



Published in final edited form as:

Dev Biol. 2022 May ; 485: 93–122. doi:10.1016/j.ydbio.2022.02.013.

The great small organisms of developmental genetics: *Caenorhabditis elegans* and *Drosophila melanogaster*

Judith Kimble^{a,**}, Christiane Nüsslein-Volhard^{b,*}

^aUniversity of Wisconsin-Madison, Department of Biochemistry, USA

^bMax-Planck-Institute for Biology Tübingen, Germany

Abstract

Experimental embryologists working at the turn of the 19th century suggested fundamental mechanisms of development, such as localized cytoplasmic determinants and tissue induction. However, the molecular basis underlying these processes proved intractable for a long time, despite concerted efforts in many developmental systems to isolate factors with a biological role. That road block was overcome by combining developmental biology with genetics. This powerful approach used unbiased genome-wide screens to isolate mutants with developmental defects and to thereby identify genes encoding key determinants and regulatory pathways that govern development. Two small invertebrates were the pioneers: the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*. Their modes of development differ in many ways, but the two together led the way to unraveling the molecular mechanisms of many fundamental developmental processes. The discovery of the grand homologies between key players in development throughout the animal kingdom underscored the usefulness of studying these small invertebrate models for animal development and even human disease. We describe developmental genetics in *Drosophila* and *C. elegans* up to the rise of genomics at the beginning of the 21st Century. Finally, we discuss themes that emerge from the histories of such distinct organisms and prospects of this approach for the future.

Keywords

Genetic screens; Mutagenesis; Embryogenesis; Morphogen; Pattern formation

1. Introduction

At the beginning of the 20th century, shortly after the rediscovery of the Mendelian laws, the German biologist Theodor Boveri performed a ground breaking experiment. By double fertilisation of sea urchin eggs, he demonstrated that the loss of individual chromosomes during development caused specific defects in particular differentiation pathways (Boveri, 1902). This first experiment in developmental genetics showed that the abstract Mendelian factors, subsequently referred to as genes, were associated with individual chromosomes.

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*Corresponding author. cnv@tuebingen.mpg.de (C. Nüsslein-Volhard). **Corresponding author. jekimble@wisc.edu (J. Kimble).

Cytological studies of invertebrates, including cleavage in a parasitic nematode and meiosis in the grasshopper, complemented the earlier study in sea urchins and provided the foundation for the chromosomal theory of inheritance (Sutton, 1903).

Based on observations of the transparent eggs of sea urchins, ascidians and nematodes Boveri suggested that initial cell decisions might depend on a graded distribution of some substance in the egg, such that different amounts of that substance would be included in the different cells formed through cleavage. The central idea was that spatial patterns exist as polar distributions of morphogenetic substances from the earliest stages, that these patterns are simple, and that genes on chromosomes act subsequently to build patterns in the final organism. The discovery of the organizer region in the newt embryo by Boveri's student Hans Spemann provided evidence for tissue induction in the amphibian embryo (Spemann and Mangold, 1924). These observations generated great excitement but were followed by years of frustrating attempts to purify factors involved in embryonic induction.

In the first half of the 20th century, *Drosophila* joined peas and maize to lay the foundation for modern genetics of multicellular organisms. *Drosophila* was originally chosen by Thomas H. Morgan as an experimental animal particularly well-suited for genetic analysis because of its rich external morphology and mutability (Morgan, 1910). *Drosophila* studies revealed the linear order of genes on chromosomes and rules of recombination, principles that apply to all organisms. Chromosomes were identified as "linkage groups", and genetic maps were constructed from recombination frequencies between linked mutations (Sturtevant, 1913). Maps could be confirmed using polytene chromosomes, whose banding patterns helped to make gene locations a physical reality long before sequencing (Bridges, 1935; Painter, 1933) (Fig. 16). The discovery that X-rays and chemicals increased the rate of mutations was crucial for generating mutants with a wide variety of phenotypes (Auerbach and Robson, 1946; Muller, 1927). In 1933 Thomas Hunt Morgan was awarded the Nobel prize for his discoveries concerning the role played by chromosomes in heredity, and in 1946 Hermann Joseph Muller for the discovery of the production of mutations by means of X-ray irradiation. The systematic construction of chromosomal deletions, translocations and duplications was essential for genetic manipulations and also for the logic used to deduce the nature of mutations. For example, if a mutation behaves genetically like a deletion, that mutation must cause a loss of gene function. Deletions covering most of the fly genome showed that most loci are able to function enough for animal survival with just one copy, a conclusion that probably holds for all multicellular organisms (Lindsley et al., 1972).

Discovery of the DNA double helix (Watson and Crick, 1953) led to the emergence of molecular biology. This new field combined biochemistry with genetics, and molecular biology with *Escherichia coli* and its bacteriophages, conducted during the 1950's and 60's, unraveled fundamental mechanisms such as the genetic code, transcription, translation, protein synthesis and DNA replication. This new field also transformed thinking of what strategy was most powerful for solving complex problems of biological regulation. In bacteria, the identification of mutations in the *cis*- and *trans*-acting regulatory elements of genes played a crucial role in the biochemical isolation of transcriptional repressors (Jacob and Monod, 1961). Although not appreciated at the time by most developmental biologists, genetic methods would ultimately provide a way to interfere specifically with

one molecular element in a developing organism without causing the gross disturbances of physical manipulation. A mutation allows one to eliminate a single component in a complex system while leaving everything else intact. Systematic mutant screens were instrumental in identifying members of biochemical pathways in bacteria and fungi (Beadle and Tatum, 1941). Discovery of key players in the control of the cell cycle by systematic mutant screens in yeast provided another example of the powers of genetic analysis (Hartwell et al., 1970; Nurse, 1975). A systematic screen for mutants defective in DNA replication by Friedrich Bonhoeffer in Tübingen identified *E. coli* DNA polymerase 3 as the replicating enzyme and demoted the Kornberg DNA polymerase 1 to a repair enzyme (Gefter et al., 1971). This last experiment convinced one of us that genetics is indispensable to understand development.

In the 1960's, some of molecular biology's most visionary pioneers decided to turn that power to understanding animal development and behavior. The animal model most amenable to genetics at the time was *Drosophila*, and several notable bacterial geneticists did indeed move to flies. For example, Alan Garen screened for mutants in *Drosophila* development (Rice and Garen, 1975), and Seymour Benzer began collecting behavioral mutants in *Drosophila* (Benzer, 1967). Alfred Gierer selected Hydra for its amazing powers of regeneration (Gierer et al., 1972). Other notable molecular biologists instead experimented with less established or novel systems. For example, Francois Jacob began work on mouse embryology to unravel developmental regulation that might be more relevant to humans; William Dove selected the slime mold *Physarum polycephalum* to tease apart cellular mechanisms of development (Haugli and Dove, 1972); Max Delbrück chose the fungus *Phycomyces* for its challenging light sensitivity (Bergman et al., 1969) and George Streisinger launched the zebrafish to study behavior (Streisinger et al., 1981). Sydney Brenner, however, searched for "an experimental organism which was suitable for genetical study and in which one could determine the complete structure of the nervous system". After testing several possibilities, Brenner settled on the nematode *Caenorhabditis elegans* – a tiny animal with a constellation of advantages for genetics (Brenner, 1974). Brenner was not the first to use *C. elegans* for genetics (see (Nigon and Felix, 2017), for a review of pre-Brenner genetic studies), but he was the first to do so with a mind honed by bacterial and phage molecular genetics.

In this review, we describe the developmental genetics of *C.elegans* and *Drosophila melanogaster*, and the advances made using this approach. Although the genetic logic is similar for both organisms, their many differences dictate separate sections to explain how mutants are generated and analysed. The worm and fly sections are organized differently, with background to genetics, molecular biology and development coming before the screens for worms but interspersed for flies. *Drosophila* developmental systems are explained in a separate Box. Each organism-specific section concludes with an "impact and outlook" discussion, and the review concludes with a more general discussion. We note that our coverage is not comprehensive and apologize to those whose work was not covered.

1.1. Developmental genetics in *Caenorhabditis elegans*

Sydney Brenner chose *C. elegans* for its strong genetics and cellular simplicity. However, with time, other advantages emerged. Its invariant lineage offered an unprecedented cell by

cell and dynamic window into development, and its small genome size facilitated molecular analyses, including completion of its entire DNA sequence. In this section, we first provide background on *C. elegans* genetics and molecular biology, its anatomy, development and cell interactions. We then describe several landmark examples of *C. elegans* developmental genetics, roughly in historical order. The first of course is Brenner's *tour de force* screen that established modern *C. elegans* genetics. Others were chosen to illustrate how key regulatory genes and pathways were identified and analysed. They are not meant to be comprehensive, but instead to touch upon the spectrum of developmental events queried with this tiny worm. We end with a brief discussion of the broader impact of *C. elegans* efforts to understand developmental mechanisms and its outlook moving forward.

2. Background

2.1. Basics of *C. elegans* genetics and molecular biology

C. elegans is a free-living non-parasitic nematode. Outside the laboratory, it typically lives in rotting fruits and vegetables though it is often called a soil nematode. The genetic power of *C. elegans* comes from its dual mode of reproduction, easy maintenance, rapid life cycle and large number of progeny. Like many animals, *C. elegans* has two sexes that mate to produce the next generation. However, one sex is special. Self-fertilizing hermaphrodites make oocytes as an adult, like a female, but they produce and store sperm before adulthood. They can therefore make self-progeny using their own sperm or cross-progeny by mating with males (Fig. 1). This capacity for self-fertilization circumvents the need for mating to reproduce, a feature that adds greatly to the power of genetic screens. But males are available for transfer of genetic information from one animal to another, which is essential for genetic analyses. Worms have five autosomes and one X chromosome; there is no Y chromosome. Sex is determined by the ratio of X chromosomes to autosomes so XX animals are hermaphrodite and XO animals are male.

C. elegans ease of maintenance is a major advantage for large scale genetic screens. Animals are tiny, ~1 mm long as an adult, and a single hermaphrodite produces ~300 self-progeny, more than enough for most purposes. They are fed a diet of *E. coli* and raised on small agar-filled Petri plates. Its life cycle of about three days from fertilization to adult quickens the pace of genetic manipulations (Fig. 2), and its short life span of about two weeks is a real benefit for aging studies. Strains are rarely lost, because larvae enter a spore-like "dauer" stage when starved, and worms can be frozen at -80° for long term storage.

The traditional bread and butter of developmental genetics is the isolation and characterization of mutants to learn how genes regulate development. The *C. elegans* genetic map began with ~100 genes scattered across all chromosomes (Brenner, 1974), and many, many more were added over the years. A mutant defect reveals a gene's biological function, and genetics analyses of mutants provide critical information about gene activity. For example, loss of function mutants lower gene activity and can be either strong mutants with no activity or weaker mutants with less activity than normal. Temperature sensitive mutants can turn a gene's activity on and off with a simple shift from one temperature to another and thereby reveal when the gene is acting, and genetic mosaics show where within

the animals the gene is acting. Genetic analyses are therefore extremely important, but one must isolate a gene's DNA to investigate its molecular function.

The small *C. elegans* genome size of only ~100 Mb (Sulston and Brenner, 1974) facilitated construction of a physical map, which is critical for most gene cloning. The first physical map was built with contiguous groups of cosmids, or contigs, and yeast artificial chromosomes to fill gaps between contigs (Coulson et al., 1986, 1988). However, sequencing the entire genome generated the ultimate physical map (Consortium, 1998) and led the way to bioinformatic and genomic analyses, which now dominate biological and biomedical research. The detailed and ongoing annotation of the *C. elegans* genome has been essential and provides wide access to its many features (<https://wormbase.org>).

As the genetic map grew and the physical map was being assembled, a few genes encoding highly conserved proteins provided anchors between the two maps (MacLeod et al., 1977). "Transposon tagging" was another early method to recognize DNA associated with a specific gene (Eide and Anderson, 1985), but today, whole genome sequencing is the favored approach (Sarin et al., 2008). To introduce DNAs or RNAs into embryos, they are microinjected into the adult gonad for delivery into multiple oocytes and thus into embryos (Fig. 3) (Mello et al., 1991). Early transgenes were carried on complex extrachromosomal arrays, but today, single copy transgenes are inserted into designer spots in the genome (Frøkjær-Jensen et al., 2008).

Forward genetics progresses from mutant phenotype to gene DNA, while reverse genetics moves the other way, from molecule to phenotype. The discovery of RNA interference, often called RNAi, was transformative for reverse genetics in *C. elegans* and a host of other organisms (Fire et al., 1998). This method delivers double stranded RNAs into the worm, and triggers degradation of mRNAs with a corresponding sequence. RNAs are delivered by feeding worms *E. coli* that carry an RNA-producing plasmid, a simple method that led to construction of an *E. coli* library with RNAs covering nearly the entire genome (Kamath and Ahringer, 2003). This library enabled genome-wide RNAi screens, with molecular identity of the gene immediately available upon detection of phenotype. By the turn of the 20th century the *C. elegans* toolkit was both broad and deep, including powerful methods for both forward and reverse genetics coupled with a complete genome sequence.

2.2. A dynamic cell by cell map of development

C. elegans development is unusually accessible because of its simple anatomy, small cell number, transparency and cell by cell developmental map. Its bilaterian body plan is much like other animals but simpler than most. The body wall has two layers: an outer "hypodermis", that secretes a cuticle, and an inner muscle layer; the body cavity houses two major internal organs, an intestine or gut and a gonad. A neuromuscular pharynx in the head pumps food into the gut, and an anterior nerve ring or "brain" extends axons into the nerve cord that runs the length of the animal. Sperm fertilize oocytes inside the mother and early embryonic cleavages begin in the uterus. The developing embryo, protected by its eggshell, is then propelled through the vulva, a specialized opening in the body wall, into the external world. The two sexes look similar superficially, but are distinguishable by both morphology and behavior. The vulva is a hermaphrodite-specific structure, and the male tail

is specialized for mating; in addition, the hermaphrodite and male gonads have sex-specific somatic structures and different numbers of elongate arms of germline tissue. Details are readily available online (<https://wormatlas.org>).

The early embryonic lineage of *Parascaris*, a parasitic nematode, was watched many years ago and found to be invariant from embryo to embryo (Boveri, 1899). After a ~75-year hiatus, lineage studies began in *C. elegans*. *C. elegans* is transparent throughout its life cycle and small enough to fit on a microscope slide to view its cells in living and developing animals with microscopy at a magnification of 1000X. Nomarski differential interference contrast (DIC) optics allows visualization of cells and their subcellular components, such as nuclei, nucleoli and mitotic spindles. John Sulston devised a simple chamber to keep a young L1 worm alive on a slide, munching on food, while its cells were watched at high power. He quickly found that cells do indeed divide in larvae and that their divisions are reproducible from animal to animal (Sulston, 1976). Sulston and colleagues then pieced together the program of cell divisions, cell migrations, cell differentiation and tissue morphogenetic changes from zygote to the adult, simply by watching cells in real time during development (Deppe et al., 1978; Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston et al., 1983). That effort generated a dynamic cell by cell map of development (Fig. 4).

The *C. elegans* cell lineage allowed fates to be assigned to each and every somatic cell throughout development. In the early embryo, four sequential asymmetric divisions generate somatic blastomeres (AB, EMS, C and D) and germline or “P” blastomeres (P1 to P4) (Fig. 4A). At each division, cytoplasmic determinants called P granules segregate to the germline blastomere (Strome and Wood, 1982). The embryonic blastomeres continue dividing to establish the major tissues (Fig. 4B; the newly hatched L1 larva contains 558 cells. Cell divisions in larvae then expand that number to 959 cells in adults (Fig. 4C). The larval lineages support tissue growth and also generate reproductive organs (gonad, vulva). The general lineage rule is that each cell divides with essentially the same timing and spindle orientation in every animal and that this reproducibility generates daughter cells at the same positions and with the same fates.

The primary exception is the germline. The P4 germline blastomere and its two daughters are born in the embryo. In larvae and adults, germline divisions vary with respect to timing, spindle orientation and daughter position. Those germline divisions produce ~2000 descendants, with a pool of germline stem cells continuing variable divisions in adults to replenish the tissue as gametes are made. The pattern of germline fates is highly reproducible: stem cells at one end of the elongate tissue, maturing meiotic cells extending along the axis and differentiating gametes at the other end. *C. elegans* development therefore employs an invariant lineage to generate 959 somatic cells and a regulative mode to generate the germline pattern.

2.3. Cell interactions that regulate somatic and germline fates

The invariant lineage produces cells with both the same ancestry and the same position. To explore the idea that cell interactions influence cell fates, John White engineered a way to focus a laser microbeam through a microscope lens and ablate individual cells during

development. If the remaining cells change their fate, an interaction could be inferred. Three different types of cell interaction were found: niche regulation of stem cells, induction of the vulva and lateral signaling (Fig. 5) (Kimble, 1981; Kimble and White, 1981; Sulston and White, 1980). In each case, the cell interaction occurs between well-defined cells.

For niche regulation, signaling from a single cell, the somatic “distal tip cell”, is required to maintain germline stem cells. This regulatory cell caps the end of the gonad in larvae and adults while adjacent stem cells divide to expand the germline tissue in larvae and maintain it in adults (Fig. 5A). When the distal tip cell is killed at any stage, germline stem cells stop self-renewing and differentiate. The distal tip cell therefore functions continuously as a stem cell niche, a concept proposed earlier without a concrete example (Schofield, 1978).

For vulval induction, signaling from a different regulatory cell, the gonadal “anchor cell”, induces a vulva in the body wall. The anchor cell normally perches above the developing vulva (Fig. 5B), and its ablation eliminates vulva formation. Vulva induction occurs over a short interval in the early L3 stage and provides a genetically tractable model for analyzing the control of tissue induction, as first seen in vertebrates (Spemann and Mangold, 1924).

For lateral signaling, signaling between equivalent progenitor cells cause them to adopt distinct fates. Indeed, breaking symmetry between equivalent progenitors turns out to be a common theme in development of all animals. Although multiple cases occur in *C. elegans*, two examples suffice. One simple example involves two equivalent precursors in the somatic gonad that randomly become either an anchor cell or continue to divide and make part of the uterus. When either is killed, the remaining precursor always becomes an anchor cell. The anchor cell therefore normally signals its neighbor, inhibits it from taking on the “primary” anchor cell fate and channels it instead into a “secondary” uterine fate. A more complex example occurs among six equivalent vulval precursor cells. Here the precursor receiving the strongest anchor cell signal adopts a “primary” vulval fate, while lateral signaling from the primary cell causes its immediate neighbors to adopt a “secondary” vulval fate. These well-defined cases of lateral signaling became paradigms for understanding the molecular basis of lateral inhibition, a concept proposed to explain the pattern of bristle spacing in insects (Wigglesworth, 1940).

Identification of cell interactions in larvae was soon followed by discovery of cell interactions in the early embryo (Fig. 5C). Most experiments either isolated embryonic blastomeres away from each other in a dish or pushed them into new places within the embryo. Here we highlight two interactions in four cell embryos, which consist of two daughters of the AB blastomere plus the P2 and EMS blastomeres (Fig. 5C). First, the AB daughters are born with equivalent potential, but P2 signals to the posterior AB daughter and breaks the equivalence of ABa and ABp, so that only one AB daughter produces pharyngeal muscle (Priess and Thomson, 1987). Second, EMS normally divides asymmetrically to make two different daughters, a mesoderm-producing anterior daughter, called MS, and a gut-producing posterior daughter, called E. When EMS is isolated, it divides symmetrically and both daughters become MS-like, but when P2 is placed next to the isolated EMS, its asymmetry is regained. Therefore, P2 signaling induces EMS to divide asymmetrically (Goldstein, 1992). This second induction relies on a divergent and likely nematode-specific

Wnt pathway that controls many and perhaps most of the branches of the fixed somatic lineage (Hardin and King, 2008).

2.4. Brenner establishes modern *C. elegans* genetics

Sydney Brenner conducted the first large scale systematic genetic screen in *C. elegans* (Brenner, 1974). He was interested in both development and behavior, and for the most part chose mutants that did not move normally, the “uncoordinated” (Unc) phenotype. In addition, he isolated mutants with various changes to body shape. Brenner only kept fertile mutants that could be maintained as homozygotes. Initially, he mutagenized hermaphrodites with ethyl-methane-sulfonate and screened for defects in their self-progeny, called Filial generation 1 (F1), or in the subsequent F2 self-progeny. Mutants were only found in the F2 generation, because recessive mutations reveal their phenotype only after two generations when they become homozygous. However, the number of progeny expanded so quickly in those early screens that scoring F2s was difficult. Brenner therefore changed his strategy and placed single F1s from a mutagenized parent onto a plate to screen for defects among its F2 self-progeny. These “F1 clonal screens” became the standard way to screen for mutants in *C. elegans* (Fig. 6).

Brenner isolated around 300 mutants in this screen. The frequency of mutations in any given gene was on average ~ 1/2000 haploid genomes or 1/1000 F1 clones (each F1 carries two sets of mutagenized haploid genomes). Once a mutation was found, its chromosomal location was determined with genetic crosses. A cross with wild-type males gave the first inkling of chromosomal linkage. Male cross-progeny receive their single X chromosome from the mutant mother so if a male appeared mutant, the gene was X-linked. Additional genetic crosses assigned autosomal genes to specific linkage groups and used recombination between genes on the same chromosome to determine their positions. If two mutations had the same phenotype and mapped to roughly the same place in the genome, they were placed in *trans* (one on each chromosome) to test for complementation. If the mutations were alleles of the same gene, the *trans* heterozygote would appear mutant; however, if they belonged to different genes, it would appear normal, or “wild-type” in genetic parlance. Genes were distributed across all five autosomes and the X chromosome. This remarkable effort founded *C. elegans* as a model and established the first genetic map.

2.5. Sex determination: discovery of a broadly conserved male-promoting gene

Hermaphrodites and males have the same basic body plan, but they are different animals with sex-specific morphologies and behaviors. To identify genes governing development as hermaphrodite or male, Jonathan Hodgkin screened for mutations that transform XX animals into males or XO animals into hermaphrodites (Hodgkin, 1980; Hodgkin and Brenner, 1977). These early screens identified four genes: *tra-1*, *tra-2*, *tra-3* and *her-1*, whose genetic analysis provides a stellar example of the logic used to understand gene functions and relationships in any organism (Fig. 7).

Recessive mutations typically reduce or eliminate gene activity, and are therefore “loss of function” or “lf” mutations. The *tra-1(lf)* mutants transform XX animals into males. Therefore, the wild-type or normal function of *tra-1* must be to promote hermaphrodite

development. By contrast, *her-1(lf)* mutants transform XO animals into hermaphrodites so wild-type *her-1* promotes male development. With mutants in different genes having opposite phenotypes, the phenotype of the double mutant can determine which gene acts first in a regulatory pathway. This approach is sometimes called “epistasis analysis”. Will *tra-1(lf); her-1(lf)* double mutants be male like *tra-1(lf)* or hermaphrodite like *her-1(lf)*? In regulatory pathways, later-acting genes are epistatic to those acting earlier. Thus, if *tra-1* acts first in the pathway, the *her-1* defect will predominate and vice versa. The result was clear: the double mutants were male, the *tra-1* phenotype. Therefore, *tra-1* acts downstream of *her-1* in the pathway. Similar tests placed *tra-2* and *tra-3* downstream of *her-1* as well. But ordering the *tra* genes with respect to each other was not possible with loss of function mutants, because all three masculinized XX animals.

Dominant mutations are rarer than recessive mutations, and typically increase gene activity or change its expression. These “gain of function” mutations often have a phenotype opposite that of recessive mutations. For example, *tra-1(lf)* masculinizes XX animals, but *tra-1(gf)* feminizes XO animals, consistent with abnormal expression of *tra-1* hermaphrodite-promoting activity. In double mutant experiments, *tra-1(gf)* feminization prevailed over *tra-2(lf)* and *tra-3(lf)* masculinization, suggesting *tra-1* acts at the end of the pathway. The *tra-1* gene, when cloned, turned out to be homologous to transcription factors previously identified in flies and mammals, *cubitus interruptus (ci)* and *GLI* respectively (Zarkower and Hodgkin, 1992). *ci* and *GLI* are the terminal regulators of the fly and human hedgehog signaling pathways. The *tra-1* gene was therefore a remnant of that ancient pathway. Indeed, *tra-2* encodes a divergent homolog of *patched*, which acts upstream of *ci/GLI* in the hedgehog pathway. Thus, a divergent hedgehog pathway regulates sex determination in worms.

Later screens identified more sex determination genes. Most important here is *mab-3*, which acts downstream of *tra-1* (Shen and Hodgkin, 1988). The wild-type *mab-3* function is to masculinize the gut and neural sensory organs. Up to this point, the worm and fly sex determination pathways had no molecular similarity, and the consensus opinion had become that the regulation of sex determination must have evolved separately in evolution. It was a big surprise therefore when *mab-3* turned out to be homologous to the *doublesex* gene that acts at the end of the sex determination pathway in flies: both encode DM-domain transcription factors (DM for *doublesex* in flies and *mab-3* in worms) (Raymond et al., 1998). Once this homology was discovered, the hunt was on and DM-proteins were found to promote male fates across the animal kingdom, including mice and likely humans (Matson and Zarkower, 2012). The parallel efforts to understand sex determination in worms and flies were thus synergistic and led to discovery of a broadly conserved genetic regulator of sex determination.

