.

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 *wgg* 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 *wgg* transgene under the control of the eye-specific *sevenless* enhancer has proven valuable for a genome-wide screen for dominant suppressors, and led to the identification of *pan*, *leg* and *pygo* (Fig. 2E).

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. A further improved, inducible version of this screen, taking advantage of a conditional *seg-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.

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).

**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.

In addition, overexpression of Fz-family proteins resulted in phenotypes similar to ectopic Wg signaling.

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*. 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 uncontrollably 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.

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. Only the above mentioned screen aimed at negative Wg components in the Bejsovec lab identified APC2 as a Wnt/Wg signaling component. Axin is another component of the destruction complex, which was first described in a Wnt-unrelated context in the mouse. cDNA microinjection experiments in Xenopus, an excellent vertebrate model for gain-of-function phenotypes, linked this protein then to Wnt/Wg signaling. In large scale screens, this system led to the discovery of Wnt-7b, Wnt-10, beta-catenin and the Wnt-inhibitor Dickkopf. Since it is ideally suited to assay for gain-of-function activities, this screening system does not suffer from the redundancy issue.

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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 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, 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 over-expression 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 flip-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.

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 over-expression 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 flip-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.
pathways. The McKay lab has created a resource of 192 inbred and sequenced lines. 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-arg, 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.

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No potential conflicts of interest were disclosed.

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