2.6. Aging: discovery of a broadly conserved regulatory pathway

Two genetic screens converged on the discovery of conserved regulators of aging. One large scale screen focused on regulation of the alternative *C. elegans* life cycles (Riddle et al., 1981) (Fig. 2). Dauer larvae, which form upon starvation or overcrowding, are thinner and denser than well-fed larvae, survive for months and are resilient to chemicals that kill other

larvae. To identify genes governing entry into the dauer life cycle stage, Riddle screened for mutants and also selected for survivors of SDS (sodium dodecyl sulfate) treatment, one of the first genetic selections done in worms. The resultant *daf* mutants (for *abnormal dauer formation*) fell into two classes. “Dauer constitutive” mutants become dauers even when well-fed, while “dauer defective” mutants cannot become dauers when starved. A smaller screen hunted for mutants with an extended life span, an assay considerably more difficult than the screen for dauer defective mutants. This smaller screen however did find the *age-1* gene (Friedman and Johnson, 1988).

A decade after isolation of the *daf* mutants, Cynthia Kenyon asked a new question: Do dauer constitutive mutants increase life span, in addition to promoting entry into the long-lived dauer stage? Remarkably, *daf-2* mutants doubled the life span and that doubling was blocked by introducing a *daf-16* mutation (Kenyon et al., 1993). Molecular cloning revealed that *daf-2*, *age-1* and *daf-16* encode components of an insulin-like pathway: *daf-2* encodes an insulin receptor, *age-1* encodes a phosphatidylinositol-3-OH kinase (PI3 kinase) and *daf-16* encodes a FOXO-like transcription factor that acts downstream of both the insulin receptor and the PI3 kinase (reviewed in (Guarente and Kenyon, 2000)). The startling conclusion was that lowering the activity of a conserved signaling pathway, the insulin pathway, extends life span. Even before molecular identities were known, the notion that mutation of a single gene could extend life span was a revelation to the aging field. Once the insulin pathway emerged as important in worms, its role in aging was validated with mutants in flies and mice and supported in humans by association of *daf-16/FOXO3A* with longevity (reviewed in (Campisi et al., 2019)). The *C. elegans daf* mutants and *age-1* thus led to discovery of a conserved genetic pathway that regulates aging with major implications for human health.

2.7. Lineage regulation and discovery of miRNAs

Genetic screens for lineage defective (“*lin*”) mutants began as soon as the first post-embryonic lineages were traced. The focus was initially broad, but development of the vulva emerged as particularly tractable (Horvitz and Sulston, 1980). A mother with a defective vulva cannot lay her eggs, and the resultant larvae hatch inside and kill her; the inert carcass full of wriggling larvae becomes a “bag of worms”, which is easily visible among siblings that move normally. This screen was a gold mine and led to identification of many developmental regulators, including members of key signaling pathways. For example, the screen for genes responsible for anchor cell induction of the vulva identified the conserved EGF signaling pathway, including ligand, receptor, *ras*, *raf* and ERK/MAP kinase in addition to other pathway components (e.g. Horvitz and Sternberg, 1991; Sternberg and Han, 1998). This discovery forged a conserved and clinically crucial link between receptor tyrosine kinases and their downstream effectors.

Here we focus on another gene, *lin-4*, that led to discovery of miRNAs. The *lin-4* mutants reiterated certain lineages, a remarkable phenotype reminiscent of *Drosophila* homeotic mutants (Fig. 8A) (Chalfie et al., 1981). The mutants had other defects as well. Most importantly, *lin-4* mutants had six larval stages instead of the normal four. A focused screen for mutants with extra larval stages next identified the *lin-14* gene (Ambros and Horvitz, 1984). The various defects of *lin-4* and *lin-14* mutants had in common major

changes in the temporal progression of developmental events, a so-called “heterochronic” effect. Lateral hypodermal precursors, for example, reiterate L1 fates in *lin-4* and *lin-14(gf)* mutants, but those precursors skip L1 fates in *lin-14(lf)* mutants (Fig. 8A). A *lin-4; lin-14(lf)* double mutant also skips L1 fates, indicating that *lin-4* negatively regulates *lin-14* (Fig. 8B) (Ambros, 1989). The *lin-14(gf)* mutations therefore likely release the gene from negative regulation.

Cloning the *lin-14* gene revealed that its gain-of-function mutations were lesions in the 3′ untranslated region (Wightman et al., 1991). This was unexpected, because few such mutations had been seen before. Cloning the *lin-4* gene revealed an even bigger surprise – a tiny non-coding RNA of only 22 nucleotides, never seen before and named a microRNA or miRNA (Lee et al., 1993). Remarkably, the *lin-4* sequence had partial complementarity to the sequences of multiple sites scattered across the *lin-14* 3′ untranslated region (Fig. 8C). This led to the proposal that the *lin-4* miRNA represses *lin-14* by binding directly to regulatory elements in its 3′UTR. Many at the time considered the *lin-4* miRNA to be an amazing finding, but likely some weird phenomenon specific to the highly divergent worm. However, seven years later, another worm miRNA, *let-7*, catapulted miRNAs onto the world stage. The *let-7* gene also controls temporal progression and encodes a miRNA of 21 nucleotides (Reinhart et al., 2000). But in addition, the *let-7* sequence is conserved in genomes across the animal kingdom, including in humans, and its temporal pattern is also conserved widely among bilaterians (worms, flies, annelids, molluscs, sea urchins and zebrafish) (Pasquinelli et al., 2000). A new and broadly conserved mode of gene regulation was born. An explosion of miRNA studies followed this breakthrough paper, which led to discoveries of many miRNAs with many biological functions, including in human physiology and disease (reviewed in (Mendell, 2005)). Their discovery in *C. elegans*, however, emphasizes the power of basic research and taking an unbiased approach.

2.8. Programmed cell death: discovery of a broadly conserved regulatory pathway

Cell deaths shape and reshape tissues during the development of virtually all animals (reviewed in (Saunders, 1966)). Cell death had also long been associated with human physiology and disease (Kerr et al., 1972). Yet molecular mechanisms governing cell death were a total mystery before *C. elegans* genetics tackled the problem. The predictability of cell deaths in the fixed lineage made them accessible to genetic screens (Fig. 9). 131 cells die in every animal and each does so at the same time and place and with a diagnostic morphology. Over the course of about an hour, a dying cell acquires a glassy appearance and then disappears as its corpse is engulfed by neighboring cells (Sulston and Horvitz, 1977). The first *ced* (for cell death) mutants were isolated by screening F2 larvae at high power for changes in cell death (Hedgecock et al., 1983). No screen prior to this one had looked for potential mutants directly with DIC optics. All earlier screens looked for a visible defect at low power (e.g. bag of worms), and then investigated its cellular basis at high power (e.g. reiterated lineages). The first *ced* mutants however looked normal at low power and would never have been found without screening at high power where *ced-1* and *ced-2* mutants were littered with persisting cell corpses, because they had not been engulfed (Fig. 9A and B). With this persistent corpse phenotype in hand, a *ced-1* mutant was mutagenized and its F2 progeny screened, again at high power, for mutants lacking cell corpses. This second screen

identified the *ced-3* gene (Fig. 9) (Ellis and Horvitz, 1986). Whereas *ced-1* mutants had an abnormally large number of corpses, the *ced-1 ced-3* double mutant had no corpses. The *ced-3* gene was therefore required for programmed cell death.

A second entrée to cell death genes came from a vulva defective mutant. Egg laying is sharply reduced when two hermaphrodite-specific neurons are killed, but treatment with serotonin releases eggs from the ablated animals. A targeted screen for mutants that not only are egg laying (*Egl*) defective but also release eggs upon treatment with serotonin identified dominant mutations in the *egl-1* gene (Trent et al., 1983). These *egl-1(dom)* mutants lack the hermaphrodite-specific neurons; however, those neurons reappear upon genetic removal of the *ced-3* gene. Therefore, aberrant cell death must have been responsible for their absence. Screens for suppressors of *egl-1(dom)* next identified two more genes required for cell death: *ced-4* (Ellis and Horvitz, 1986) and *egl-1* itself (Conradt and Horvitz, 1998). Another screen identified the *ced-9* gene, which had a distinct phenotype: the expected deaths did not occur in *ced-9(gf)* mutants, while extra deaths occurred abnormally in *ced-9(lf)* mutants (Hengartner et al., 1992). The explanation of those phenotypes was that wild-type *ced-9* normally protects cells from programmed death. Double mutant analyses established a genetic pathway that converged on *ced-3* as the final regulator. Cloning *ced-3* revealed sequence similarity to a mammalian gene encoding the interleukin-1 β -converting enzyme, also known as caspase-1 (Yuan et al., 1993). Based on that homology, Yuan and colleagues proposed that CED-3 functions as a cysteine protease to execute programmed cell death, a proposal that has stood the test of time. This breakthrough led to a host of studies demonstrating that the cell death pathway and its molecular mechanism of cell death are conserved from worms to humans (Fig. 9C) (reviewed in (Vaux and Korsmeyer, 1999)). The initial discovery of *ced* genes underscores the power of an invariant lineage in a transparent animal.

2.9. Cell polarity in the early embryo: discovery of the broadly conserved PAR network

The early embryo is governed by gene activities delivered from the mother. Mutants in those genes are therefore first seen in F3 embryos, born from a homozygous mutant F2 mother. Jim Priess took advantage of the *lin-2* “bag of worms” phenotype to spot maternal effect mutants in the F2 (Fig. 10A) (Priess et al., 1987). A *lin-2* mutant mother dies when her embryos live, but she survives when they die and is instead filled with dead F3 embryos. A living and moving mother full of dead embryos is easy to recognize among the bag of worm carcasses of her siblings. This trick opened the door to easy and straightforward isolation of maternal effect mutants and led to identification of many genes that regulate the early embryo. Here we highlight genes that regulate polarity of the first asymmetric divisions.

The first division of the *C. elegans* zygote establishes the future anterior-posterior axis. That markedly asymmetric division produces a larger AB blastomere and a smaller P1 germline blastomere, with P granules segregating to P1 (Fig. 10B). Ken Kemphues scored the dead early embryos of maternal effect mutants for defects in blastomere size, mitotic spindle position and orientation plus P granule segregation (Kemphues et al., 1988). Kemphues found mutants in six *par* genes (for *partitioning* defective), and their molecular identities suggested functions in intracellular signaling (reviewed in (Kemphues, 2000)). For example,

PAR-1 is a serine-threonine protein kinase. Immunostaining of the PAR proteins was more immediately informative: PAR-3 and PAR-6 are enriched in the cytoplasmic cortex of the larger AB blastomere, together with PKC-3, while PAR-1 and PAR-2 localize to the cortex of the smaller P1 blastomere (Fig. 10C) (reviewed in (Kemphues, 2000)). Moreover, PAR proteins affect each other's localization. For example, the posterior enrichment of PAR-1 depends on activity of anterior PAR-3.

This PAR network thus establishes polarity of the one-cell embryo and the anterior-posterior axis. How PARs induce an asymmetric division is a fascinating story entwined with regulation of microtubules and basic cell biology, largely beyond the scope of this review. However, as a taste, pulling forces on the anterior and posterior spindle poles are normally unequal, with a greater force on the posterior pole; that force difference is lost in *par-2* mutants (Grill et al., 2001). PAR asymmetry also regulates localization of MEX-5/6 cytoplasmic determinants, as explained in the next section. Soon after molecular identification of the *par* genes, a regulator of *Drosophila* oocyte polarity was found to be homologous to *par-1*, and the biochemical activity of a mammalian PAR-1 homolog, known as MARK, was found to phosphorylate microtubule-associated proteins (reviewed in (Wodarz, 2002)). The *par* genes are now recognized as having uncovered an ancient mechanism that governs cell polarity broadly in embryos, epithelial cells and neuroblasts throughout the animal kingdom.

2.10. The soma-germline distinction and its regulation

The distinction between somatic and germline fates was recognized over 100 years ago, but its regulation has been a longstanding puzzle. In the *C. elegans* early embryo, each successive asymmetric division creates one somatic founder cell and a P germline blastomere, leading to P4 as the founder for all germ cells. Several "germline" regulators segregate to P blastomeres at each of these division, including P granules and their many associated RNAs and proteins (Updike and Strome, 2010). We focus here on one germline regulator, called *pie-1* (for pharynx and intestine in excess) and two paralogous somatic regulators, called *mex-5* and *mex-6* (for muscle excess). Normally, the first germline blastomere P1, produces a somatic EMS blastomere and another germline blastomere, P2. However, in *pie-1* mutants, P1 loses its germline character and makes two somatic daughters (Fig. 11A) (Mello et al., 1992). The PIE-1 protein has two tandem CCCH zinc fingers, but is otherwise novel; PIE-1 segregates to germline blastomeres, where it is predominantly nuclear and disappears from somatic blastomeres soon after they are born (Mello et al., 1996). The *pie-1* molecular function was discovered by assaying transcription in early embryos. A reporter transgene and 16 other zygotically expressed genes are normally only expressed in somatic blastomeres, but in *pie-1* mutants, they were transcribed in all blastomeres (Seydoux et al., 1996). PIE-1 therefore silences transcription in germline blastomeres, which protects them from somatic differentiation.

The nearly identical MEX-5 and MEX-6 proteins are enriched in somatic blastomeres, a pattern reciprocal to that of PIE-1 (Fig. 11B) (Schubert et al., 2000). Their somatic enrichment is lost in *par* mutants and likely depends on PAR-1 phosphorylation (Tenlen et al., 2008). Loss of MEX-5/6 allows germline regulators to persist in all blastomeres,

while increased MEX-5/6, driven by a heat shock promoter, eliminates germ-line regulators from all blastomeres (Fig. 11C) (Schubert et al., 2000). MEX-5/6 was therefore proposed to promote degradation of germline regulators in somatic blastomeres. The mechanism involves the MEX-5/6 translational regulation of a factor that recruits E3 ubiquitin ligase to germline regulators (DeRenzo et al., 2003). The importance of translational regulation in the soma-germline decision persists into adults, where two RNA binding proteins, GLD-1 and MEX-3 function redundantly in germ cells to repress somatic differentiation (Ciosk et al., 2006). Indeed, transcriptional quiescence and translational regulation are also critical to the soma-germline decision in flies and likely more broadly (Strome and Lehmann, 2007).

2.11. C. elegans Notch and its control of multiple types of cell interactions

Genetic screens for three entirely different developmental defects identified two genes, *lin-12* and *glp-1*, that control cell interactions in both embryos and larvae (Fig. 12). The *lin-12* gene was identified in screens for vulva mutants (Greenwald et al., 1983). Dominant *lin-12(gf)* mutants had multiple vulvae, while recessive *lin-12(lf)* mutants were sterile with a constellation of defects. A role for *lin-12* in cell fate specification was deduced from its effects on cells that use lateral signaling to adopt distinct primary and secondary fates. In *lin-12(lf)* mutants, both cells adopt primary fates, but in *lin-12(gf)* mutants, both adopt secondary fates. Therefore, standard genetic logic tells us that the *lin-12* gene promotes the secondary fate.

The *glp-1* gene (for germline proliferation defective), on the other hand, was identified in screens for genes regulating cell interactions. Numerous *glp-1(lf)* alleles came from a screen for genes affecting niche maintenance of stem cells (Austin and Kimble, 1987) and a *glp-1(gf)* allele was found in a later screen (Berry et al., 1997). Germline stem cells stop self-renewing in *glp-1(lf)* mutants and instead differentiate, while a germline tumor forms in *glp1(gf)* mutants. Therefore, the *glp-1* gene promotes stem cell self-renewal. Genetic mosaics revealed that *glp-1* acts in the germline and therefore on the receiving side of niche signaling. In addition, other *glp-1(lf)* alleles were found in a parallel screen for maternal effect genes, looking for a defect diagnostic for loss of the P2 to ABp embryonic induction (Priess et al., 1987). Temperature sensitive alleles were used to show that *glp-1* functions in larvae and adults to maintain germline stem cells and in the early embryo to mediate embryonic induction.

The *lin-12* gene was cloned and found to encode a transmembrane protein with multiple EGF repeats in its extracellular domain (Greenwald, 1985). The *Drosophila* Notch gene was cloned the same year (Wharton et al., 1985), and remarkably, the *lin-12* and Notch genes were homologs. Notch was one of a group of “neurogenic” genes in *Drosophila* with roles in neural development and wing morphogenesis (Lehmann et al., 1981). The lack of any common biological function between *lin-12* and Notch made their homology totally unexpected. The *glp-1* gene, cloned a few years later, encodes a *lin-12* homolog so *C. elegans* has two genes in this family, *lin-12* and *glp-1* (Austin and Kimble, 1989; Yochem and Greenwald, 1989). The *lin-12* vulval defects were the first evidence for a role of Notch family proteins in lateral signaling, the *glp-1* stem cell defect was the first evidence that

Notch can control stem cells and the *glp-1* mosaic analysis was the first evidence that they might be receptors.

Whereas *Drosophila* genetics had uncovered multiple neurogenic genes, *C. elegans* genetics had not found other genes with the same defects. This was puzzling. Perhaps other genes work with both *lin-12* and *glp-1* and have a different phenotype. Indeed, *lin-12 glp-1* double mutants arrest as L1 larvae with a unique Lag (*lin-12* and *glp-1*) phenotype (Lambie and Kimble, 1991). A simple F1 clonal screen for Lag mutants identified multiple alleles of two more genes, *lag-1* and *lag-2*. These *lag* genes, when cloned, had homologies with *Drosophila* neurogenic genes. Worm *lag-1* and fly *Su(H)* encode homologous transcription factors that bind the same DNA consensus sequence (Christensen et al., 1996), and worm *lag-2* and fly *Delta* encode homologous transmembrane proteins (Henderson et al., 1994). Expression of *lag-2* was restricted to the signaling distal tip cell, consistent with suggestions at the time that Delta might function as a Notch ligand. Additional screens, one genetic and one molecular, identified a final member of the pathway, LAG-3/SEL-8, which has weak homology to yet another neurogenic gene, *Drosophila* Mastermind (Doyle et al., 2000; Petcherski and Kimble, 2000a). Despite their weak homology, however, their proteins have similar central roles in the Notch-dependent transcription factor complex in worms, flies and mammals (Petcherski and Kimble, 2000a, b). The core Notch pathway thus emerged from a strong synergy between efforts in worms and flies. Identifying the key genes benefited from *Drosophila*'s relative lack of gene redundancy, and understanding their functions benefited from the well-defined cell interactions in *C. elegans*.

Notch signaling is best known to occur between cells in close contact with their membranes touching. This is certainly the case for P2 to ABp induction and lateral signaling. However, GLP-1/Notch signaling from the niche regulates cell fates across a distance to determine which remain stem cells and self-renew and which are triggered to differentiate. The most likely model is that niche signaling is graded with stem cell pool size determined at a given threshold (Fig. 12B). More importantly, the *C. elegans* Notch pathways govern multiple types of developmental signaling and this holds true of Notch signaling in other organisms as well.

2.12. Stem cells, redundancy and an RNA regulatory network

Our final example combines genetic and molecular approaches to overcome redundancy and pleiotropy, the two Achilles heels of genetics. It also highlights the importance of serendipity in discovery, and the significance of RNA regulation during development. This effort began with a genetic selection for sex determination regulators (Barton et al., 1987) and led years later to finding broadly conserved stem cell regulators (Crittenden et al., 2002) and an RNA regulatory network governing the balance between self-renewal and differentiation (reviewed in (Kimble and Crittenden, 2007)).

The story begins with temperature sensitive *fem-1* mutants, which are self-fertile hermaphrodites when grown at one temperature, but self-sterile females (spermless hermaphrodites) at another temperature. Mutagenized *fem-1* mutants were shifted to the restrictive temperature and their F1s tested for rare self-fertile animals that carry a dominant suppressor. This selection generated nine *fem-3(gf)* mutations at a frequency of 1/200,000

haploid genomes (Barton et al., 1987). The *fem-3(gf)* mutants had masculinized germlines that made only sperm, while *fem-3(lf)* mutants made only oocytes. Therefore, the normal *fem-3* activity must promote the sperm fate. Upon cloning, each *fem-3(gf)* mutation was a single base pair change, with all changes mapping to a five nucleotide region of the *fem-3* 3'UTR (Fig. 13A) (Ahringer and Kimble, 1991). The mutations therefore identified key residues in a 3'UTR regulatory element. The idea was that a repressor must bind the element, and that *fem-3(gf)* mutations release the mRNA from repression.

Large scale genetic screens failed to identify a candidate repressor, but searching for a protein that bound the element was successful. Using the *fem-3* 3'UTR element as bait for a library of worm cDNAs, a yeast three hybrid screen identified one cDNA with binding activity and found the repressor, called FBF (for *fem-3* binding factor) (Fig. 13B) (Zhang et al., 1997a). The FBF cDNA sequence revealed homology with *Drosophila* Pumilio, a translational repressor required in embryos (Lehmann and Nüsslein-Volhard, 1987; Wharton et al., 1998). The *C. elegans* genome sequence revealed two nearly identical genes, *fbf-1* and *fbf-2*, which had caused genetic screens to fail. RNAi against *fbf-1* and *fbf-2* masculinized the germline, as expected, but RNAi was new at the time and not as effective as it became later. An *fbf-1 fbf-2* double mutant, generated afterwards, revealed a second defect: germline stem cells stop self-renewing and differentiate instead (Crittenden et al., 2002). Therefore, FBF-1 and FBF-2 were not only redundant but also pleiotropic: they govern both germline stem cells and the sperm/oocyte fate decision. Moreover, Puf proteins (for *Pumilio* and *FBF*) regulate germline stem cells in flies, neoblasts in planaria, and neuroblasts in mice (Forbes and Lehmann, 1998; Salvetti et al., 2005; Zhang et al., 2017). These sequence-specific RNA-binding proteins are therefore ancient stem cell regulators.

Discovery of FBF was part of a larger effort to understand the regulatory network governing the balance between self-renewal and differentiation in the *C. elegans* germline (Fig. 13D). The entire network is riddled with redundancy and pleiotropy, and the story of its unraveling is too much for this review. However, the major idea is that the network has four major regulatory nodes or “hubs”. FBF-1 and FBF-2 are central to the hub that drives self-renewal, and other RNA-binding proteins and RNA regulators are central to hubs that drive differentiation, including entry into the meiotic cell cycle and choice of sexual fate (reviewed in (Kimble and Crittenden, 2007)). The regulators in each hub integrate multiple inputs and act on hundreds of mRNAs. Removal of the self-renewal hub causes stem cells to differentiate; removal of two major hubs driving meiotic entry creates a germline tumor; and removal of the sexual fate hub transforms sexual fate. The concept therefore is that an RNA regulatory network can govern key developmental decisions. The role of RNA regulation in development is far from new (reviewed in (Wickens et al., 2000)), but RNA networks are less well appreciated and likely a common developmental mechanism.

2.13. *C. elegans* developmental genetics: Impact and outlook

When *C. elegans* was launched in the 1970's, no one predicted its many breakthroughs over the next 20 years. One simple measure of *C. elegans* impact is its three Nobel prizes: Brenner, Horvitz and Sulston in 2002 for organogenesis and cell death, Fire and Mello in 2006 for RNAi, and Chalfie, Shimomura and Tsien in 2008 for GFP. But only the first prize

recognized a discovery made by developmental genetics. The other two recognized methods of universal import, both of which enhance the genetic approach tremendously but were not the direct result of genetic screens. So what are the contributions of *C. elegans* genetics to our understanding of developmental mechanisms? The cell death pathway of course is among them, but also the discoveries of miRNAs and broadly conserved regulators of aging, cell polarity, stem cell self-renewal, cell signaling and sex determination. Genetic analyses of the soma-germline distinction led to understanding that transcriptional quiescence is critical for initial establishment of the germ lineage and translational repressors are critical for its later maintenance. Other contributions, not described in this review, include fundamental insights into broadly conserved regulators of somatic cell fates, neuronal guidance, cell migration, chromatin and the meiotic cell cycle (see (Corsi et al., 2015) for review). And what contributions did not come from genetics but underscore the impact of the worm? These include the first complete cell lineage of any metazoan, discovery of the first stem cell niche, the first complete genome sequence of any metazoan and the first complete wiring diagram of an organism, the hermaphrodite nervous system (White et al., 1986). The wiring diagram together with neurogenetics led to understanding the circuitry of *C. elegans* movement and certain aspects of behavior.

This review emphasizes developmental genetics begun in the 1970's and 1980's and culminating in the discovery of conserved regulators by the turn of the century. But what about the next 20 years and the future? What recent advances have transformed research with this little worm and shape its future? Many are new methods. Aided by its short generation time, CRISPR/Cas9 gene editing is remarkably fast in *C. elegans* (Dickinson et al., 2013). This breakthrough technology allows genome manipulations with tremendous precision, and methods become increasingly effective each year. On a different note, a host of new fluorescent reagents take live imaging to an entirely new level, and their power is exceptional in this microscopic transparent animal. A prime example is discovery of liquid droplet behavior with GFP-tagged P granules in the early embryo (Brangwynne et al., 2009), which led the way to realizing the general importance of liquid phase separation in cells. Other technical advances apply broadly to any genetically manipulable organism, for example using light or chemicals to turn genes and proteins on or off at specific times and in specific places. Other changes over the past 20 years involve addressing new biological questions that, together with studies of development and neurobiology, set the stage for the future. Cell biology, physiology, evolution and bacterial pathogenesis, for example, have all emerged as areas of intense focus over the past 20 or so years. These studies all have potential for discoveries that will impact human health, much like earlier studies of development and neurobiology. Indeed, *C. elegans* has now been adopted as a model for finding and investigating human disease genes (Apfeld and Alper, 2018) with promise for cost effective approaches to drug discovery (Giunti et al., 2021). The outlook for research in *C. elegans* has thus expanded considerably. Its robust foundation of genetics, genomics and cell biology will empower research on a spectrum of problems and continue to solve fundamental questions with impact on human health (see Discussion).

3. Developmental genetics in *Drosophila*

3.1. Traditional genetics: the adult fly

Drosophila produces many offspring, can be raised in bottles on simple food in the laboratory, does not normally suffer from diseases and has a life cycle of 12 days (Fig. 14). The most important features are a body that is covered with a pigmented exoskeleton decorated with a stereotypic pattern of bristles and hairs, red compound eyes, and a pair of wings with characteristic veins providing a wealth of markers that can change upon mutations, which could be fortuitously isolated in large numbers. Classical *Drosophila* genetics focused on the structures of the adult fly; however, since the 1970's of the last century targets of genetic screens increasingly were embryos, oogenesis, imaginal discs, the compound eye and internal organs (see BOX on *Drosophila* developmental systems).

Drosophila has two large autosomes, one tiny one, and a pair of sex chromosomes, the X- and Y-chromosomes. Its phenotypic sex is determined by the autosome to X-chromosome ratio, and male fertility depends on a Y-chromosome (Morgan, 1910). Males are distinguished from females by a characteristic pigmentation of the posterior abdomen and the presence of prominent bristles (sexcombs) on the foreleg. Much of the early work focused on sex-linked phenotypes because they can be readily seen in males, which have only one X-chromosome. The isolation of a white-eyed male by TH Morgan in 1910 was the start of *Drosophila* genetics (Fig. 15). Females carrying the two X-chromosomes attached to each other allowed the identification of X-linked mutant phenotypes in the males of the Filial generation 1 (F1) because in this case the F1 males will receive their X-chromosome from the mutagenized father.

Conveniently, there is no meiotic recombination in males, which facilitates the maintenance of stocks with linked mutations. Genetic screens for morphological phenotypes have been performed using X-rays or chemicals to increase the mutational frequency (Auerbach and Robson, 1946; Lewis and Bacher, 1968; Muller, 1927), and a large collection of stocks with chromosomal aberrations, including deletions (in flies called deficiencies), translocations and inversions, as well as mutations with visible phenotypes were collected over the years. These provided a rich tool kit for further screens and the genetic analysis of many biological processes.

Most of these mutants displayed phenotypes that did not significantly affect the viability of the fly (in the laboratory); many proved useful as cell autonomous markers in studies following groups of cells through development in clones produced by mitotic recombination (Stern, 1936), or as markers for the maintenance of lethal and sterile mutants. A nomenclature was adopted in which the mutant phenotype was briefly described. For example, *white* is a gene that, upon loss-of-function mutation, changes the wild type red eyes to white; therefore, a functional *white* gene is required for the production of red eyes. A catalog describing all genetic aberrations including multiple alleles at the same loci and their origins was updated regularly ((Lindsley and Grell, 1968); now <https://FlyBase.org>). In addition to mutants with morphological visible phenotypes, mutations affecting simple behavior were collected (vision, hypo-hyperactivity, phototaxis, flight, walking) (Homyk, 1977). It was recognized, though, that many more genes of the fly mutated to lethality

than to a visible phenotype (Hadorn, 1955). By screening for lethal mutants uncovered by a chromosomal deletion, it was calculated that the number of essential genes corresponded roughly to the number of visible chromosomal bands which is 5000 (Judd et al., 1972). This correspondence is a coincidence, however, and we now know that the total number of genes is about 14,000 which means that most genes are not essential.

Some general conclusions from the early fly work relate to the complex relationship between genes and phenotypes: each organ or structure depends on many genes (for example numerous genes affect the eye color, often with indistinguishable phenotypes such as “eyes bright red” (*cinnabar*, *vermillion* and *scarlet*), and an individual gene can affect several, frequently quite unrelated features. A small number of genes were identified that display a dominant visible phenotype in individuals heterozygous for a deletion of the locus (haploinsufficient), but most dominant mutations represent gain-of-function alleles of the respective gene. These could be reverted by inactivating the gene with a second, loss-of-function, mutation in the same gene. Recessive alleles reflecting a loss-of-function of the gene may be weak (hypomorph) or strong, behaving like a deletion of the locus (amorph). Lethal or sterile mutations are kept as heterozygotes over so called balancer chromosomes (carrying a lethal mutation as well as multiple inversions to prevent recombination) thus eliminating the need to select for carriers in each generation. A draw-back of *Drosophila* is that the stocks have to be propagated live by transfer in every generation, as attempts to keep them frozen have not been successful in providing practicable protocols.

A small collection of so-called homeotic mutants attracted the attention of developmental biologists because they show characteristic transformations of body parts that acquired the character of another body part - most famous the *bithorax* mutants that display wing structures instead of halteres, and *Antennapedia*, in which the antennae form leg-like bristle patterns (Lewis, 1978), (Lewis et al., 1980). A haploinsufficient dominant mutation, *Krüppel*, causes flies to develop aberrant patterns including deletions and duplications of pattern elements (Gloor, 1950).

3.2. Behavior

Seymour Benzer and colleagues pioneered screens for behavioral mutants located on the X-chromosome, in which F1 male progeny of mutagenized males hemizygous for a mutagenized chromosome were scored using clever devices by which individual flies affected in phototaxis could be separated from those with normal behaviors (Benzer, 1967). In a famous paper, the mutant foci were mapped relative to the adult structures using gynandromorphs (see BOX imaginal discs for further explanation) (Hotta and Benzer, 1972). The Benzer lab also isolated mutants affecting learning and memory (Dudai et al., 1976) as well as the first clock mutants affected in the circadian rhythm (Konopka and Benzer, 1971). Recently, this field was honored in 2017 by the Nobel Prize for Jeffrey C Hall, Michael Rosbash and Michael W Young “for their discoveries of the molecular mechanism controlling the circadian rhythm”.

3.3. Lethal mutants

Although the importance of mutations causing lethality for the understanding of development was noted early, only few larval or pupal lethal phenotypes were analysed before the 1970s (Hadorn, 1955). One example is *lethal (2) giant larva*, in which the homozygous mutants showed dramatic tumor-like overgrowth of the brain. The lack of transparency made the analysis of embryonic mutants difficult. Nevertheless, a group of scientists around Don Poulson at Yale collected the first embryonic mutants. Poulson described the lethal recessive phenotype of the *Notch* locus, which previously was known from its dominant haploinsufficient phenotype of notches on the wing; he also mapped *Notch* to a single band on the X-chromosome using deficiencies uncovering the locus (Fig. 16). Lethal *Notch* embryos lack ventral epidermis with its characteristic denticle belts and instead develop nervous tissue (Poulson, 1940). Poulson also provided the chapter on embryogenesis ((Poulson, 1950) in the classical Handbook: Biology of *Drosophila*, edited by M. Demerec 1950), in which he presented a fate map of the embryo deduced from sectioned material.

In comparison to other insects, embryogenesis in *Drosophila* had not been studied much experimentally, although methods for transplantations of egg cytoplasm had been established. In one famous experiment, transplantation of pole plasm from the posterior of donor embryos induced the formation of germ cells in the anterior of recipient embryos, suggesting the existence of “cytoplasmic determinants” for germ cells in the pole plasm (Illmensee and Mahowald, 1974). The maternal effect lethal phenotype of an eye-colour mutant, deep orange, was shown to be partially rescuable by the injection of cytoplasm from wildtype embryos (Garen and Gehring, 1972). A few embryonic lethal phenotypes were identified with specific patterning defects. Reduction of the number of segments was described for homozygous *Krüppel* mutants (Gloor, 1950). A curious maternal mutant, *bicaudal*, occasionally developing duplications of the posterior abdomen anteriorly in embryos from homozygous mutant females was described (Bull, 1966; Nüsslein-Volhard, 1977a). However, the general notion was that there would be very many genes mutating to embryonic lethality and that the phenotypes probably would just reflect early stops in development, and not be informative, which precluded systematic approaches to analyse embryogenesis genetically (Wright, 1970) and the general interest persisted for the development of the adult fly.

3.4. Maternal homeotics

Screens for maternal mutants affecting adult morphology were performed. These screens were designed based on the belief in localized cytoplasmic determinants instructing cells to become particular imaginal discs, as exemplified by the pole plasm transplantations. The expectation was that the adult progeny of a homozygous mutant female would show defects, or lack a particular structure, such as a leg or a wing. Although a number of mutants were isolated in which the embryonic progeny of homozygous females developed defective blastoderms, or embryos that failed to hatch with undefined defects (Gans et al., 1975; Mohler, 1977; Perrimon et al., 1986; Rice and Garen, 1975; Zalokar et al., 1975), these screens were considered unsuccessful: “A desirable mutant which was not recovered was one with normal fertile females producing descendants which, regardless of their genotype,

bore specific morphological abnormalities" (Gans et al., 1975). However, several mutants produced progeny lacking germ cells, a "grandchildless" phenotype suggesting that the germ cell determinants were affected.

3.5. Classical screens for larval patterning

How to find the genes that control early embryonic decisions? *Drosophila* embryos are not transparent, as are worm or fish embryos. They are covered in an opaque chorion, and filled with yolk. The result of embryogenesis is the first instar larva, a segmented headless maggot with a much simpler body organization than the adult fly. The big breakthrough of *Drosophila* developmental genetics was to focus on the larval pattern instead of the adult and to screen lethal embryos for structures of the cuticle secreted by the larval hypodermis. Conveniently, this cuticle remains intact even after death and can be scored following longer collection periods. The hypodermis is derived from a rather large anlage in the embryo and provides an excellent readout of the pattern. The cuticle is richly decorated with denticles and hairs with characteristic polarities and patterns, and the cuticle preparations make the three thoracic and 8 abdominal segments easy to recognize via prominent ventral denticle belts and allow for the analysis of other patterned features along the dorsoventral axis. Cuticle preparations were first applied to analyse the phenotypic spectrum of two maternal mutants, *bicaudal* (Nüsslein-Volhard, 1977a) and *dorsal* (Nüsslein-Volhard, 1979). The dominant and recessive phenotypes of embryos from *dorsal* mutant females display clearly interpretable partial or complete dorsalization of the entire embryo, demonstrating the usefulness of cuticle patterns to interpret larval morphological phenotypes. To screen for mutations affecting larval patterning, efficient clearing protocols for the larval cuticle were applied (Schubiger and Wood, 1977; van der Meer, 1977).

Screens for embryonic lethal mutants requires collecting eggs from large numbers of individual mutant families for inspection. Two low-tech but very efficient procedures greatly reduced the work required to score embryonic mutants: Eggs were collected on transparent agar plates instead of fly food, and their opaque chorions were rendered transparent by applying a special halocarbon oil (Voltalef), already used in transplantations. This allowed direct inspection of the embryos on the agar plates with a stereomicroscope and transmitted light. To collect eggs, flies were placed into test tubes glued together in a block of tubes, such that eggs could be collected simultaneously from several different lines, and on successive days (Nüsslein-Volhard, 1977b; Wieschaus and Nüsslein-Volhard, 1986). Cleared eggs were mounted for inspection with a compound microscope using dark field and phase contrast optics. Importantly, the shift of paradigm to study the cuticle pattern of the larva was the secret of success and the start of a new field.

Two sets of genes are required for larval patterning. Maternal genes expressed in the female during oogenesis deliver products to the zygote for early embryonic decisions. Zygotic genes, transcribed in the embryo, generate products for later decisions. Mutations in both classes would cause defects visible in the larval cuticle (Fig. 17). In the late 70s and early 80s large-scale mutant screens were performed to identify most genes of both classes producing specific phenotypes visible in the cuticle of mutant embryos.

3.6. The Heidelberg screens for zygotic genes affecting the larval pattern

The first systematic screens of the entire genome were for zygotic mutants performed in the lab of Christiane Nüsslein-Volhard and Eric Wieschaus at the European Molecular Biology Laboratory EMBL in Heidelberg in 1979–1980 (Jürgens et al., 1984; Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984; Wieschaus and Nüsslein-Volhard, 2016; Wieschaus et al., 1984). A screen for embryonic patterning mutants requires the establishment of inbred families of flies derived from individual F1 progeny arising from mutagen-treated males (sperm) and scoring F3-eggs from F2 brother-sister matings. For mutagenesis a standard protocol uses 25 mM EMS which leads on average to about one hit in each gene per thousand mutagenized sperm, and 50% and 90% lethal mutations on the X- or autosome, respectively. Therefore, to aim at saturation, about 5 times as many lines had to be examined. Because following all chromosomes in a single experiment was difficult, three separate screens were performed—two for the large autosomes and one for the X chromosome. The use of dominant temperature-sensitive mutations (Suzuki, 1970) helped to kill unwanted progeny, selecting carrier flies by simple temperature shifts (for the crossing schemes see Wieschaus and Nüsslein-Volhard, 2016). After two generations, females and males heterozygous for the mutagenized chromosome were obtained. Among their progeny, a quarter should be homozygous for a mutation. These were scored for embryonic lethality. Eggs were collected from lethal lines and unhatched embryos were processed for cuticle preparations.

Initially, the screen had been designed to detect mutations affecting segmentation. However, very soon it became obvious that the opportunity to screen through the F3s of such a large number of mutant lines should not be missed; consequently, mutants with other specific and consistent cuticle phenotypes were kept and analysed because they could be of interest for scientists working on other developmental processes (reviewed in (Wieschaus and Nüsslein-Volhard, 2016)).

In the three screens, embryonic lethal mutants occurred at a frequency of about 25% of all lethal lines (representing about 1200 loci). A total of 600 mutants displayed a specific cuticle phenotype, which by complementation tests defined 120 loci, corresponding to 10% of embryonic lethal loci and 2.4% of lethal genes. This small number came as a surprise. It means that most essential genes either are dispensable for pattern determination or have roles in this process that do not require precise temporal and spatial control of gene expression. Almost all lethal mutants retained some remnants of structures, indicating that the eggs are provided with house-keeping functions and the products of genes that need not be transcribed in a precise pattern in the embryo are sufficient to develop to an advanced stage of embryogenesis.

The small total number of loci allowed them to be grouped into classes and candidate developmental processes. In several cases, multiple alleles with different strengths were identified, which helped the interpretation of the phenotypes. For example, strong loss of function alleles of *even skipped* do not show any segmentation, whereas the weak loss of function alleles skip every other segmental boundary. In several instances, mutations turned out to be lethal alleles of genes found earlier with viable phenotypes, e. g. *wingless*, *cubitus interruptus* (*ci*), *engrailed*, *hairy*, *thick vein*, and *eyes absent*.

Significantly, most genes display unique phenotypes that can be distinguished from those produced by other genes in the same general phenotypic group. The degree to which the larval pattern was affected suggested sequential processes of patterning the egg along the anteroposterior or the dorsoventral axis. Mutants in which the larval pattern appeared normal but the epidermis showed specific defects, were also isolated. Such defects ranged from holes to various degrees of lack of structures and differentiation. In several cases, the mutations did not directly affect epidermal development or patterning, but the cuticle reflected abnormalities in the arrangement of the embryo inside the egg case or failures in final differentiation.

The phenotypes classified the mutants as affecting the following developmental processes:

Anteroposterior axis: Segmentation, segment number, segment polarity and pattern. Segment identity (homeotic mutants). Head and posterior terminal structures.

Dorsoventral axis: Gastrulation movements (ventral furrow formation, germ band extension), midline patterning, neurogenesis, dorsal closure.

Epidermal integrity: epidermal cell number, cuticle synthesis, cuticle pigmentation, denticle formation and patterning.

A first account of the segmentation genes was published (Nüsslein-Volhard and Wieschaus, 1980), followed by publications of the screens of the three chromosomes (Jürgens et al., 1984; Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984) which included map positions, images and brief description of phenotypes. In these papers, no attempt was made to classify the genes according to similar phenotypes beyond a rough description. These screens reached saturation for non-redundant genes that affect a given process and identified strong loss of function mutations in most of the essential patterning genes that are used throughout development. It provided the basis for identifying the transcription factors and signalling molecules that generate positional information in the embryo.

The segmentation genes: The Heidelberg screens were carried out with the primary aim of understanding the logic and complexity of embryonic pattern formation in terms of its genetics and the phenotypes. The advent of recombinant DNA technologies and positional cloning allowed for the molecular isolation of the genes and the identification of the gene products. Because the mutants from the Heidelberg screen were made available to the scientific community even prior to publication, many of them were cloned and sequenced and their expression patterns characterized in the years that followed. The banding patterns observed in giant salivary gland chromosomes of *Drosophila* were especially useful in these early molecular analyses because mutants could be assigned to visible bands and thus to physical positions on chromosomes. The discovery of the transposable P-element and its use in mutagenesis and genetic transformation provided another advantage, speeding up the exploration of the collection (Rubin and Spradling, 1982). Many genes were cloned using P-element induced alleles from which a genomic walk of overlapping clones was initiated. In several instances, microdissection of a chromosomal band helped isolating clones located in the region of interest (Preiss et al., 1985). Genetic transformation was performed by injecting the cloned gene flanked by P-element sequences as vector into the pole plasm of early embryos (Rubin and Spradling, 1982). Complementation of the mutant allele by such

a transgene came to be regarded as standard proof of having isolated a complete functional copy of the gene.

At this time the first *Drosophila* patterning genes had been cloned, among them the *bithorax* and *Antennapedia* complexes (Bender et al., 1983; McGinnis et al., 1984), which, excitingly, turned out to encode transcription factors and resulted in the discovery of the homeobox, a DNA binding domain with homology to yeast transcription factor alpha, and to many genes in all animals (Carrasco et al., 1984; Hafen et al., 1984; Laughon and Scott, 1984; McGinnis et al., 1984). Many of the segmentation genes of the Heidelberg collection also encode transcription factors, often with a homeobox. The molecular data helped classifying the genes into groups with similar phenotypes, because their expression patterns could now be determined using molecular probes that were hybridised *in situ* to sectioned or whole mounted fixed embryos (Tautz and Pfeifle, 1989). Strikingly, the transcription pattern of these genes in blastoderm-stage embryos generally corresponded to the patterns of deletions observed in the mutants (Hafen et al., 1984; Knipple et al., 1985).

Three classes of segmentation genes could be distinguished (Fig. 18):

1. Mutations in five gap genes (*giant*, *hunchback*, *knirps*, *Krüppel*, and *tailless*) lead to gene-specific loss of large contiguous regions along the anterior-posterior axis.
2. Mutations in the eight pair rule genes showed deletions in every other segment. Surprisingly, each of these eight mutants had a distinct frame of deletions, skipping specific elements in the even or odd-numbered denticle bands and adjacent naked cuticle (*even-skipped*, *odd-skipped*, *hairy*, *fushi tarazu*, *runt*) or displaying characteristic pairwise fusions of the denticle bands (*paired*, *odd-paired*, *sloppy-paired*).
3. Mutations in the segment polarity genes displayed deletions associated with duplications in each segment. In this class, phenotypes of duplicated anterior denticle bands in four of the five genes (*hedgehog*, *armadillo*, *gooseberry/cubitus interruptus* (*ci*), *wingless*) were quite similar, whereas one phenotype (*patched*) was distinct from the other phenotypes.

The genes of the gap and pair rule classes encode transcription factors that are expressed in a series of molecular prepatterns, triggered by maternal gradients (see below). By regulating each other via gradients and combinations, the patterns become more and more refined until the molecular pattern directly determines the individual segments (Fig. 19) (reviewed by (Pankratz and Jäckle, 1993)). It is striking that the first periodic pattern of pair rule genes results from determination of each individual stripe by a different combination of gap gene expression (what Michael Akam described as: “making stripes inelegantly”) rather than a system producing periodicity, such as the segmentation clock in Vertebrates or the segment addition zone in other insects).

Some segment polarity genes encode transcription factors (*gooseberry*, *ci* and *engrailed*), whereas others produce secreted signalling molecules that are active in feedback loops to establish segmental compartments (*hedgehog*, *wingless*) (Fig. 20). A fourth class of segmentation gene displays specific deletions in each segment without duplications (*arrow*, *lines*, *midlines*).

Other gene groups: The Heidelberg screen identified lethal alleles in many components of the major cell signaling pathways. Among the segment polarity and segment pattern genes are key components of the Hedgehog (*hedgehog*, *smoothened*, *patched*) and Wnt (*wingless*, *armadillo*, *naked*) pathways. The pattern along the dorsoventral axis depends on transcription factor prepatterning of *twist*, *snail* and *zen*, as well as on two signaling systems with molecules of the transforming growth factor (TGF) class: Patterning of the dorsal side depends on BMP (bone morphogenetic protein)-related proteins (*decapentaplegic* (*dpp*), *tolloid*, *screw*, *punt*, *thickveins*) (Padgett et al., 1987; Ray et al., 1991). *dpp* encodes the first secreted protein discovered that acts as a morphogen by determining position in a concentration dependent manner (Ferguson and Anderson, 1992). It is homologous to growth factors in vertebrates that participate in the Spemann organizer. TGF- β like proteins specify dorsoventral pattern in both vertebrates and insects, albeit in reverse orientation (see Fig. 21).

Ventral epidermal development is regulated by molecules of the epidermal growth factor (EGF) class (*single minded*, *spitz*, *faint little ball*, *Star*) (Mayer and Nüsslein-Volhard, 1988). Neurogenesis depends on the Notch-pathway (*Notch*, *Delta*), where both ligand and receptor are anchored in cell membranes and mutual activation and repression results in singling out neuroblasts from the underlying ectoderm, a prominent example of lateral inhibition. This process involves, a number of “neurogenic” genes with similar phenotypes (*mastermind*, *big brain*, *neuralised*, (Lehmann et al., 1983).

Components of the same pathway could often be recognized on the basis of their shared phenotypic features in fly embryos as described above, however many downstream components were missed in the screen, presumably because the maternal supply lasted through embryogenesis (see below). The genes affecting epidermal integrity encode components involved in the cell cycle (*string*, *pebble*), apical-basal cell polarity (*crumbs*, *bazooka/par-3*), or cell adhesion (*shotgun*). Mutants with altered denticle, hair and bristle morphology are affected in cyto-skeletal proteins (*crinkled*). Other groups of genes encode enzymes involved in the final steps of cuticle differentiation (*knickkopf*), or cuticle pigmentation (*faint*). Final epidermal differentiation is defective in another group of genes controlling the synthesis of ecdysone (*haunted*). Recent review: (Wieschaus and Nüsslein-Volhard, 2016).

3.7. Other zygotic screens for embryonic morphology

Subsequent F3 saturation screens were performed in which the embryos were stained with antibodies to detect specific structures in internal organs, for example abnormalities in the axonal pathways of the ventral nerve chord (Seeger et al., 1993). This screen identified several additional interesting loci (*commissureless*, *roundabout*) but many mutants turned out to be alleles of already known genes, which had been identified in the Heidelberg screen among the ventral development class of EGF signalling molecules. With the increase in molecular markers and antibodies available for specific cell types, numerous screens were performed which, however, mostly were not published as such, instead, focusing only on the most relevant genes and their description.

To detect genes acting very early in development, one approach made use of compound chromosomes, deficiencies, and translocations to generate embryos lacking large chromosomal regions (Merrill et al., 1988). This approach was undertaken in the hope of identifying genes that affected very early embryonic morphology and that might have been missed because their morphological effects did not result in a cuticle phenotype. These screens showed that there are just 8 genes that are required zygotically prior to the formation of the cellular blastoderm.

3.8. The screens for maternal effect genes affecting larval patterning

To identify the genes that encoded the maternal factors present in the egg and guiding the expression of the zygotic genes, large-scale screens were performed in Princeton for maternal mutants on the autosomes (2nd chromosome, (Schüpbach and Wieschaus, 1989, 1991)) and Tübingen (3rd chromosome, (Anderson and Nüsslein-Volhard, 1984; Nüsslein-Volhard et al., 1987)) after the Heidelberg screen. In addition, mutants from the screens on the X-chromosomes from the Gans- and Mohler Laboratories were re-examined (Gans et al., 1975; Mohler, 1977; Perrimon et al., 1986). In these screens, F3 females homozygous for a mutagenized chromosomes were tested for fertility and the production of eggs or embryos. Starting by setting up individual families with F1 progeny of mutagenized males, they required one further generation of inbreeding compared to the zygotic screens. The screens were facilitated by the introduction of a dominant temperature-sensitive lethal mutation (as in the zygotic screens) as well as a dominant female-sterile mutation to avoid sorting flies in the last inbreeding generation (Schüpbach and Wieschaus, 1989). The mutagen dose was reduced to obtain about 50% viable lines for the two autosomes. Eggs which did not hatch were scored for the shape of the chorion. Early development through gastrulation was observed in living embryos and the cuticle of developed larvae was analysed (for normal development see BOX on embryogenesis (Fig. B1), the larval fate map (Fig. B2) and oogenesis (Fig. B3)). The phenotypes allowed the mutants to be classified as affecting the following developmental processes:

Female-steriles, no eggs produced: Stem cells and egg chamber formation, meiosis, follicle cell migration, vitellogenesis.

Abnormal egg shape: egg polarity along both AP and DV axis. Dorsal appendage formation, chorion formation, egg turgor, fertilisation.

Maternal effect lethals with normal egg shape: cleavage, blastoderm formation, embryonic patterning along the anteroposterior or dorsoventral axis. Gradient formation. RNA localization, Signalling between follicle cells and oocyte.

Only the second chromosomal screen was quantitatively evaluated allowing the estimation of numbers of genes required maternally for the development of a morphologically normal larva (Schüpbach and Wieschaus, 1989, 1991). In this near-saturation screen, it was estimated that the number of female sterile genes reflects about 10% that of genes mutating to lethality. A total of 140 loci were identified, of which about one third each fell into each of the classes of female-steriles producing no eggs, eggs with abnormal shapes, and maternal effect lethals with normal egg shapes. The female-steriles displayed various stops during oogenesis, produced tumours, or failed to select one oocyte in the 16-cell germ cyst

(*BicaudalD*, *egalitarian*). Many of the “abnormal egg shape” class produced cup-like small eggs in which apparently the nurse cell content had not been dumped into the oocyte. A small number displayed a dorsalization of the follicle and embryos displayed a dorsalisated phenotype (*fs(1) K10*, *cappuccino*, *spire*) or a ventralisation (*gurken*, *torpedo*, *cornichon*). A third class of spindle shaped eggs variably produced bipolar oocytes with micropyles at both ends. About 50 maternal effect lethal complementation groups with normal egg shape were identified, which failed to develop a normal cellular blastoderm but showed various defects in cleavage and cellularisation (*aurora*, *giant nuclei*, *polo*).

A large-scale screen for female sterile mutants induced by a P-element insertion was carried out in which mostly alleles of previously identified genes were found, which conveniently provided entry points for cloning the genes (Cooley et al., 1988). In more recent years, screens for female-sterile mutants with more sophisticated set ups featured oogenesis as an important target for mutagenesis in which many cell-biological processes have been analysed and studied in detail (Review: (Bastock and St Johnston, 2008)) (see BOX and Figure B3). Interestingly, most of the major players in oogenesis and maternal control of egg development have been identified by their strong phenotypes in the first EMS-screens; and the later screens, while turning up alleles of the previously identified genes, enriched the collection mainly by modifiers and modulators with less characteristic phenotypes.

3.9. Axis determination

Mutants of a total of about 30 loci (combined from the screens of all three major chromosomes) developed a normal cellular blastoderm, but were affected in the establishment of the anteroposterior or dorsoventral embryonic axes. The total number of these maternal patterning genes, which are specifically required for axis determination of the embryo, is less than 40 (Review: (St Johnston and Nüsslein-Volhard, 1992)). One striking result was that there was a much smaller set of observed phenotypes than identified genes, and groups of genes shared a common or very similar phenotype, which is in contrast to the observation on zygotic mutants. In one group of genes (the dorsal-group of eleven genes (*dorsal*, *nudel*, *pipe*, *snake*, *easter*, *spätzle*, *pelle*, *tube*, *Toll*, *windbeutel*, *gastrulation defective*) loss of function mutations lead to a dorsalisated phenotype, like *dorsal*, or a partially ventralised phenotype (*cactus*), like the dominant *Toll* alleles. These dorsal-group genes operate in a complex pathway via the activation of the Toll-receptor in the ventral egg membrane to finally result in a gradient of nuclear Dorsal-protein that determines position along the dorsoventral axis in a concentration-dependent manner (Anderson and Nüsslein-Volhard, 1984) (Review (Chasan and Anderson, 1993; Nüsslein-Volhard, 2022)).

In contrast, patterning along the antero-posterior axis depends on three systems defining distinct spatial domains (Nüsslein-Volhard et al., 1987). The three AP-groups, largely independently, control the development of the head and thorax, the abdomen, and the terminal regions of the larva, the acron and telson (Fig. 22). In the anterior group (*bicoid*, *exuperantia*, *swallow*, *staufer*) head and thorax do not develop, and in *bicoid* the acron is replaced by a telson (Review: (Driever, 1993)). The posterior group develops a phenotype lacking the abdomen, while head, thorax and telson are present (*oskar*, *vasa*, *staufer*, *valois*, *tudor*, *nanos*, *pumilio*). In mutants of this group, with the exception of *nanos* and *pumilio*,

also the pole cells are lacking (Review: (St Johnston, 1993)). Mutants of the terminal group (*torso*, *torsolike*, *trunk*) lack the unsegmented termini, the acron (anterior) and telson (posterior) (Review: (Sprenger and Nüsslein-Volhard, 1993)).

The analysis of these genes using cytoplasmic transplantations between mutant and wild type embryos revealed the existence of localised activities consisting of *bicoid* and *nanos* mRNA at the anterior and posterior egg pole, respectively ((Berleth et al., 1988; Driever et al., 1990; Frohnhöfer and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1986, 1991)). These control the anterior pattern, and the abdomen, respectively, by acting as localised sources for the proteins that spread and form a morphogen gradient determining position in a concentration dependent manner (Driever and Nüsslein-Volhard, 1988a, b; Wang and Lehmann, 1991). The transcription factor Bicoid activates different target genes above certain thresholds, and provided the first demonstration of the long-postulated morphogens (Fig. 23).

Chimeras, in which a mutant germline is surrounded by wild type somatic follicle cells during oogenesis, and vice versa, revealed signalling between follicle cells and oocyte and identified localised cues from the follicle cells that trigger gradient formation in the cases of the dorsoventral and terminal systems (Stein et al., 1991; Stevens et al., 1990). The *pipe*-signal, expressed in the ventral follicle cells, in turn depends on two genes that affect the polarity of the egg chamber (*gurken*, *Torpedo*) (Neuman-Silberberg and Schupbach, 1993). This extracellular signal is transmitted via receptor signalling cascades to the interior of the egg. *Toll* activation on the surface of the embryo results in the graded nuclear uptake of the Dorsal transcription factor. Dorsal activates a number of zygotic target genes, e.g. the transcription factors *twist* and *snail* that determine ventral furrow formation (Leptin and Grunewald, 1990; Roth et al., 1989), *short gastrulation (sog)*, an antagonist of *decapentaplegic*, and *rhomboid*, a member of the EGF signalling group of the midline genes that determine the ventral epidermis (see Fig. 21). Dorsal also represses the transcription of the zygotic genes *tolloid*, *zen* and *decapentaplegic*.

In the case of the terminal system, a signal produced by the somatic border cells and the posterior follicle cells triggers the activation of the Torso-receptor tyrosine kinase, resulting in short range gradients controlling gap gene expression at the termini (Fig. 24).

It came as a surprise that the determination of the acron and telson in the larva by the terminal system occurs independently from patterning of the trunk (with head, thorax and abdomen), and without mutational analysis this would have probably remained undetected.

3.10. Modern developmental genetics: reporter screens, clonal screens of all tissues

Transgenic reporter lines with the *LacZ* gene under the control of a *hsp70* minimal promoter and distinctive enhancers enabled tissue-specific labelling by staining for beta-galactosidase activity. Such lines were systematically produced in so called enhancer trap screens, in which the transgene was mobilised and progeny screened for specific expression in particular cells or tissues of interest (O’Kane and Gehring, 1987). Transgenic lines expressing fluorescent proteins in specific cell types, first applied in *C. elegans* (Chalfie

et al., 1994), allowed easy detection of morphological deviations in the tissue of interest without staining in genetic screens.

The yeast transcriptional activator Gal4 was introduced to regulate gene expression in *Drosophila* by inserting the upstream activating sequence (UAS), to which it binds, upstream of a gene of interest (Brand and Perrimon, 1993). Tissue specific expression of GAL4 then drives the transcription of a reporter, or a transcriptional activator (or, in fact, any gene of interest) in a specific cell type. Such systems were widely used, for example, the peripheral nervous system was analysed genetically in much detail (Review: (Jan and Jan, 1993)).

3.11. Cell migration

Germ cell migration in *Drosophila* provides a model system for the study of cellular movements and cell-cell interactions (BOX Fig. 1) An F3-zygotic saturation screen of the second and third chromosome was performed monitoring germ cell migration in embryos transgenic for the *fat-facet-lacZ* gene to stain germ cells easily (Moore et al., 1998). In this screen, many new alleles of known patterning genes such as segmentation genes, genes affecting gut development (*huckebein*, *serpent*) and mesoderm formation (*tinman*, *heartless*) were identified because they affect the normal development of the path or target of migration. Interestingly, all mutants were zygotic lethal suggesting pleiotropic effects on other migratory processes. Further, all loci were expressed in the mesoderm rather than the primordial germ cells, which demonstrated that the embryonic germ cell program is controlled by maternally preloaded RNAs.

Among the genes that do not affect patterning of migration route, the gene *columbus* is expressed in the somatic gonad, attracting the germ cells to this tissue. It encodes HMG-CoA reductase, an enzyme which in insects is not involved in cholesterol biosynthesis, but instead, for germ cell migration, leads to the synthesis of a secreted, isoprenoid attractant for germ cells. Another gene, *wunen*, identified by a screen of homozygous deficiency embryos (Zhang et al., 1997b), which encodes a *Drosophila* homolog of mammalian lipid phosphatase, has been proposed to produce a repellent for the germ cells. In a misexpression screen (Rorth, 1996), to identify genes that lead to germ cell migration defects when activated either in the germ cells or in the mesoderm, a homolog of the *Drosophila wunen* gene, *wunen-2* was identified (Starz-Gaiano et al., 2001) demonstrating the use of this kind of screens to identify redundant genes. Lipid phosphatases act in both the surrounding and the migrating cells. This screen also revealed the nature of the main germ cell-expressed guidance receptor in the germ cells, the G-protein-coupled receptor (GPCR) Tre1 (Kunwar et al., 2003).

Parallel to these, systematic studies on border cells, a group of specialized cells that migrate toward the oocyte and are important for the differentiation of the egg (BOX Fig. 3), revealed cellular and molecular mechanisms of a different type of migration, called collective migration, where groups of cells move as a functional unit (Pocha and Montell, 2014). Many of the cellular and molecular principles learnt from the study of single and collective cell migration in models such as fly germ cells or border cells are found in other systems and defects in migration are a major cause of disease in humans, from immune deficiency to cancer.

3.12. Zygotic lethals with specific maternal effect phenotypes

The screens for maternal-effect mutations identified key players of pattern forming processes that are only required for embryonic patterning. Genes that are also essential at other times in development escaped detection because homozygous females would be lethal. To identify genes that maternally affect patterning of the embryo, but also are required for viability at later stages, clones of homozygous mutant cells were screened in a background of heterozygous somatic tissue. To facilitate the detection of such clones, they were induced in females carrying a dominant female sterile mutation, *ovo^{DI}*, which acts in the germ line (Perrimon and Gans, 1983). In heterozygous *ovo^{DI}* mutant females oogenesis is blocked very early. Females heterozygous for a mutagenized chromosome and a chromosome carrying the *ovo^{DI}* mutation were X-irradiated to induce mitotic recombination in the germ line and only females with clones would produce normal ovaries or eggs, which could then be scored for a maternal-effect phenotype. The screens (performed systematically for the X-chromosome) turned up a large number of mutants with rather variable phenotypes, and few with embryonic pattern phenotypes, which, in general, looked more irregular than those of viable maternal effect mutations. Fertilization with mutant sperm resulted in embryos that lacked both the maternal as well as the zygotic contribution. Two classes could be distinguished, those that were rescuable by a wildtype copy from the father, and those that were purely maternal. In this screen, a number of missing downstream components of signalling pathways (*dishevelled*, *corkscrew*, *hopscotch*) were found (Perrimon et al., 1989).

3.13. Clonal screens for female steriles and maternals with the FLP-FRT system

Because in the embryonic screens only the first essential function of the gene is detected, clonal screens proved to be very useful to detect the participation of lethal genes in later developmental processes such as oogenesis and imaginal disc development. Screening of mutant clones induced by X-rays is hampered by a rather low frequency. To increase the frequency of recombination, the Flp/FRT system of site-specific recombination from yeast was adopted. The site-specific recombinase (flippase, Flp) under the control of a heat shock promoter was introduced into the *Drosophila* genome (Golic and Lindquist, 1989), and strains were constructed that carried the Flp recombination target (FRT) sites close to the centromere for all major chromosome arms (Chou and Perrimon, 1992). To screen for maternal effects of zygotic lethal mutations a copy of the dominant female-sterile *ovo^{DI}* allele was placed on the chromosome arms already carrying the FRT sites. Germ line clones induced by heat-shock during late larval or pupal stages were scored for maternal effects. The same method was used in combination with *GFP-Staufen* as a read out for oocyte polarity (Martin et al., 2003). In this screen the *Drosophila* homolog of *C. elegans par 4* was identified as an important factor in establishing oocyte polarity (Martin and St Johnston, 2003).

Another screen for maternal effects in germ line clones of single F1 females was aimed at isolating mutants that were missed in previous maternal screens as well as zygotic lethals with maternal phenotypes (Luschnig et al., 2004). Females heterozygous for an EMS mutagenized FRT-chromosome and an FRT-chromosome carrying a ubiquitously expressed GFP insertion were raised and heat-shocked. Eggs were collected from single females and clones were recognized by the absence of GFP. These screens turned up alleles of

most of the previously identified maternal effect patterning genes (with the exception of those expressed in somatic cells), as well as a small number of novel genes with similar phenotypes to the previously identified genes of the four classes (*weckle*, *krapfen*, *didum*).

FLP-*ovo^{D1}* screens were done in numerous variations, aimed at specific developmental processes to be best studied in the female ovary (Reviewed in (Bastock and St Johnston, 2008)). The establishment of male and female germ line stem cell by a niche was dissected genetically (Fuller and Spradling, 2007; Xie and Spradling, 2000). Many signalling processes between the somatic follicle cells and germ cells operate during various stages of oogenesis, and many factors identified in the zygotic screens are also involved in oogenesis, such as *unpaired*, *decapentaplegic*, *Notch*, *Delta*, *bazooka/par3* and others.

Localization of *gurken* mRNA plays important roles in the establishment of both, the anterior-posterior and the dorsoventral axes during oogenesis. *gurken* encodes a secreted ligand of the TGF α class, its mRNA is closely associated with the oocyte nucleus and the resulting protein sequentially signals (via the EGF-receptor *torpedo*) first to the posterior and then to the dorsal follicle cells to determine the posterior pole and dorsal side of the egg chamber respectively (González-Reyes et al., 1995, Roth et al., 1995, Neuman-Silberberg and Schupbach, 1993). Following posterior *gurken* signaling, a polarisation of the oocyte occurs which depends on the *par*-genes, first discovered in *C. elegans*. As a consequence, microtubules reorganize, and mRNAs of *bicoid* (Berleth et al., 1988; St Johnston et al., 1989) and *oskar* (Ephrussi et al., 1991) are localised at the anterior and posterior egg pole of the growing oocyte. Oskar recruits other mRNAs to localize in the pole plasm such as *nanos* mRNA, required for abdominal development in the embryo and the Vasa protein, a germ cell determinant (see Box and Figure B3).

The Flp/FRT system was further explored and widely used in *Drosophila* for mutational analysis of homozygous clones in heterozygous individuals by many researchers investigating later developmental processes such as oogenesis, organogenesis and imaginal disc development. The Flp/FRT system makes it possible to carry out screens in almost any cell and at any stage of development, a unique strength of *Drosophila* (Germani et al., 2018).

3.14. Clonal eye screens

The *Drosophila* compound eye is a particularly useful target for mutant screens because it is not required for viability, and it has a sophisticated cellular pattern that allows identification of components in signalling systems between individual cells in an ideal way. The compound eye develops from an imaginal disc that produces about 800 ommatidia, that is 20-cell units of regular composition. Each ommatidium is composed of 8 photoreceptor cells labelled R1–R8, surrounded by 12 accessory cells. The receptor cells are arranged in a hexagonal stereotyped regular pattern, starting with the centrally located R8 cell. The R7 cell is added as the last cell, which makes this cell particularly useful as target, because its absence does not influence the arrangement of the other cells. The semi-crystalline arrangement of the ommatidia allows easy detection of any irregularities as a rough eye phenotype.

The first screens for mutants affecting R7 used the fact that R7 is the only photoreceptor cell that responds to UV light, and absence of phototactic behaviour resulted in the identification of two genes, *sevenless* (*sev*) and *bride of sevenless* (*boss*). Mosaic analysis showed that *sev* and *boss* are required in R7 and R8, respectively. Cloning revealed that *sev* encodes a receptor tyrosine kinase (RTK) and *boss* its ligand (Cagan et al., 1992; Hafen et al., 1987). A temperature sensitive-allele of *sev*, which is just sufficient to produce a near regular eye pattern, provided a sensitised genetic background to screen for dominant enhancers, which produced, already in F1- heterozygotes, a rough eye phenotype (Simon et al., 1991). Likewise, screens were performed for suppressors of a rough eye phenotype induced by overproduction of *sevenless*. These screens have been very successful in identifying a number of downstream components of the *sevenless* signalling pathway, which together with parallel studies of vulval development in worms (reviewed in Horvitz and Sternberg, 1991), made the first link between RTKs and the Ras/Raf/MAP-Kinase pathway, now recognized as major drivers in many human cancers. Furthermore, these components were found to participate generally in receptor tyrosine kinase signalling such as by the Torso receptor tyrosine kinase or the *Drosophila* epidermal growth factor receptor.

To dissect the formation of the ommatidia and the role of individual cell types further, FRT-lines were created with distally inserted marker genes that are cell autonomously expressed in all cells of developing and internal tissue, and in particular allow the detection of small clones in the eye, but also other tissue. As markers, chimeric proteins were designed with Myc peptides that are detectable by monoclonal antibodies and targeted to the nucleus or cell membrane respectively with proteins from *Drosophila* (P-element nuclear localization signal, or Notch transmembrane domain, respectively). They are placed under the control of a heat-shock promoter. This system is used to detect mutants in F1-genetic screens in homozygous clones (Xu and Rubin, 1993).

The rough eye phenotype allows an easy analysis of suppressors and enhancers of any RTKs mis-expressed in the eye. For this purpose, the gene to be examined is placed under the control of a *glass* promoter or the *sevenless* promoter to specifically allow expression in the eye. Depending on the phenotype, suppressor or enhancer screens can be done to identify ligands or downstream components of the respective RTKs, also from humans or other animal systems.

3.15. Imaginal discs

Imaginal disc development and genetics is reviewed by Cohen (1993) (Cohen, 1993). Numerous visible mutant alleles from traditional collections and screens are affected in structures derived from imaginal discs such as those affecting wing and leg development and integrity, *engrailed*, *wingless*, *vestigial*, *Notch*, *Serrate*, *cubitus interruptus* (*ci*), *dachsous*, *distalless*, *decapentaplegic* (*dpp*). These mutants frequently turned out to be viable weak partial loss of function alleles of embryonic lethals. The positioning of the anlage of the imaginal discs and abdominal histoblasts on the fate map is determined by the segmental expression of *wingless* in the anterior compartment of each segment, and *decapentaplegic*, which is expressed in longitudinal stripes bounding the lateral side of the epidermal anlage (St Johnston and Gelbart, 1987). The initial organization of the discs is derived from the

embryonic segmental organization, in which the secreted factors *hedgehog* and *wingless* provide a positive feedback loop resulting in the expression of the transcription factors Engrailed in the posterior and Cubitus interruptus in the anterior compartment. In the wing, a Dpp morphogenetic gradient with maximum along the anteroposterior compartment boundary specifies the wing pattern (Fig. 20).

By expressing FLP in larvae under the control of a heat-inducible promoter, any embryonic phenotype can be bypassed by specifically inducing clones in the imaginal discs. Flp/FRT screens used this approach to identify mutants that affect the growth and patterning of the discs on the basis of their visible adult phenotypes (Böhni et al., 1999; Tapon et al., 2002) and also investigated the role of other genes in twin spot clones. They also identified novel components of the integrin adhesion complexes that hold the two surfaces of the wing blade together (Prout et al., 1997). Clones induced in a genetic background with compromised cell proliferation due to dominant mutations called *Minute* can get very large and easy to score.

Further numerous screens have been performed for internal organs, mesoderm, flight muscles, heart, Malpighian tubules, tracheae using the methods described above. As screens have become more sophisticated, they have used more and more complex genetic backgrounds. There is an endless spectrum of possibilities for further unbiased discoveries using genetic screens. With the advent of genomic tools and analyses ends our survey, as the novel approaches of genomics, reverse genetics and gene editing have widened and extended the field beyond the scope of this article.

3.16. *Drosophila*: Impact and outlook

What are the most significant contributions to biological research? The big breakthrough in *Drosophila* was to focus on the larva instead of the adult fly and screen the structures of the cuticle secreted by the larval hypodermis. The first saturating screen for pattern mutants in a multicellular organism, the Heidelberg screen, resulted in a collection of mutations in 120 genes that affect the patterning or structure of the larval cuticle. These genes provided a core collection, a rich resource for investigating many fundamental processes in the development of embryos and attracted many researchers to a fast-growing *Drosophila* community. They include components of the major intercellular signalling pathways: *Notch*, *decapentaplegic* or *BMP*, (the protein was first described), *spitz* or EGF, (first molecularly identified), *Toll*, *Hedgehog*, and *Wingless*, now called *Wnt* because of its homology with a mammalian oncogene called *int*. These signalling systems are all conserved and play key roles in vertebrates. Developmental genetics of *Drosophila* led to the discovery of the homeobox, and the co-linearity of Hox genes determining anteroposterior patterning in invertebrates and vertebrates. These achievements were honoured with the Nobel Prize awarded to Edward Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus in 1995 “for their discoveries concerning the genetic control of early embryonic development”.

In many respects, *Drosophila* development is quite special. In particular, *Drosophila* embryogenesis is characterized by an initial period in which the cleavage nuclei are not separated by cell membranes (BOX Fig. 1). This syncytial stage allows the hierarchy of transcription factor prepatterns to develop; these processes do not have parallels in *C. elegans* nor in early vertebrate development. The establishment of the embryonic axes by

localized determinants in the form of RNA or extracellular cues is not even conserved in closely related insect species. In the analyses of the four systems of axis determination many details were elucidated by experiments involving transplantation of cytoplasm or mRNA from and between mutant and wildtype embryos. The transcription factor Bicoid, which is distributed in an exponential gradient in the early *Drosophila* embryo, is the first clear case of a morphogen that determines position in the embryo by its concentration, which in turn depends on its source of localised RNA. The Bicoid gradient is special as it distributes in a syncytial embryo without cell walls. The Dorsal morphogen, again a transcription factor, determines position along the dorsoventral axis by a gradient of nuclear localization. Decapentaplegic or BMP is the first described secreted morphogen which distributes in the extracellular space; it determines the dorsal pattern of the embryo as well as numerous features in the development of imaginal discs in a concentration dependent manner. BMP related proteins are components of the Spemann organiser orienting the gastrulation pattern in vertebrate embryos. The four systems of axis determination belong to those most thoroughly understood, offering many aspects of molecular and cellular diversification to unravel with modern biophysical and biochemical approaches such as following single molecules using optogenetics.

Drosophila as a holometabolous insect develops a plethora of adult structures, which arise in the 15 imaginal discs and allow numerous investigations of patterning and structure differentiation unparalleled in other organisms. The ability to analyse the function of genes with multiple roles in development in mitotic recombination clones produced by the Flp/FRT system (which is unique in *Drosophila* as it is based on its small number of chromosome arms, and pairing of homologous chromosomes during mitosis) is a powerful achievement as it allows to investigate the contribution of early lethal genes in adult structures, in fact in any stage of development. This type of research in *Drosophila* has been instrumental in analysing many components that underlie growth and differentiation in various developmental processes. These pathways participate in the structuring of organs and tissues in all metazoans.

The ability to create mutants in known genes in mice by homologous recombination in embryonic stem cells allowed to test the function of genes in a vertebrate organism. Reverse genetics in mice in fact profited much from the availability of gene sequences from *Drosophila*. Most genes discovered in the Heidelberg screen have human orthologs, and a significant fraction have homologs causing congenital diseases in humans or are known as human oncogenes (reviewed in (Wieschaus and Nüsslein-Volhard, 2016)).

Driven by the homology of many players in similar developmental processes in vertebrate organisms, investigations in flies revealed details that could then be verified and elaborated on. An interesting example is the similarity in regulation of trachea development with blood vessel formation in vertebrates. In contrast, segmentation, reflected in the formation of the metameric somite pattern, occurs differently in vertebrate embryos compared to *Drosophila*. Genes active in somite formation encode *Delta* and *Notch* homologs that act in conjunction with a segmentation clock that subdivides the AP axis into metameric units (Holley et al., 2002; van Eeden et al., 1996). The Hedgehog pathway is involved in shaping the vertebrate

somites. Only one *Drosophila* segmentation gene, a *hairy* homolog (*her-1*), participates in the segmentation clock (Holley et al., 2002).

Although in most cases the anatomy and function suggest where to look for homologies, there have been surprises of seemingly unrelated functions of homologous pathways such as the Toll-dorsal pathway, which maternally controls the formation of the dorsoventral pattern in the embryo, and zygotically acts as innate immunity system against pathogens in *Drosophila* and in most metazoans (honored by a Nobel prize for Jules Hoffmann 2011 for discoveries concerning the activation of innate immunity). This pathway does not operate in *C.elegans*, the single *Tol-1* gene has a different function. How the pathway has been adopted for dorsoventral patterning in *Drosophila* is still a mystery. This process is not highly conserved even among arthropods (Reviewed in (Nüsslein-Volhard, 2022)).

With the whole genome sequencing (WGS) projects (Adam et al., 2000) and RNAi induced reverse genetics, the paradigm of genetic approaches changed considerably, as any gene known by its sequence can now be tested by creating loss of function conditions for its participation in particular pathways. In *Drosophila* the WGS allowed the silencing of selected genes, until libraries covering almost the entire genome were constructed (Dietzl et al., 2007). An important aspect of these whole genome approaches is that in addition to finding missing components, components can also be excluded from participation in a given pathway. More recently, CRISPR-Cas9 gene editing complements the repertoire of tools to maintain *Drosophila* as one of the best-understood organisms in our world.

4. Discussion

4.1. Large-scale genetic screens

When *Drosophila* and *C. elegans* took off as genetic models of development, they were chosen as organisms simple enough to hope for answers to difficult questions in development, and complex enough to offer interesting developmental processes for genetic dissection. At this time, the general notion was that the diverse animals studied by developmental biologists had individual strengths but not much in common. Communities working on each organism – ascidians, chickens, flies, frogs, slime moulds, snails, sea urchins and worms – were largely independent, and it was not expected that analyses of development in *Drosophila* or *C.elegans* would yield results of importance beyond the specific organism, let alone human biology.

Though worms and flies are vastly different organisms, the common approach was to conduct large scale screens for identification of genes controlling development. Genetic screens ask the genome which molecules are important for a given process. A chemical mutagen makes changes in the genome without regard to whether they affect the coding sequence of a transcription factor, a regulatory element or some apparently meaningless bit of sequence between genes. A mutant that alters the spatial pattern of a fly embryo or the lineage of a worm simply must encode something important, and that mutant therefore provides the “foot in the door” to molecular mechanism. This unbiased approach had the potential to find any genetically encoded regulator of developmental consequence.

The prime example is identification of a gene not encoding a protein, and leading to the discovery of a new mode of genetic control via non-coding miRNAs.

Genetics studies of flies and worms benefited greatly from the ground breaking successes of bacterial, phage and yeast genetics, which emphasized efficient experimental design and rigorous interpretation. This background set a high bar for those taking the same approach in more complex systems and shaped their efforts. The idea of large-scale saturation screens to discover most, if not all genes controlling a developmental process in a multicellular animal came from work with these simpler systems. Unlike most scientific advances, genetic screens did not depend on new technology: they are low tech and could have been done in the 30ties! However, their impact would not have been the same in an earlier era. Although the initial driving force was to understand the logic and complexity of development, the concurrent rise of methods for gene cloning and DNA sequencing soon expanded that initial goal to include molecular identification of key regulatory genes and their biochemical functions.

4.2. Ancient mechanisms of development

Arguably the most important principle to emerge from developmental genetics is the ancient nature of developmental pathways in the animal kingdom. Discovery of the homeobox in *Drosophila* and the co-linearity of hox-clusters patterning the anteroposterior axis of metazoan animals was the first example and a huge surprise. However, many examples followed. Worm *lin-12* and fly *Notch* were homologs, also a big surprise; EGF signalling pathways were found in both flies and worms; and perhaps most remarkably, BMP-related growth factors act in flies and vertebrates to orient the dorsoventral axis of these vastly different organisms. In retrospect, one wonders why this was so surprising. Scientists accepted that metazoans have a common ancestor, but did not expect the molecular regulation of their development to be ancient. At that time, the extraordinary range of animal developmental strategies and morphologies made it seem more likely that the underlying molecular principles would be totally different. Once developmental genes were cloned, the many homologies quickly overturned that idea. It came as a revelation that the spatial distribution of the gene products in embryos often displayed relationships much better than their morphologies and opened up a new field of Evolution and Development (Evo-Devo). Most invertebrates are protostomes and belong to one of two large clades, Ecdysozoa and Lophotrochozoa. Worms and flies belong to the Ecdysozoa clade, which are moulting animals that grow in each larval stage predominantly by endoreplication and an increase in cell size rather than cell number. The evolutionary split between Protostomia and Deuterostomia, which separated worms and flies from chordates (vertebrates) occurred soon after the last common metazoan ancestor, the Urbilaterian. Urbilaterians must therefore have already been organised by a basic developmental “toolkit” of regulators and signalling systems. The ability to use genes from *C.elegans* and *Drosophila* to understand vertebrates, despite the phylogenetic distance between the Ecdysozoans and vertebrates (humans), had tremendous impact.

4.3. Flies and worms have complementary strengths

Both worm and fly are rather special organisms and many aspects of their development may not serve as a general model. Both have a somewhat stingy body organisation, many fewer cell types than vertebrates, and are streamlined for fast reproduction in favourable conditions. However, both also have features that can be used to analyse complex pathways and regulatory networks in molecular depth. Worms have little external morphology with no eyes or limbs, but a rich interior with defined cell lineages, migrations and interactions, allowing development to be explored in exquisite detail. They also have a short generation time and self-fertilization, which allows screening large numbers quickly. That strength promoted detection of rare alleles, such as gain of function dominant mutations or mutations in tiny genes. Flies, on the other hand, as holometabolous insects, have a rich external morphology. They are more difficult to breed in large numbers, and the effort to set up enough lines for saturating F3 screens is considerable. However, the history of fly genetics provided a set of balancer chromosomes and other genetic tools to facilitate screens, and the Flp/FRT recombinase system allows analysis of phenotypes in almost any tissue at all stages of development in clones produced by mitotic recombination. Even though many structures and processes are restricted to these organisms (such as the vulva and dauer formation in *C. elegans*, or the compound eye and syncytial embryos in *Drosophila*), the underlying regulatory mechanisms, such as cell signalling and gradient formation, teach us about how complex animals develop. They also allow us to admire the richness of regulatory mechanisms, feedback loops, repair processes, and the different ways by which similar body plans can be achieved.

Early concepts of development, such as cytoplasmic determinants, cell- and tissue induction, morphogen gradients and asymmetric cell division have been validated in the small invertebrate models, but in addition, their cellular and molecular underpinnings have been revealed for the first time. Although each mechanism is used in a unique way in worms and flies, work from the two organisms demonstrates how major developmental principles operate. In addition, certain developmental mechanisms were simply more accessible in flies or worms. The Hox-clusters, conserved transcription factors of anteroposterior patterning, and long-range morphogen gradients were found in the fly with its larger embryo and imaginal discs. By contrast, programmed cell death was discovered in the worm, a transparent animal with a fixed lineage; a stem cell niche was found first in worms with its dramatically reduced cell number; and discovery of genes controlling life span was facilitated by its short life span. The importance of these unique features for finding fundamental regulatory mechanisms seems logical in retrospect. Finally, the parallel efforts in worms and flies quickened the pace of discovery. Their short life cycles and simple development were certainly factors in accelerating progress, but more interestingly, findings in one animal often aided interpretations in the other as pathways were unraveled.

4.4. Relevance to vertebrate development

The relevance of *Drosophila* and *C.elegans* to vertebrate development extends far beyond molecular homologies. Vertebrates continuously replenish many tissues using stem cells, and invertebrates use stem cells to replenish their germline tissue as gametes are made. Indeed, the stem cell niches in worms and flies together laid the groundwork for identification

of stem cell niches in vertebrates; both fly and worm niches rely on conserved signalling pathways that also control stem cells in vertebrates, albeit different ones in worms and flies. Flies also have stem cells in their intestine, which has become another model for stem regulation. A vertebrate-specific evolutionary innovation is the neural crest (NC), multipotent highly migratory cells that add body elements to organs derived from the three germ layers such as the head bones and the pigment cells. *Drosophila* and *C. elegans* do not have a neural crest, but their studies of cell migration may serve as models for collective migration. In recent years, organoids, derived from mouse or human induced pluripotent stem cells, have emerged as new models to study mammalian development, and human organoids in particular provide a unique window into human biology. In addition, gene editing now allows unbiased screens to be done directly in mammalian cells. But the days for screens in flies and worms are not over! Compared to mammalian models, these little invertebrates are much easier to grow, faster to analyse and more powerful for dissecting underlying cellular and molecular mechanisms. They are also much cheaper. When genes or processes cannot be investigated in flies or worms, the zebrafish *Danio rerio*, a vertebrate model with transparent development, a short life cycle and strong genetics may help bridge the gap between the invertebrates and mammals.

4.5. Whole genome sequences and human disease

The era of whole genome sequencing transformed research in biology and medicine. The first metazoan genomes sequenced – worms (1998), flies and humans (2000) – were a huge achievement. Whereas their genomes range 30-fold in size, they have comparable numbers of protein-coding genes (~20,000 in worms, ~14,000 in flies and ~2 4000 in humans). Moreover, genome comparisons revealed that flies and worms have many thousands of homologous genes, both with each other and with humans.

It was a big surprise that *C. elegans* and *Drosophila* are so close in gene number and core proteome (about 9000, Rubin et al., 2000), despite their considerable differences in morphological complexity. The worm has an anterior nerve ring that serves as its brain, instead of the much larger and more complex brain in flies. Moreover, worms lack segments, limbs, eyes and pigmentation, have many fewer cell types than flies and a simpler body organization. Why do worms have thousands more genes than flies? The answer appears to lie, at least in part, in the number of duplicated genes – flies have thousands fewer than worms (Rubin et al., 2000). The lower number of duplicated genes benefits fly genetics, because traditional screens do not identify functionally redundant genes. On the flip side, the lower redundancy is coupled to higher pleiotropy: an unduplicated gene often acts at multiple points in development and in more than one tissue, whereas duplicated genes often have evolved to assume stage- and tissue-specific roles. Luckily, new methods help to circumvent these traditional problems of redundancy and pleiotropy. For example, RNAi and CRISPR/Cas9 gene editing can deplete the functions of two or more nearly identical genes and can also be targeted in a tissue-specific manner.

The *Drosophila* and *C. elegans* communities have developed sophisticated genetic and molecular tools that make these two small invertebrates primed to make new discoveries in both fundamental science and human disease. RNAi and CRISPR/Cas9 gene editing

have revolutionized their genetics and new fluorescent probes have transformed the ability to see cells, organelles and molecules. Although many genes in the genome have known functions, many more remain complete mysteries. The age of discovery in the biological sciences is far from over. Researchers working with worms and flies continue to explore fundamental developmental biology of course, with an eye to finding what mechanisms are broadly conserved and what mechanisms lead to unique morphologies. The incorporation of new methods and new approaches continue to enrich this area. For example, exciting efforts couple development with physics, optogenetics, single molecule approaches, mass spectrometry and mathematical modelling. Researchers are also expanding into additional areas of basic research that complement developmental biology, such as physiology and environmental effects on animal biology.

The idea that flies and worms might be relevant to human disease was not on the horizon fifty years ago, but their relevance is now well established. Worms and flies not only share many proteins with humans, their genomes also encode orthologs of many disease genes – ~40% in worms and nearly 70% in flies from early estimates. Many conserved developmental regulators, first discovered in flies and worms, cause human disease when defective. The central roles of developmental genes in cancer became clear from their homology with mammalian oncogenes, but functions in tissues and organs once they have been formed in development, followed soon thereafter. The canonical signalling pathways are prime examples of relevance to both human cancer as well as tissue and organ health. Many ongoing efforts now rely on worms and flies as powerful tools to investigate human health and disease. One approach launched over the past 20 years or so is to conduct screens to identify the key genes affecting devastating diseases, such as obesity or bacterial pathogenesis. And finally, both worms and flies have been adopted as models for cost effective approaches to drug discovery. These new efforts promise a rich future for these great small model organisms.

Acknowledgements

The authors thank Uwe Irion, Ruth Lehmann, Siegfried Roth, Trudi Schüpbach, Susan Strome and Eric Wieschaus for comments on the manuscript, and Laura Vanderploeg, Eric Wieschaus and Uwe Irion for assistance with illustrations; Figs. 14–24 are redrawn by Laura Vanderploeg from Nüsslein-Volhard, C.: *Coming to life. How genes drive development*. Kales Press 2006. Eric Wieschaus provided BOX Fig. 1, and Uwe Irion BOX Fig. 3. JK is supported by NIH R01 GM134119; CNV is supported by the Max-Planck-Society.

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5.

BOX: Developmental systems covered by systematic mutagenesis screens in *Drosophila***Embryogenesis (BOX Fig.1)**

The *Drosophila* embryo develops outside the maternal organism in a relatively large egg ($500 \times 180 \mu\text{m}$) which is produced during oogenesis by the germ line-derived oocyte-nurse-cell complex surrounded by somatic follicle cells (BOX Fig. 3). The somatic cells produce the extraembryonic vitelline membrane and chorion, which cover the egg cell. The egg is filled with an opaque yolk. After fertilization, the cleavage nuclei migrate to the periphery and divide synchronously 13 times without forming cell membranes between them. This stage is called the syncytial blastoderm. Prior to that the pole cells, the future germ line cells, are forming in a clear cytoplasmic region (the pole plasm) at the posterior pole. At 3 h of development, the first cellular state is reached, consisting of a uniform layer of 6000 cells. At this stage, the cellular blastoderm, the cells by and large all look the same. During gastrulation, the anlage of the mesoderm folds in forming a furrow along the ventral side of the embryo. Cell groups at the front and back end of the embryo which will develop the intestine and the head, also begin to fold in. On its dorsal side the embryo stretches forward. In the stretched state the embryonic cells divide further, the nervous system is established, and the organization of organs and segments begins to appear. Further movements of tissues to their final positions characterise later development. Finally, differentiation takes place with cells specializing according to their function within the individual organs. After approximately 24 h the larva, about 1 mm long, hatches.

The heart of the larva develops on the dorsal side. There are no arteries or veins, but the colourless blood, the hemolymph, that carries nutrients circulates freely around the inner organs of the larva. Oxygen is not carried by the blood but rather enters the larva through an extensive network of air-filled tracheae, which is connected to the outside by paired stigmata situated at the front and back. The central nervous system of the larva consists of a brain and a rope-ladder shaped ventral nerve chord, both of which develop from individual cells derived from the ectoderm, called neuroblasts. The ectoderm develops the outer covering of the headless larva, called hypoderm, which secretes a cuticle richly decorated with a characteristic segmental pattern of rows of denticles on the ventral- and fine hairs at the dorsal side of the larva.

The primordial germ cells develop from the pole cells (BOX Fig. 1 in red) which migrate and localize in the fat body, the organ with functions analogous to a mammalian liver, together with the somatic gonadal precursor cells derived from abdominal segments. During gastrulation, the pole cells move along the dorsal surface of the embryo, along with the posterior midgut primordium. They then migrate through the wall of the posterior midgut to the dorsal side of the embryo and toward lateral mesodermal cells. As the germ band retracts, primordial germ cells associate and align with mesodermal cells that will give rise to the somatic component of the gonad. Finally, the primordial germ cells and gonadal mesoderm coalesce to form the embryonic gonad.

Fate maps of the larval hypoderm at the blastoderm stage have been constructed using radiation of small groups of cells with a laser microbeam which cause local defects in the larval cuticle (Lohs-Schardin et al., 1979), as well as using larval gynandromorphs in which the female part of the larval hypodermis was visualized by histochemical staining for aldehyde oxidase, lacking in male *maroonlike* mutant cells (Szabad et al., 1979). The anlagen of the epidermis cover a large continuous region from 15% to 60% egg length, with each segment occupying a horizontal strip of 3–4 cell diameters. At the ventral side the anlage of the mesoderm that involutes during gastrulation separates the hypodermal anlagen of the right and left side (BOX Fig. 2).

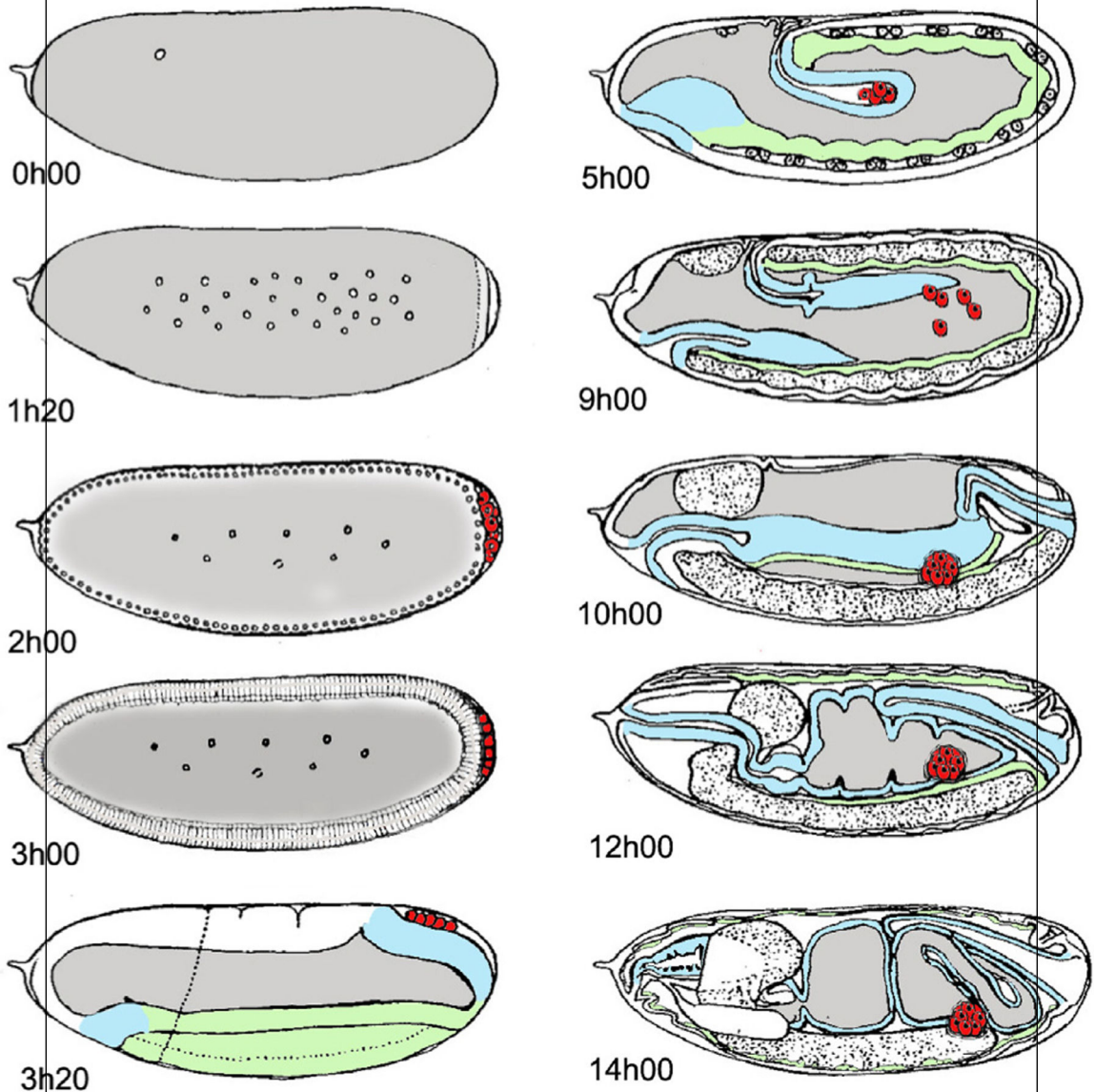


Figure B1. Embryogenesis.

During cleavage, a syncytial blastoderm is formed in which all nuclei share the same cytoplasm. By 3 h, cell membranes have formed, separating the nuclei to give rise to the cellular blastoderm. During the process of gastrulation groups of cells giving rise to

the endoderm (light blue) and mesoderm begin to invaginate. The embryo forms a germ band that stretches anteriorly at the dorsal side. In this stage, the neuroblasts form in the epidermis, and the pole cells, which are incorporated in the posterior midgut, migrate through its wall towards the posterior mesoderm. In later stages, the germ band retracts, the nervous system connected to the brain develops along the ventral side and the organs further differentiate.

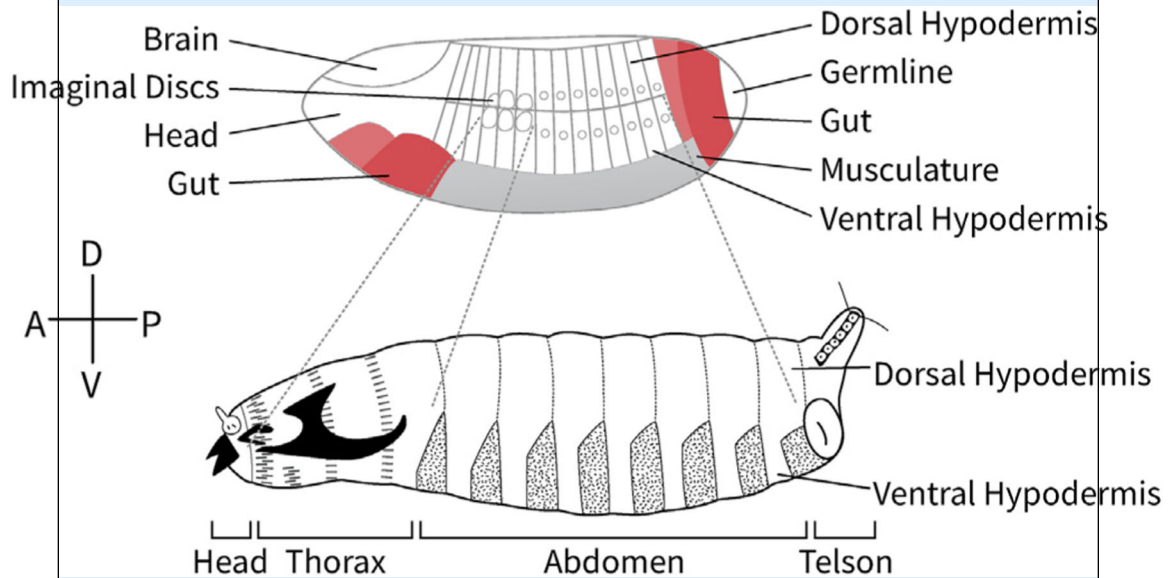


Figure B2. Fate map of the embryo.

The upper figure indicates which regions of the embryo give rise to which larval or adult structure. The segmented area of thorax and abdomen is derived from a large central region of the embryo. Within this region, each segment consists of a strip of just three cells wide. Most of the anterior and posterior regions of the embryo move inside the embryo during gastrulation to form inner organs such as muscles (grey) and digestive tract (red). Most of the head structures also involute to result in an essentially headless maggot.

Oogenesis (BOX Fig. 3)

Oogenesis takes place in two paired ovaries composed of about 18 strings each of egg chambers in successive stages of development (ovarioles), the anterior tip being the youngest reaching the posterior as mature eggs. Two germ line derived stem cells lie at the tip of the ovariole next to two somatic cap cells, they divide asymmetrically such that one of the daughter cells remain associated with the cap cell and remains stem cell while the other cell divides four times to form a cyst of 16 cells interconnected by channels, so called ring canals.

One of these 16 cells will develop into the oocyte, while the others become polyploid nurse cells, synthesising nutrients and cytoplasmic components to be transported into the oocyte. The cysts are enveloped by an epithelium of somatic follicle cells and bud off as egg chambers while the oocyte, which lies at the posterior, grows. The oocyte undergoes meiosis. The egg chamber increases in size and the follicle cells proliferate. At

mid-oogenesis, the nurse cells occupy about as much space as the oocyte, and columnar follicle cells cover the oocyte. The oocyte nucleus has now moved to the dorsal anterior corner of the oocyte. A group of about 6 somatic cells, called border cells, migrate from the anterior tip of the follicle through the nurse cells to collect at the anterior of the oocyte. Follicle cells at the posterior also differentiate as border cells, while the follicle cell layer stretches over the growing oocyte, under the nurse cells to form a shell that encompasses the entire oocyte at later stages. Dorsal anterior follicle cells produce the dorsal appendages of the chorion (Fig. B3).

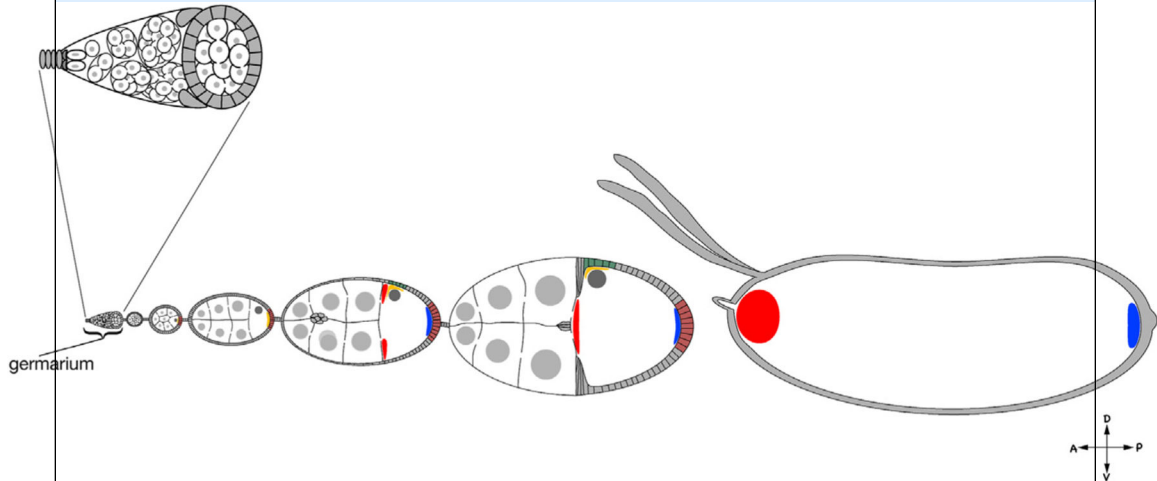


Figure B3. Oogenesis, RNA localization.

In the germarium of an ovariole (enlarged) two germ line stem cells are located at the anterior tip (left) close to two somatic cap cells. The progeny from asymmetric stem cell division form cysts of 16 germ line derived interconnected cells. The cysts are enveloped by an epithelium of somatic follicle cells (grey) and bud off as egg chambers while the oocyte, which lies at the posterior, grows. At the beginning of oocyte maturation, the *gurken* RNA (yellow) is located at the posterior tip. At midoogenesis, it has moved together with the oocyte nucleus to the dorsal anterior of the oocyte. Signalling to the follicle cells determines their posterior and dorsal fate. A ring of localised mRNA of the *bicoid* gene is appearing anteriorly, and at the posterior mRNA of the posterior group genes such as *oskar* are localised (blue). The mature egg (right) is covered by a vitelline membrane and chorion with its prominent dorsal appendages produced by the follicle cells.

Imaginal discs (BOX Fig.4)

Drosophila is a holometabolous insect, and the adult structures develop from sac like imaginal discs. These are two-dimensional sheets of undifferentiated epithelial cell populations set aside in the embryo and proliferating during larval growth, while most somatic cells of the larva just increase in volume without cell divisions. Most imaginal discs come in pairs, one each for the right and left side of the fly. There are three pairs for the legs, two pairs for wing, haltere and thorax, two pairs for most of the head with eyes and antenna, and a few smaller ones for head parts, genitalia and abdomen. Fate maps of the location of disc primordia in the embryo were constructed using

gynandromorphs (Garcia-Bellido and Merriam, 1969; Hotta and Benzer, 1972). These are mosaic flies arising from the loss of a special ring-shaped X-chromosome during the first cleavage division and consist of half male, half female tissue in random composition. The frequency by which two structures are of different genotype was used as a measure for the distance of the primordia at the blastoderm stage. These maps indicated that the distance between right and left disc primordium of a given type is large while the anlagen of the discs of the three thoracic segments of the same side are very close to each other (BOX Fig. B2). During pupation, which occurs after two larval molts, the imaginal discs differentiate into the various external body parts which fuse to create the seamless adult body of the fly (BOX Fig. B4).

By transplanting specific sections of a disc into larvae, where they undergo metamorphosis, fate maps have been produced of the various imaginal discs such as genital, leg, wing and eye discs. These imaginal discs can be cultured in the abdomen of adult females and tested for their developmental potential by transfer into larvae. Specific imaginal discs maintain their programming even when cultured in vivo for long periods of time. The phenomenon of “transdetermination” was discovered (Hadorn, 1963): cultured leg imaginal disc cells, for instance, could occasionally ‘transdetermine’ to form wing structures, similar to the homeotic transformations observed in mutants. The molecular basis for this phenomenon, however, remains elusive.

The relationship of the imaginal discs and the embryo was studied by cultivation of dissociated cells of anterior and posterior halves of embryos at the first cellular state, the blastoderm, in adult females. Metamorphosis in host larvae suggested that there may already be a restriction of the potential to form anterior or posterior structures at this early stage (Chan and Gehring, 1971). The induction of clones of marked cells by mitotic recombination at successive stages of development and gynandromorph mapping estimated that the number of imaginal disc progenitor cells at the cellular blastoderm stage is about 9–12 for the major discs, and that these progenitors divide about once in 11 h to produce mature discs of 22000–40000 cells in the third instar larva, whereas cells of the surrounding larval epidermis divide only twice, during embryogenesis. Interestingly, clones induced at the blastoderm stage overlap between leg and wing of the same segment, but not between adjacent segments, although by gynandromorph mapping the distance was estimated to be equal. This suggested a determination of segmental identity at this early stage (Wieschaus and Gehring, 1976). Clones induced in a genetic background with compromised cell proliferation due to dominant mutations called *Minute*, showed spectacular restrictions either to the anterior or posterior part of the imaginal disc, so called compartments (Garcia-Bellido et al., 1973). Mutations in “Selector” genes such as the homeotic genes respect these compartments as a unit of determination, suggesting that the early embryo is organized in “parasegments” bounded by the anterior-posterior compartment borders instead of the segmental boundaries. Clones homozygous for the selector mutant *engrailed* cross the compartment boundary in the wing, suggesting that the mutation causes posterior compartment cells to behave like anterior (Morata and Lawrence, 1975).

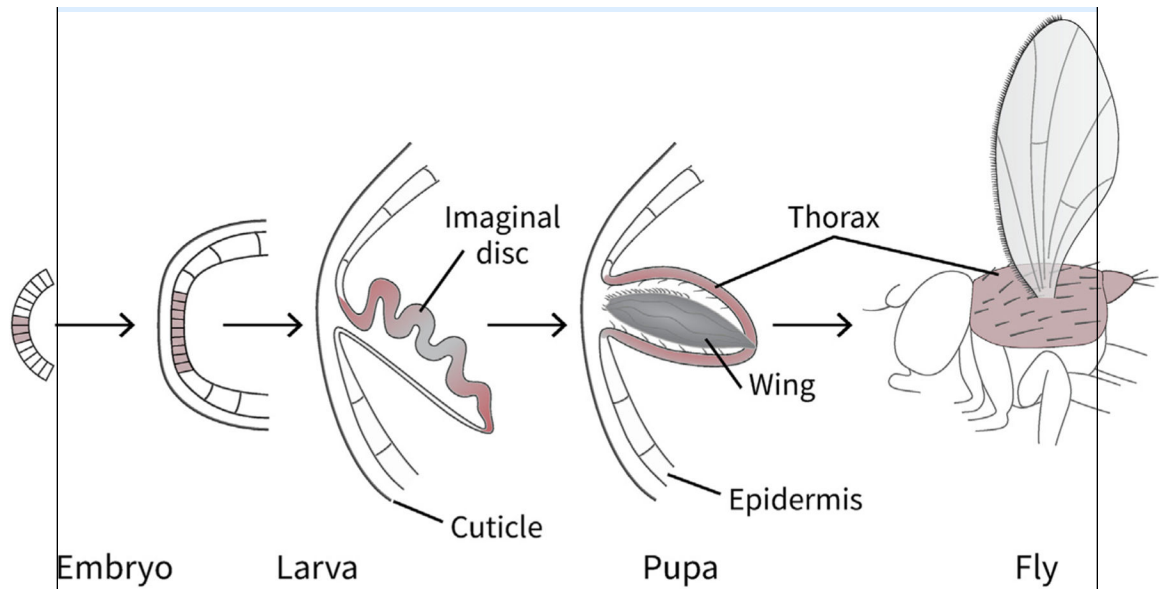


Figure B4. Imaginal discs.

The imaginal discs arise from a small number of cell located in the hypodermis of the larva. During larval development, the imaginal disc cells remain small and divide, whereas the surrounding larval cells increase in size without cell divisions. The discs develop into sac like folds and differentiate during metamorphosis in the pupa the adult structures which they unfold to connect with the structures derived from the other discs to form the seamless body of the adult fly.

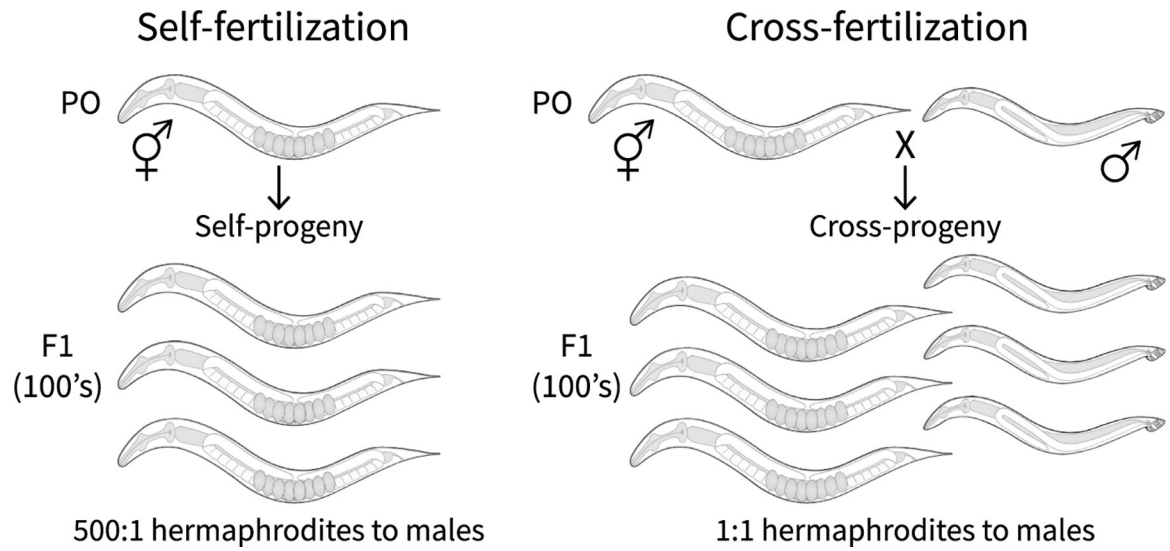


Fig. 1. *C. elegans* two modes of reproduction.

Left, self-fertilization generates self-progeny with a 500:1 sex ratio of hermaphrodites to males; right, cross-fertilization generates cross-progeny with a 1:1 sex ratio.

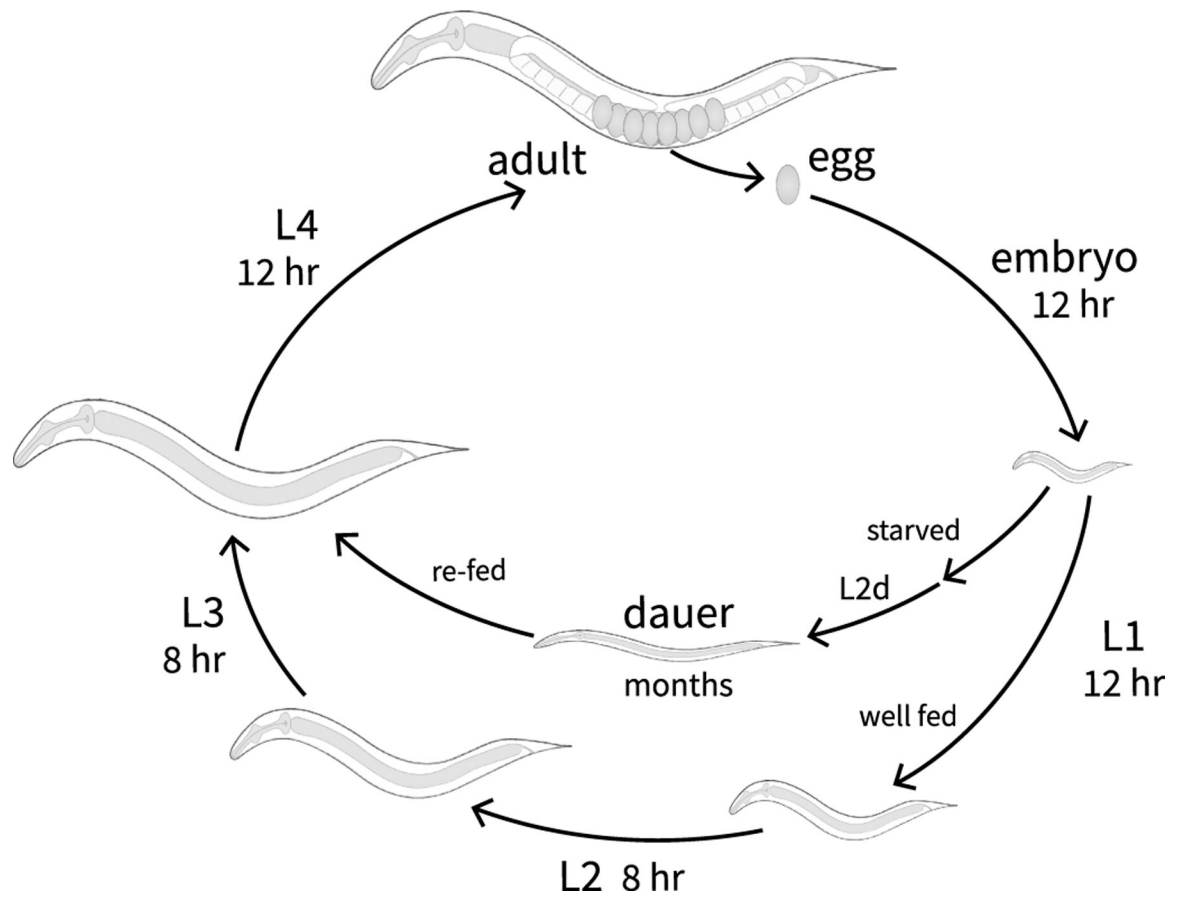


Fig. 2. *C. elegans* life cycles.

The life cycle of well-fed worms (outer) takes about 3 days; when starved, worms enter the alternative dauer larval stage with a much longer life cycle.

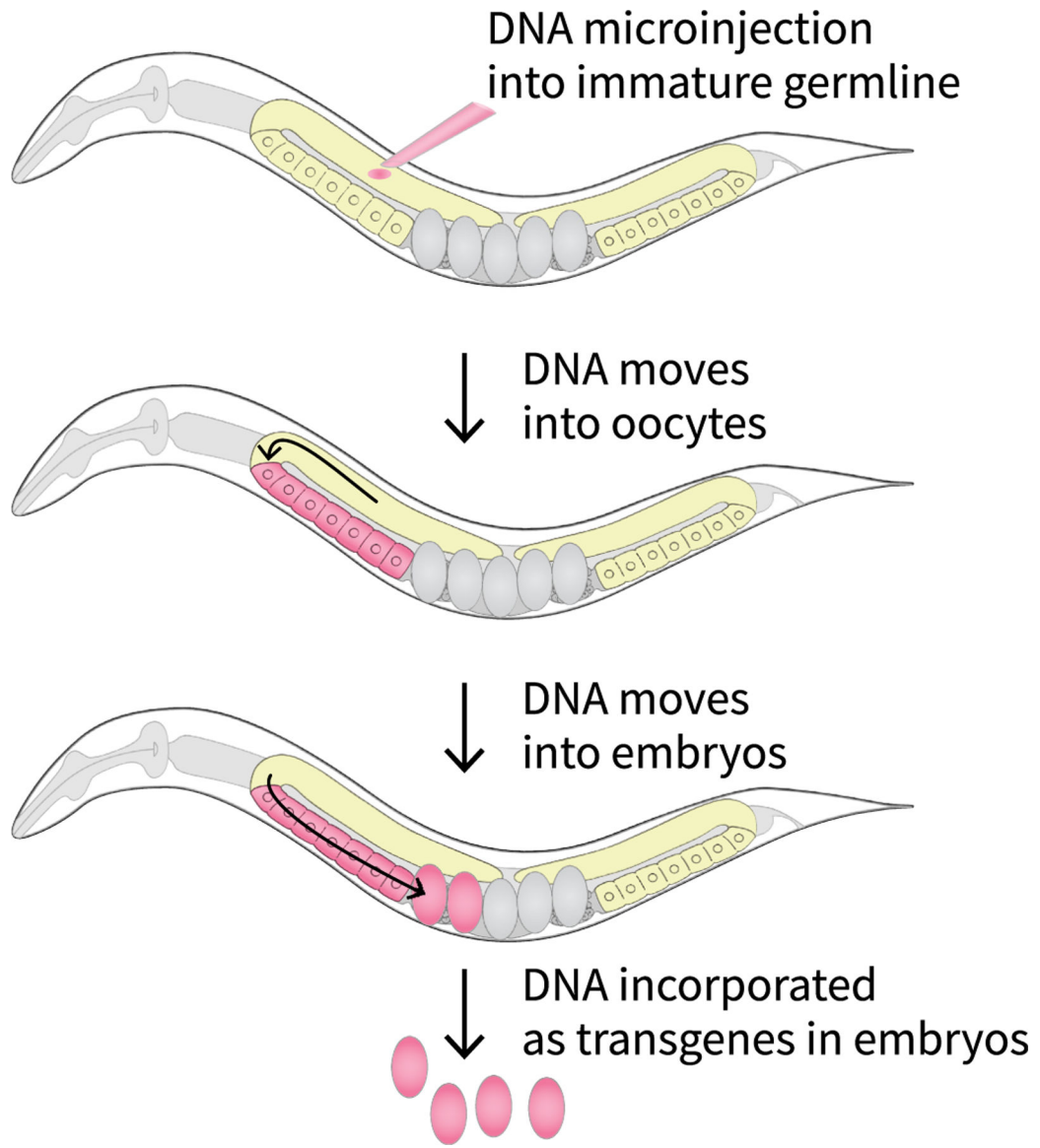


Fig. 3. Method for introduction of DNA into *C. elegans*.

DNA (pink) is injected into the germline (top), becomes incorporated into oocytes (middle) and finally into embryos (bottom).

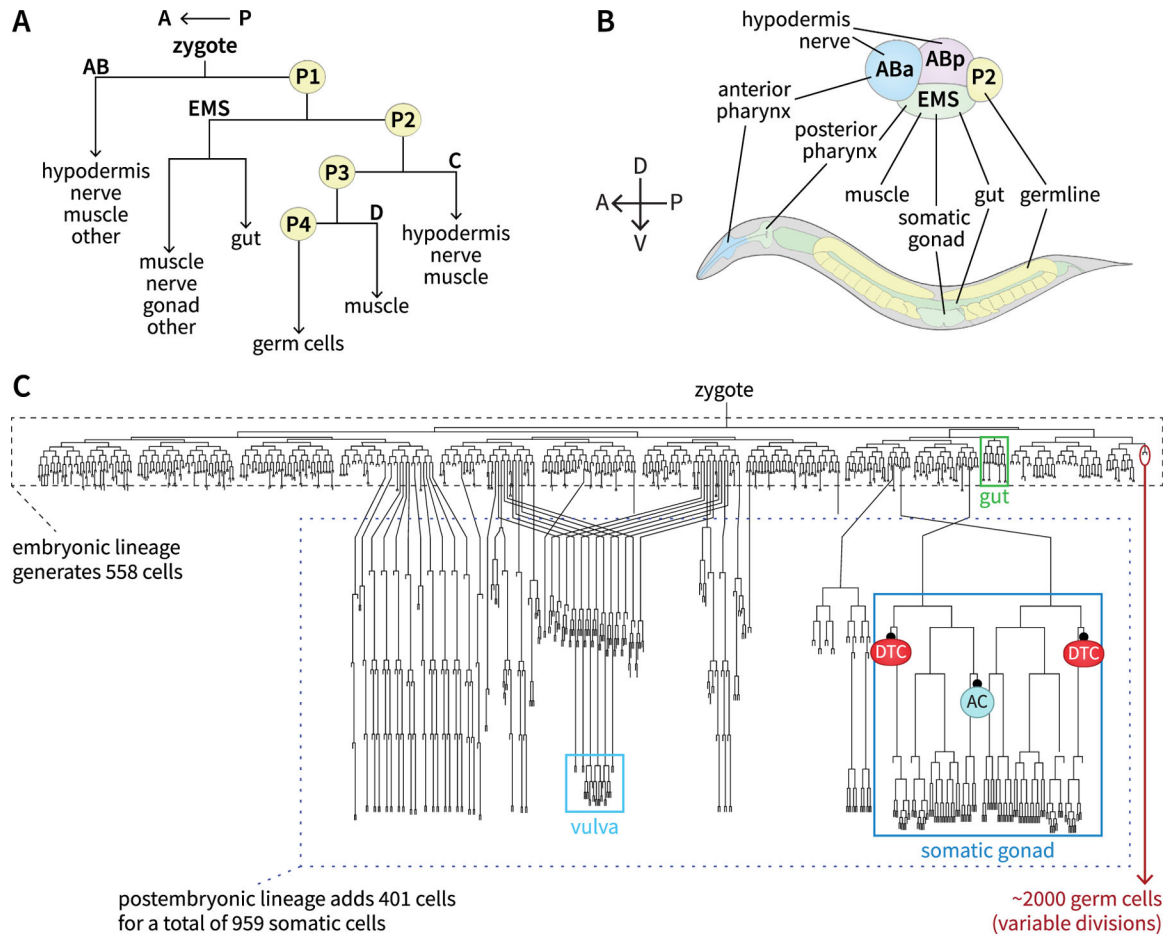


Fig. 4. *C. elegans* development and lineage.

A. Early embryonic asymmetric divisions generate somatic blastomeres (AB, EMS, C and D) and germline blastomeres (P1–P4). Arrow depicts anterior-posterior axis. B. Blastomeres at the four-cell stage (top) generate major tissues in the adult (bottom). Arrows depict anterior-posterior and dorsal-ventral axes. C. An invariant lineage generates somatic cells (black lines), while variable divisions generate the germline (red line). Two distal tip cells (DTC) and one anchor cell (AC) are born in the somatic gonadal lineage (dark blue square), which is far removed from the vulval lineage (light blue square).

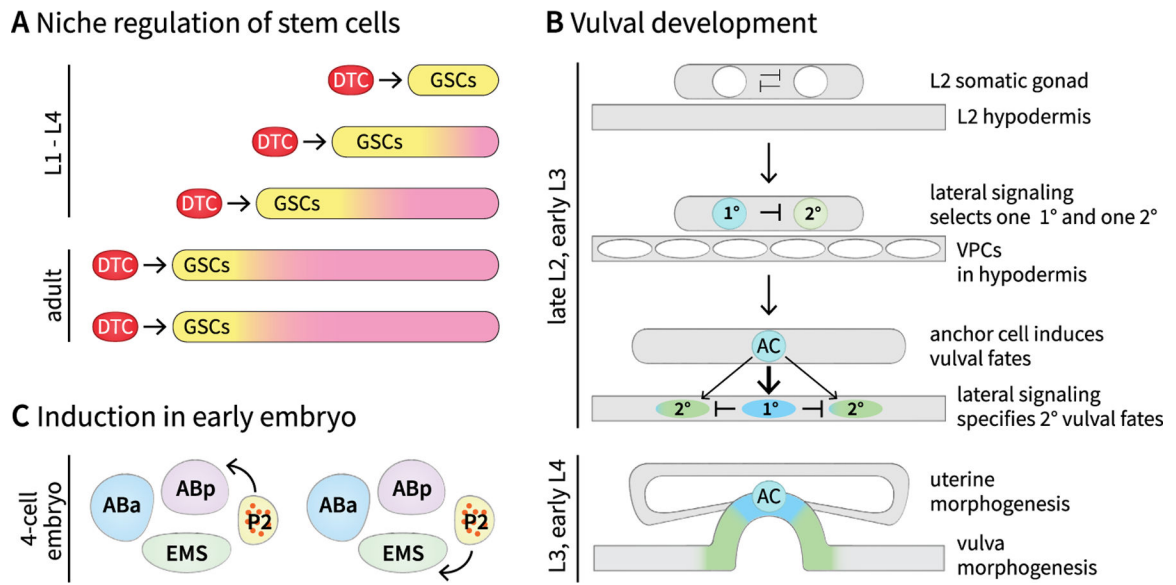
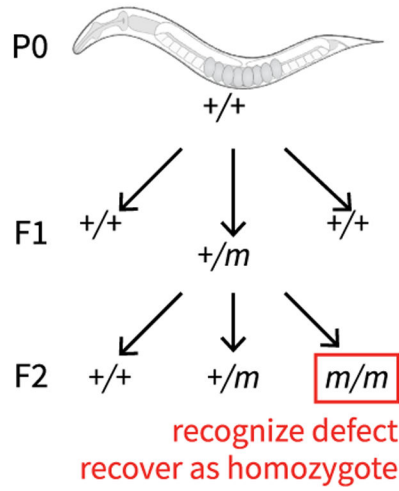


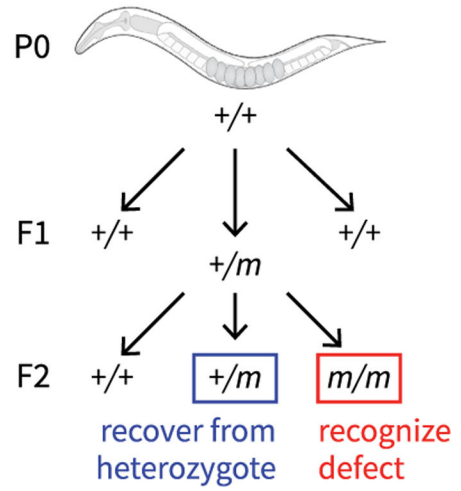
Fig. 5. *C. elegans* cell interactions (all (highly schematized)).

A. The distal tip cell (DTC) signals to the germline (elongate oval) during larval development (L1 – L4) and adults to maintain germline stem cells (GSCs). B. Vulval development. The anchor cell (AC) in the gonad induces vulval precursors in the hypodermis (arrows). Lateral signaling (⊥) occurs between equivalent AC precursors in the gonad, and equivalent vulval precursors in the hypodermis leads to distinct fates. C. Two cell interactions in the four-cell embryo. Left, P2 induces nonequivalence in Aba and ABp sisters. Right, P2 polarizes EMS to divide asymmetrically.

Zygotic screen, viable and fertile



Zygotic screen, lethal or sterile

**Fig. 6. F1 clonal screens.**

Parental P0 hermaphrodites generate F1 progeny with one chromosome carrying mutation (m) and the other carrying the normal or wild-type version of the gene ($+$). The heterozygous ($+/m$) F1 progeny generate homozygous (m/m) F2 progeny that are screened for defects. Left, if the homozygous mutant is viable and fertile, it can be used to generate a mutants strain. Right, if the homozygous mutant is lethal or sterile, its heterozygous siblings can be used to recover the mutant.

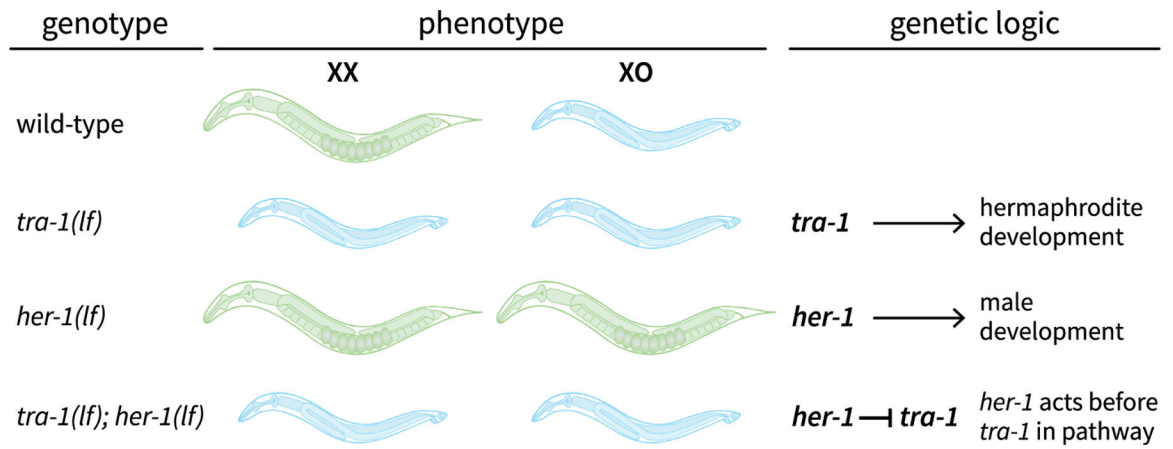


Fig. 7. Sex determination and genetic logic.

Development as a hermaphrodite (green) or male (blue) depends on the activities of regulatory genes. Wild-type XX animals are hermaphrodite and XO animals are male. Interpretations of single and double loss-of-function mutations (lf) in sex determination genes are shown.

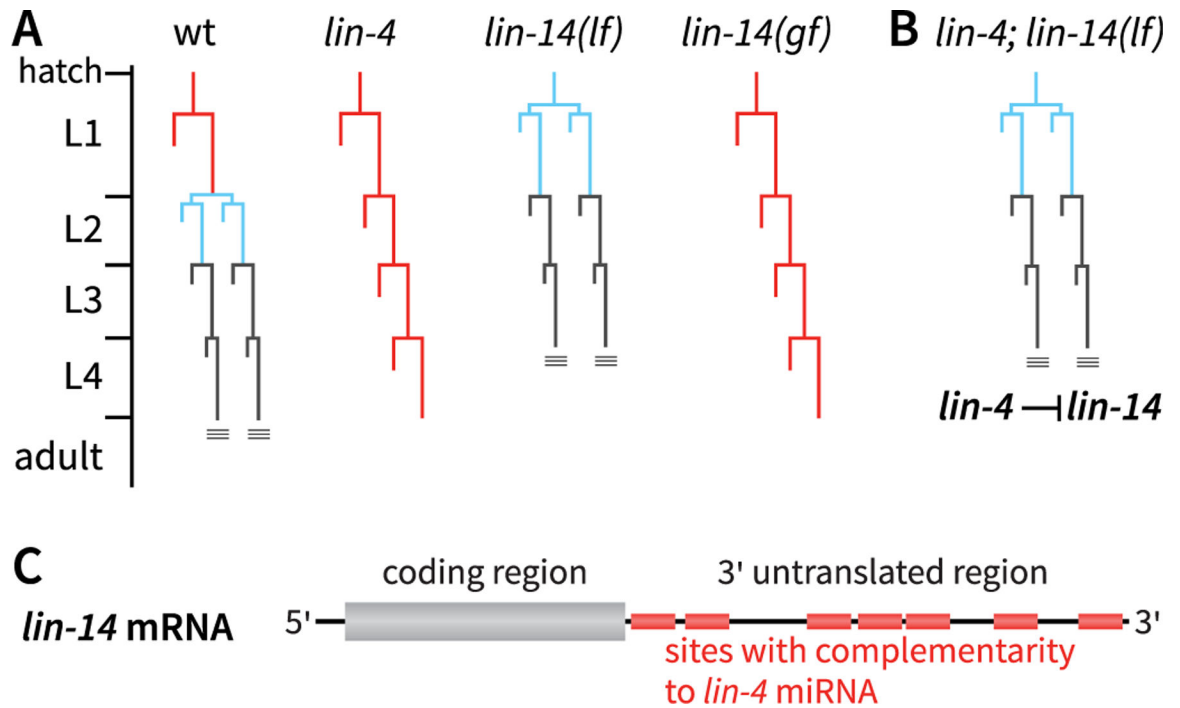


Fig. 8. Discovery of miRNAs.

A. The wild-type (wt) lineage includes L1-specific (red) and L2-specific (blue) divisions. L1 divisions are reiterated in *lin-4* mutants but they are skipped in *lin-14(lf)* mutants; *lin-14(gf)* mutants are similar to *lin-4* mutants. B. The *lin-4; lin-14(lf)* double mutant suggests that *lin-4* represses *lin-14*. C. The *lin-14* 3' untranslated region has seven sites of partial complementarity to the *lin-4* miRNA.

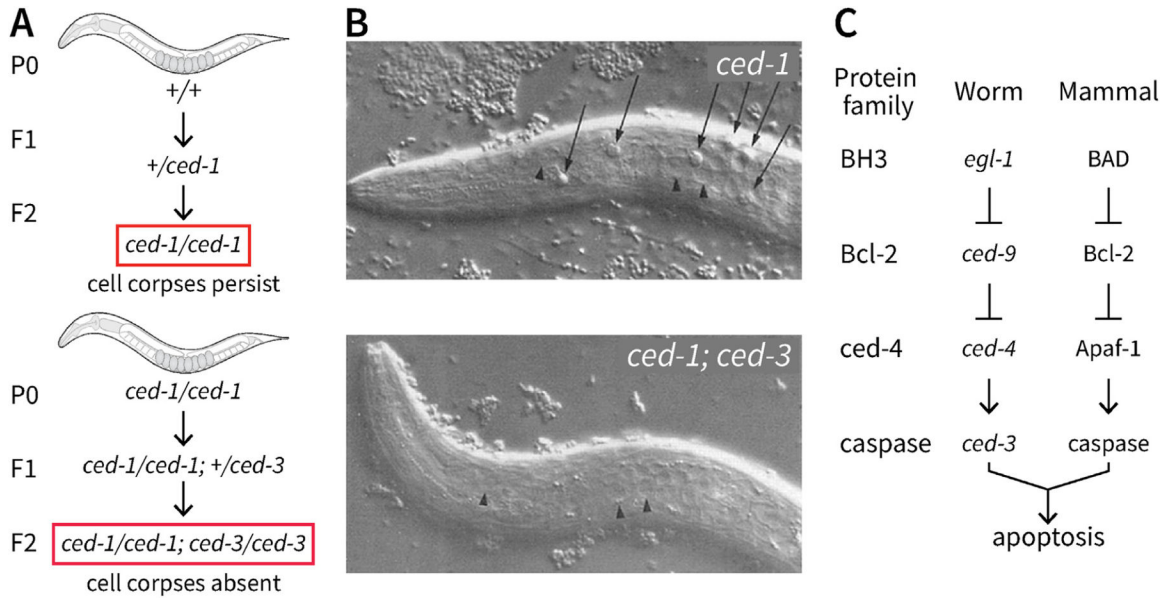


Fig. 9. Discovery of genes that regulate programmed cell death.

A. Two staged genetic screen. Above, first screen identifies *ced-1* mutants with persistent cell corpses; below, second screen begins with a *ced-1* mutant and identifies *ced-3* mutants without cell corpses B. DIC images of living animals, focusing on the head. Above, *ced-1* mutant with persisting cell corpses (arrows); below, *ced-1; ced-3* double mutant lacks cell corpses. Image reproduced from Ellis and Horvitz, 1986) with permission from Elsevier. C. Conserved pathway of cell death regulation.

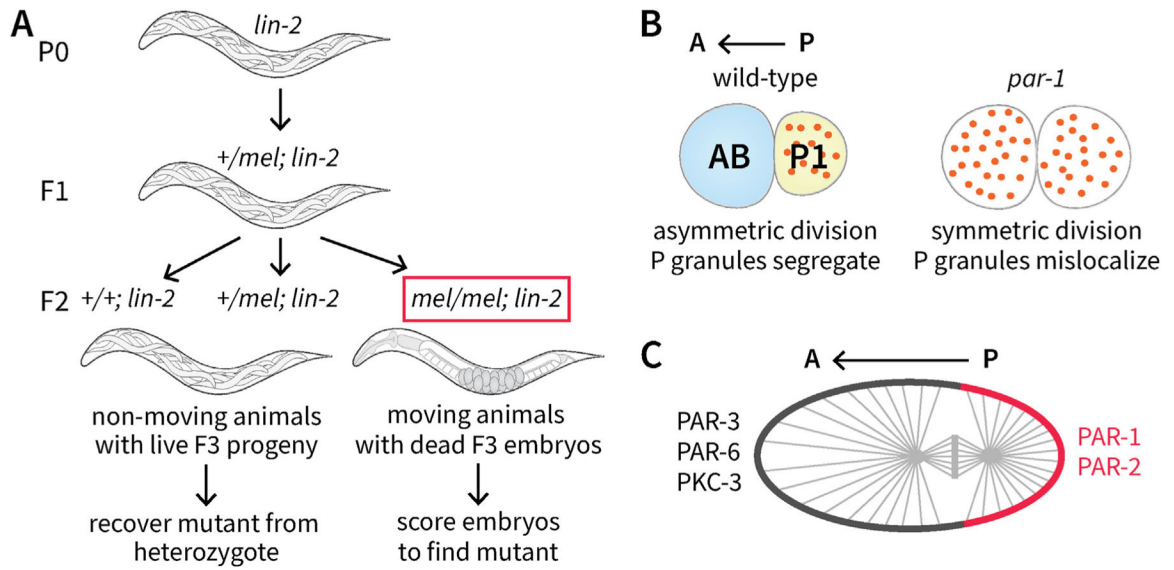


Fig. 10. Discovery of the *par* genes.

A. Scheme for detecting maternal effect lethal (*mel*) mutants among F2 progeny. Most *lin-2* mutants fill with living progeny and die, but F2 *lin-2* mutants that are also homozygous for a maternal effect lethal (*mel*^Δ) mutation fill with dead embryos and live. B. Left, wild-type one-cell embryo divides to generate differently sized blastomeres with P granules (red dots) segregated to the smaller one; right, *par-1* mutant embryo generates same sized blastomeres at first division with P granules (red dots) found in both. C. PAR proteins are localized reciprocally in the dividing one-cell embryo to its anterior cortex (PAR-3, PAR-6, PKC-3) (thick black line) or its posterior cortex (PAR-1, PAR-2) (thick red line).

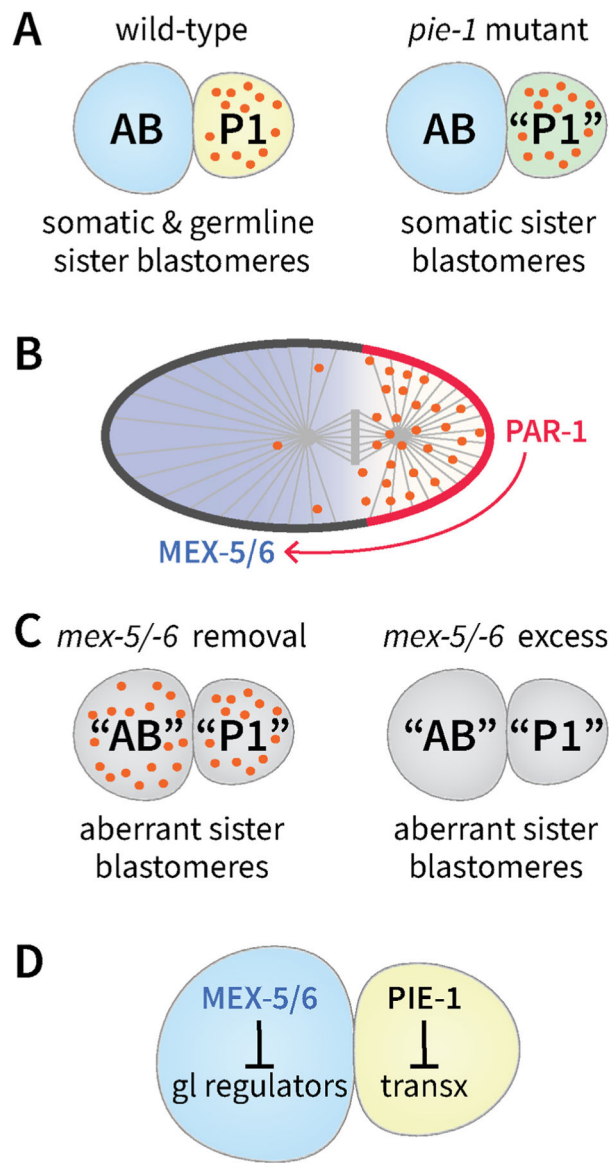


Fig. 11. Genes regulating the germline-soma dichotomy.

A. Left, wild-type one-cell embryo generates a larger somatic (blue) blastomere and smaller germline (yellow) blastomere. Right, *pie-1* mutant one-cell embryo generates two somatic blastomeres, one normal (blue) and one abnormal (green). P granules (red dots) segregate to the smaller blastomere in both wild-type and the *pie-1* mutant. B. Dividing one-cell embryo with cortical PAR proteins as in Fig. 10C. MEX-5 and MEX-6 proteins (blue) are enriched in the anterior, due to PAR-1 phosphorylation in the posterior. C. Left, upon RNAi depletion of *mex-5* and *mex-6*, blastomeres both retain P granules and have aberrant fates; right, with excess *mex-5* and *mex-6*, two blastomeres both lose P granules and have aberrant fates. D. Soma-germline dichotomy relies on MEX-5/-6-dependent degradation of germ-line (gl) regulators, including PIE-1, in the somatic blastomere (blue) and PIE-1 transcriptionally silencing in the germline blastomere (yellow) as well as action of other germline regulators.

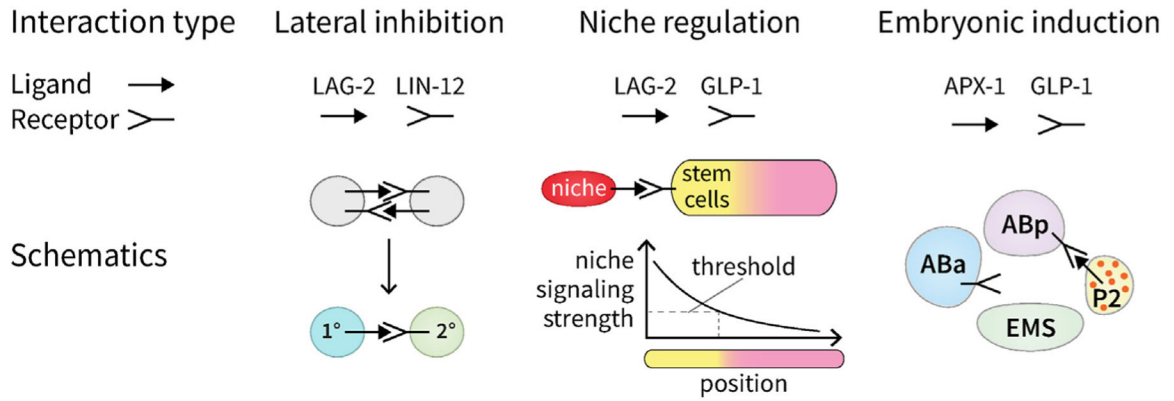


Fig. 12. Notch signaling is central to multiple types of cell interaction.

C. elegans Notch ligands, either LAG-2 or APX-1 (arrow); *C. elegans* Notch receptors, either LIN-12 or GLP-1 (Y). Left column, lateral inhibition. Above, equivalent precursors express both ligand and receptor; below, precursors resolve so that one signals and one receives. Middle column, niche regulation. Above, the niche expresses the ligand and stem cells express the receptor; below, model for regulation of stem cells over a distance, with graded signaling and a threshold marking the site where stem cell transition to differentiation. Right column, embryonic induction. The P2 blastomere expresses the ligand, while equivalent sisters ABa and ABp both express the receptor. ABp receives the P2 signal, but ABa is too far away.

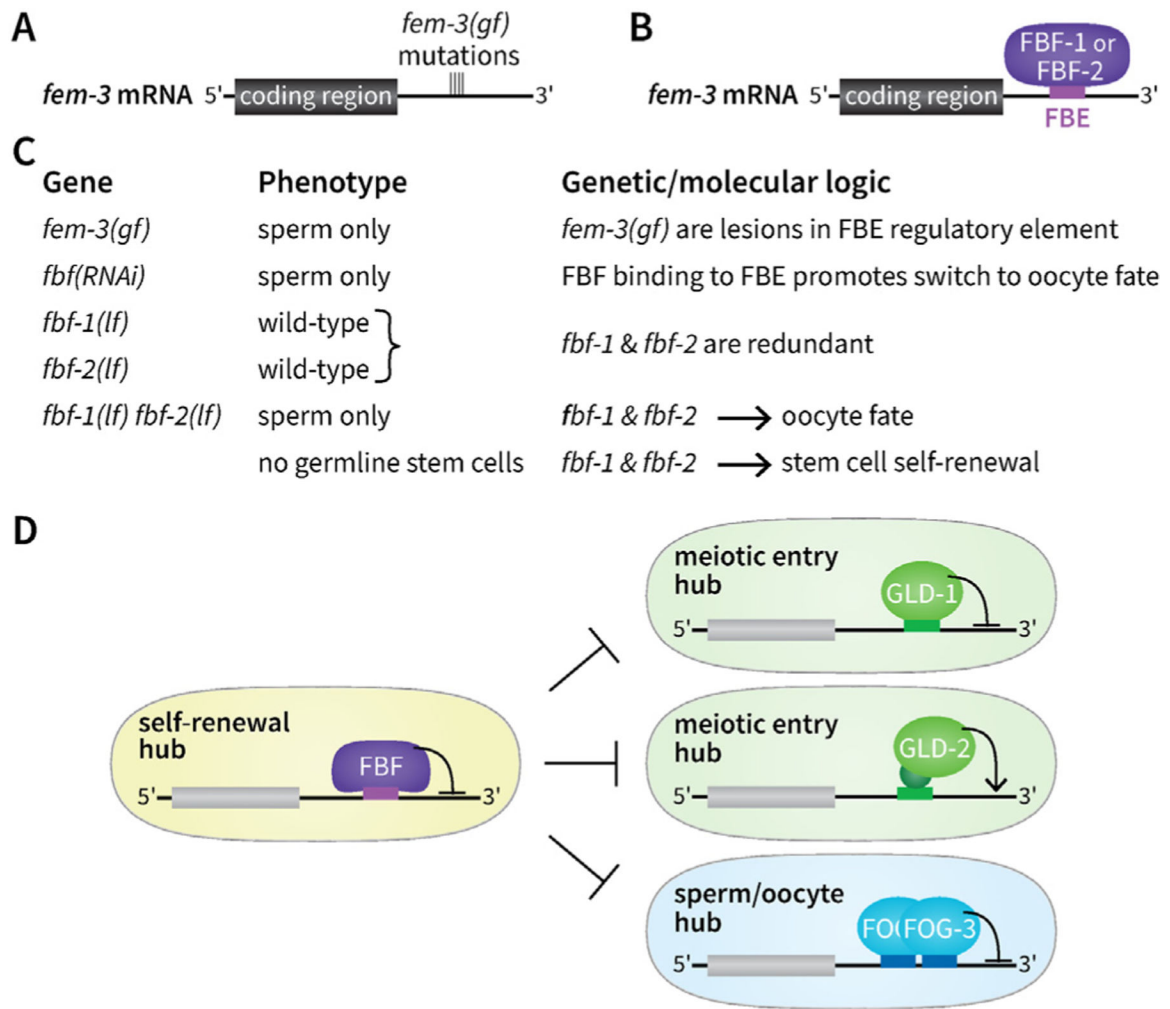


Fig. 13. Discovery of RNA network controlling germline self-renewal and differentiation.

A. *fem-3(gf)* mutations map to a small 5 base pair region in the 3' UTR. B. FBF-1 and FBF-2 bind to *fem-3* 3' UTR regulatory element. C. Genetic phenotypes reveal redundancy and pleiotropy of the *fbf-1* and *fbf-2* genes. D. Post-transcriptional regulation is central to each hub in the network controlling self-renewal and differentiation. FBF-1 and FBF-2 act redundantly in the single self-renewal hub; GLD-1 and GLD-2 are distinct proteins, one repressing and one activating target RNAs – they act in two differentiation hubs that redundantly promote entry into the meiotic cycle; FOG-3 is central to another differentiation hub that promotes specification to the sperm fate at the expense of oogenesis.

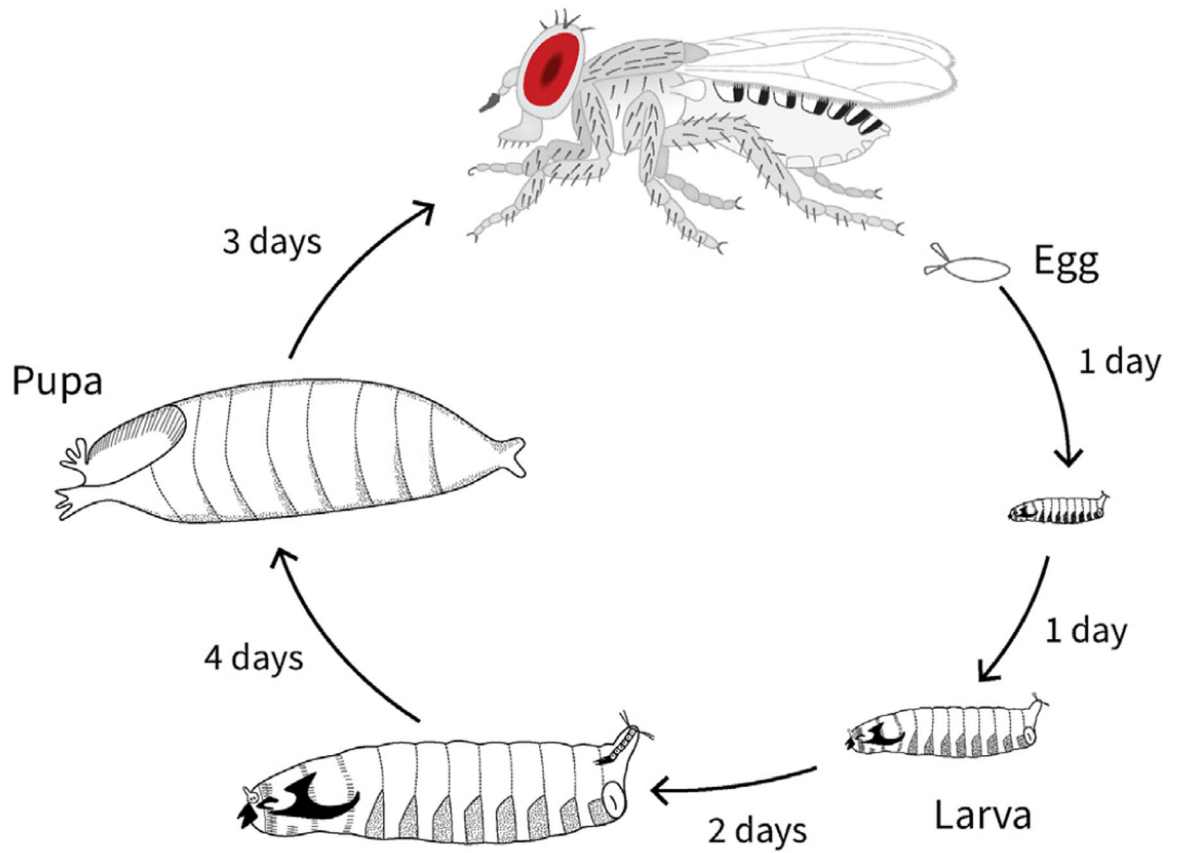


Fig. 14. The life cycle of *Drosophila*.

Female flies lay about 50 eggs per day, the larvae are simple segmented maggots without legs and head. They grow fast, molt twice without changing their morphology. During a pupal stage, metamorphosis takes place and an adult fly emerges.

x-linked inheritance

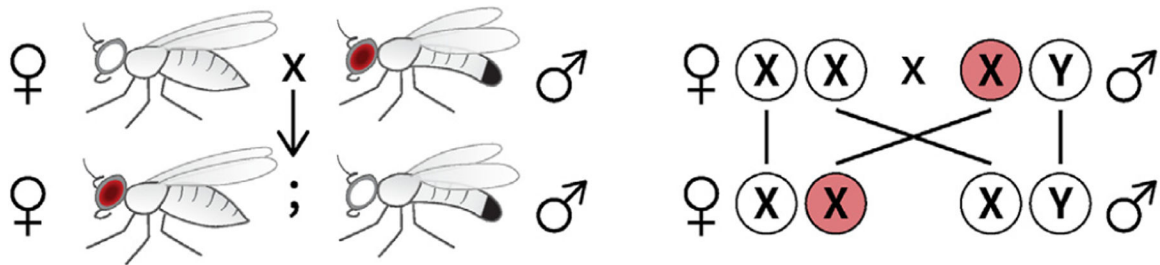


Fig. 15. X-linked inheritance.

A mutation in the *white* gene causes flies to have white instead of red eyes. The gene is located on the X-chromosome, and males have only one X-chromosome and a Y chromosome. If a white eyed female is crossed to a red eyed male, all the sons will receive the *white* allele from the mother, and the Y-chromosome from the father. All the daughters will be red eyed because they received the wildtype allele from their father. If the two X-chromosomes of the females are physically attached to each other, they are transmitted together to the daughters. In this case, the male progeny will receive their X-chromosome from their father and a Y-chromosome from the mother. Any mutation that has been induced in the father can be recognized in the male progeny. This crossing scheme has for example been used by Benzer to isolate mutants with behavioral defects.

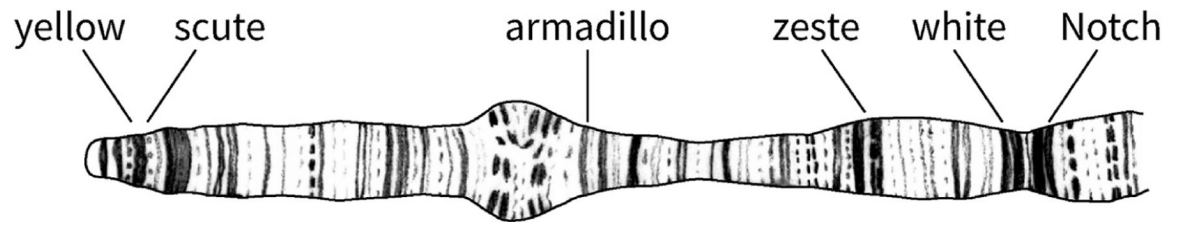


Fig. 16. Chromosomal maps.

In the salivary glands, the chromosomes are polytene and composed of strings of about a thousand copies such that a stereotyped banded pattern appears on which the genes can be localized. Shown here is the tip of the X-chromosome covering about 150 of the 5000 bands that have been characterized by the map of Bridges.

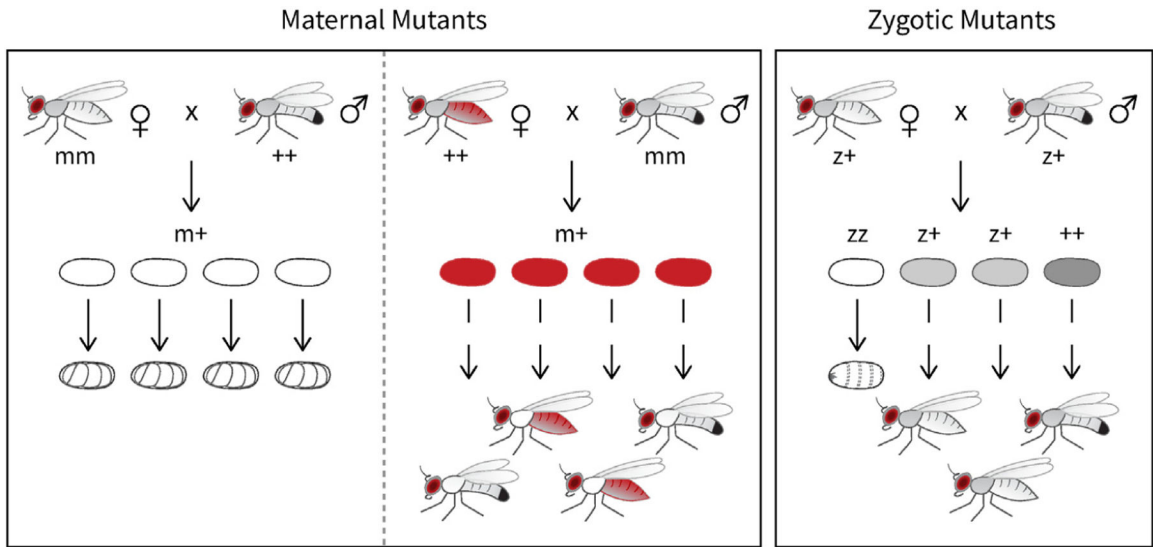


Fig. 17. Maternal and zygotic mutants.

Females that are homozygous for a maternal mutation produce eggs which lack the gene product (here in red) even if the paternal genome contributes the wildtype allele. The mothers genotype alone decides whether the mutant phenotype will show as the embryonic genotype is identical. In zygotic mutants, a cross between heterozygous carriers results in one quarter of the embryos receiving both mutant alleles that will show the phenotype, the other three quarters will receive at least one wild type copy and will survive.

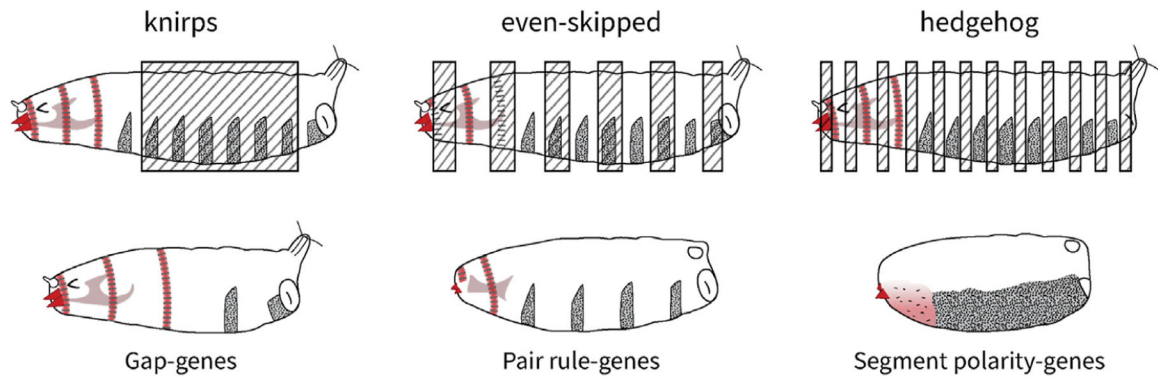


Fig. 18. Three classes of zygotic segmentation genes.

In the gap genes, a large region is deleted from the pattern, the example here is *knirps* in which the central abdominal segments are missing. The two other classes show a periodic deletion of pattern elements. In the pair rule mutants a portion of a segment is deleted in every other segment, whereas in mutants of the segment polarity class each segment misses a substantial portion, and the remainder is duplicated. Examples are *even-skipped* and *hedgehog*, in which the remainder of the segments fuse together to a lawn of denticles.

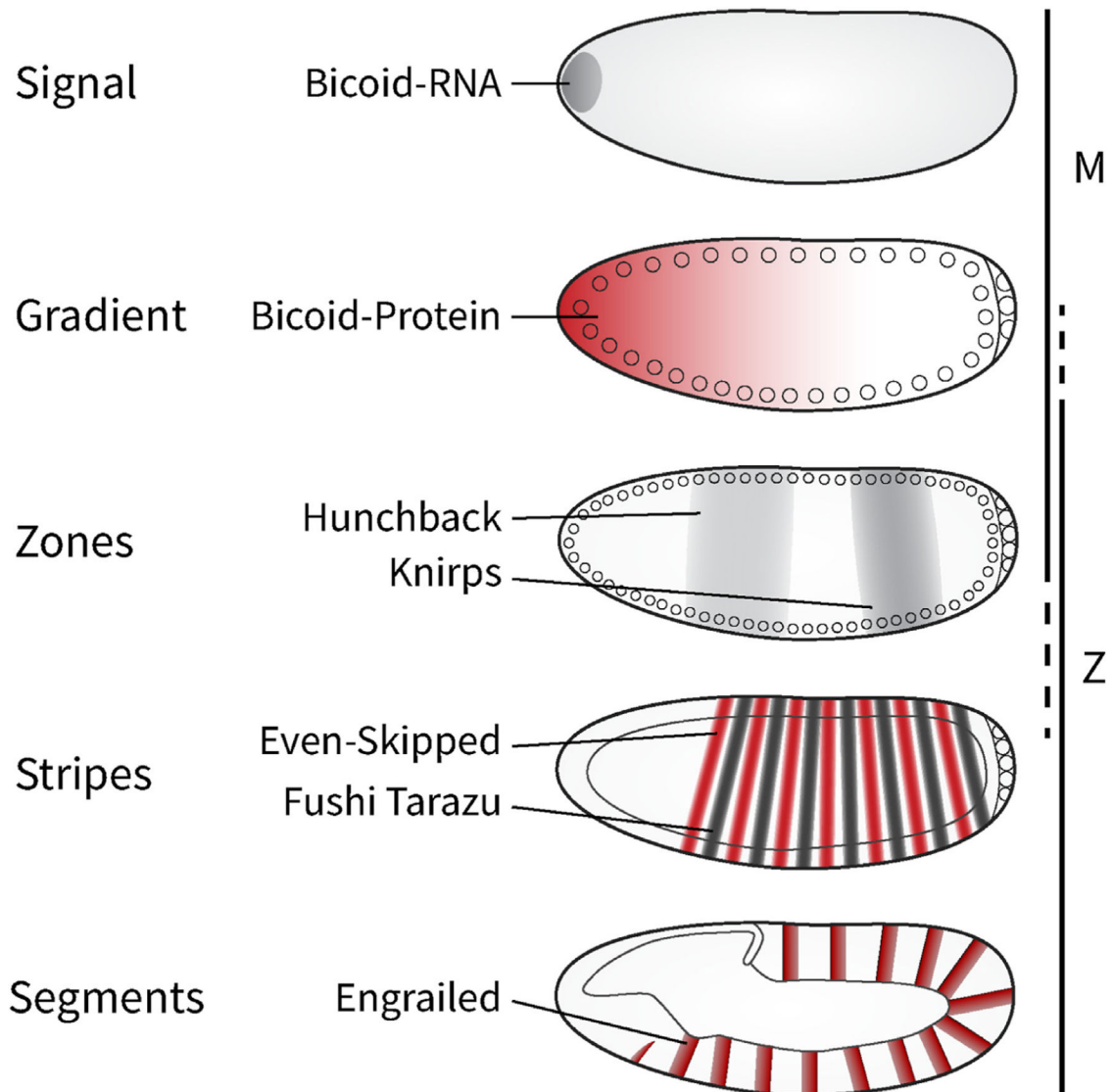


Fig. 19. Molecular prepatterns organizing segmentation.

A series of molecular prepattern determines segmentation. In the egg, the mRNA of the *bicoid* gene is localized at the anterior pole. The mRNA is translated and the Bicoid protein spreads posteriorly to form an exponential gradient. A similar gradient emanates from the posterior pole (not shown, see Fig. 24). In response to the concentration of these gradients, the gap genes are expressed each in one or two distinct domains, as examples are shown *knirps* and *hunchback*. A combinatorial activity of all the gap genes determines the pair rule genes to be expressed in seven stripes each, as examples are shown *even-skipped* and *fushi tarazu*. This is an important transition to a periodic pattern, which then is resolved into 14 stripes, here represented by the engrailed gene which is expressed in each segment, prefiguring the segmented pattern of the larva.

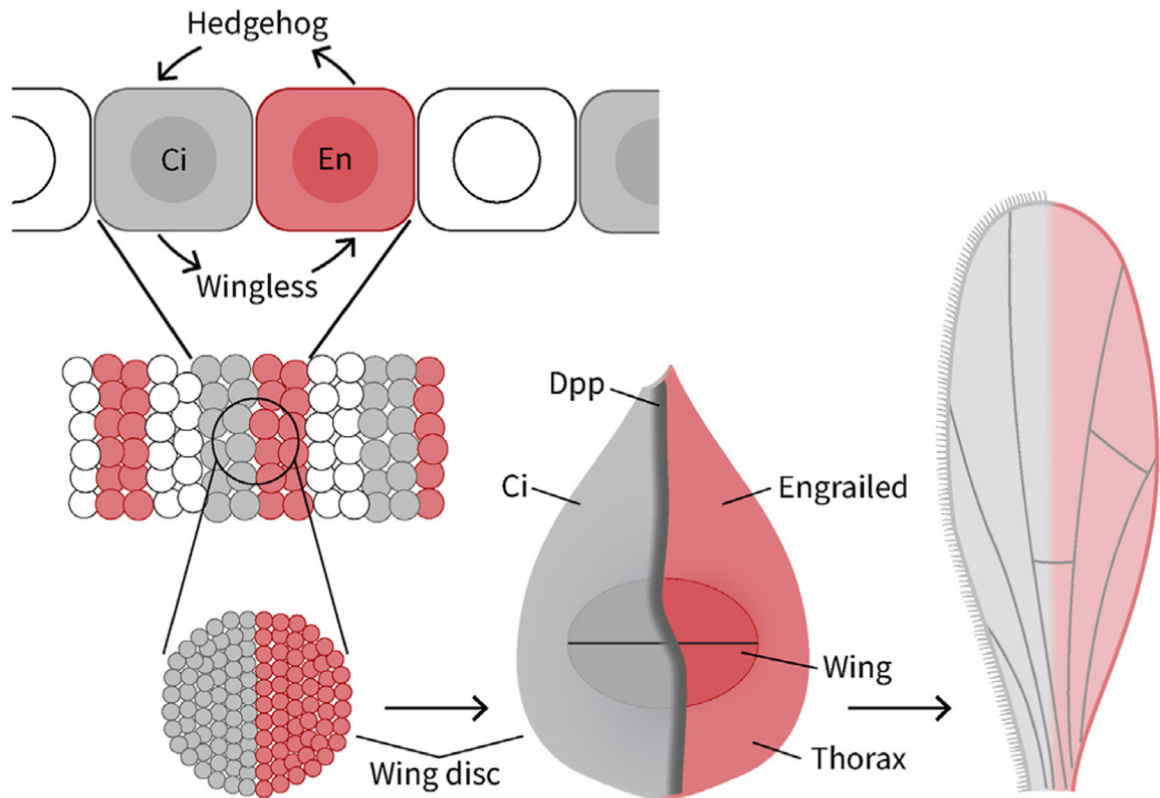


Fig. 20. Compartment formation in segment and imaginal disc.

During segmentation, neighboring epidermal cells express Wingless and Hedgehog, secreted proteins that enhance each other and form stable relationships which are maintained by the transcription factors Ci and Engrailed. The imaginal discs develop from this double row of cells and stably maintain the relationship during their growth. At the boundary between Engrailed- and Ci expressing cells, the morphogen Dpp is expressed. The transverse line separates the front and back of the anlagen of the wing blade.

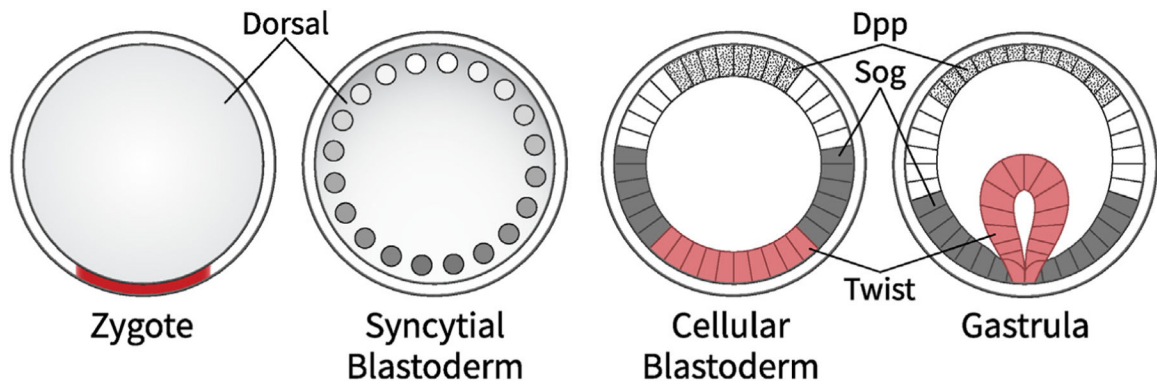


Fig. 21. The Dorsal gradient.

The maternal gene *dorsal* encodes a transcription factor that is uniformly distributed in the egg. The Dorsal protein is localizing to the nuclei on the ventral, and stays outside in the cytoplasm at the dorsal side of the embryo, forming a gradient of nuclear localization. This gradient is the result of a signal transduction process in which Toll functions as a receptor which is activated ventrally in the egg membrane by an outside signal in the vitelline membrane (here in red). The Dorsal transcription factor activates target genes, shown here *twist* and *sog*, in a concentration-dependent manner, it acts as a repressor on the transcription of *dpp*, such that *dpp* is transcribed at the dorsal side of the egg where the dorsal nuclear concentration is low. The cells expressing *twist* invaginate to form the ventral furrow.

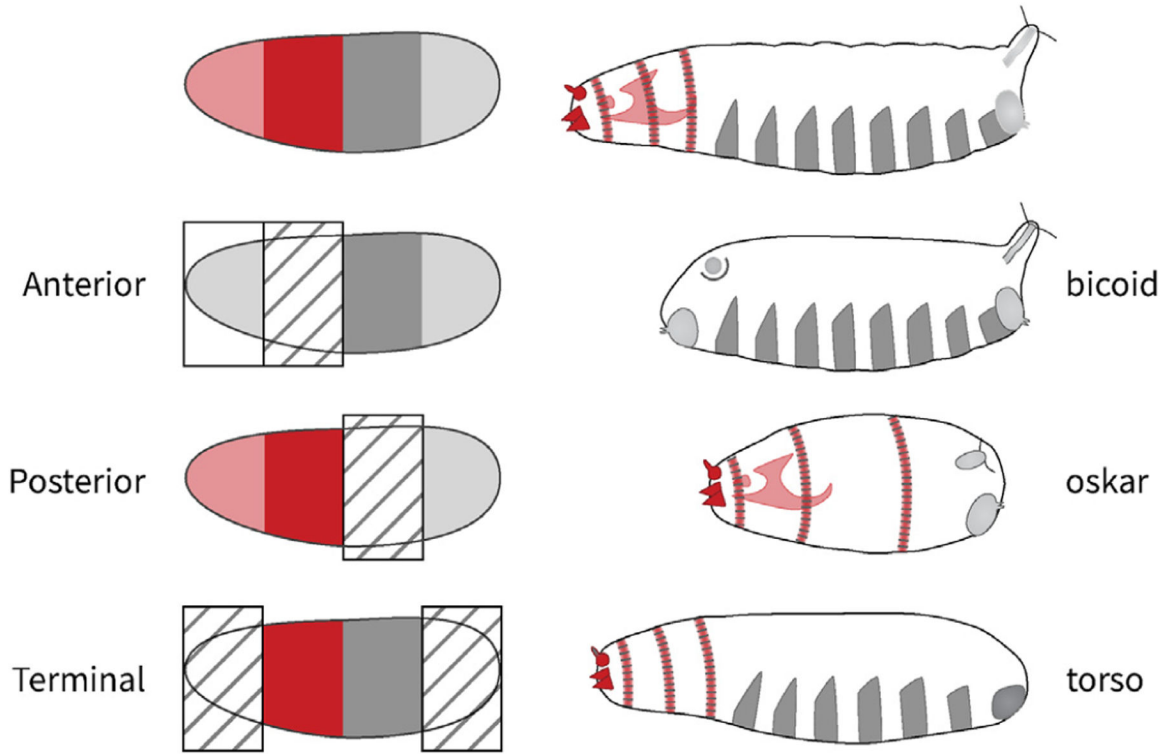


Fig. 22. Maternal gene groups of the anteroposterior axis.

Embryos produced by maternal mutants lack characteristic parts of the segmented pattern. In the anterior group, mutant *bicoid* embryos lack head and thorax, the acron is replaced by a telson. Mutants of the posterior-group genes (*oskar*) delete the entire abdomen, whereas a third group of genes is required for the anterior and posterior termini, the acron and telson, of the embryo (*torso*).

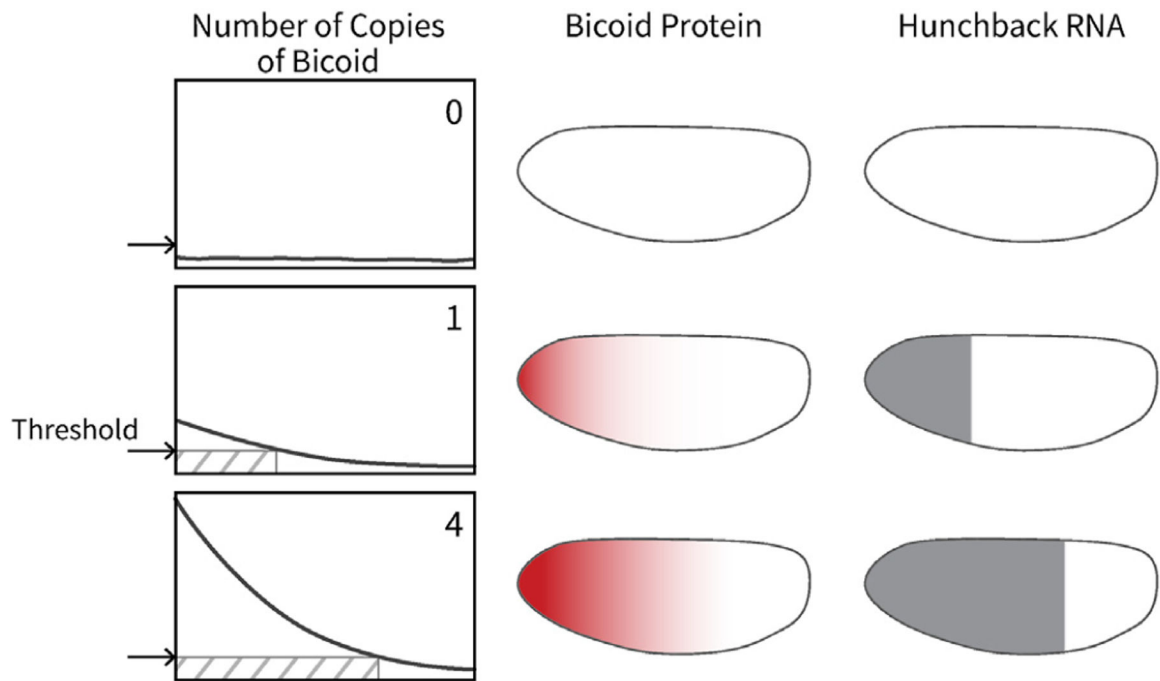


Fig. 23. Bicoid gradient: concentration dependent positional information.

The Bicoid protein is a transcription factor that activates a target gene *hunchback* at the anterior above a certain threshold concentration. It is distributed in a gradient with its maximum at the anterior tip of the egg, where the *bicoid* mRNA is localized. If this RNA-source is increased by additional copies of the *bicoid* gene in the genome, the gradient gets steeper, and the threshold for *hunchback* activation is shifted towards a more posterior position.

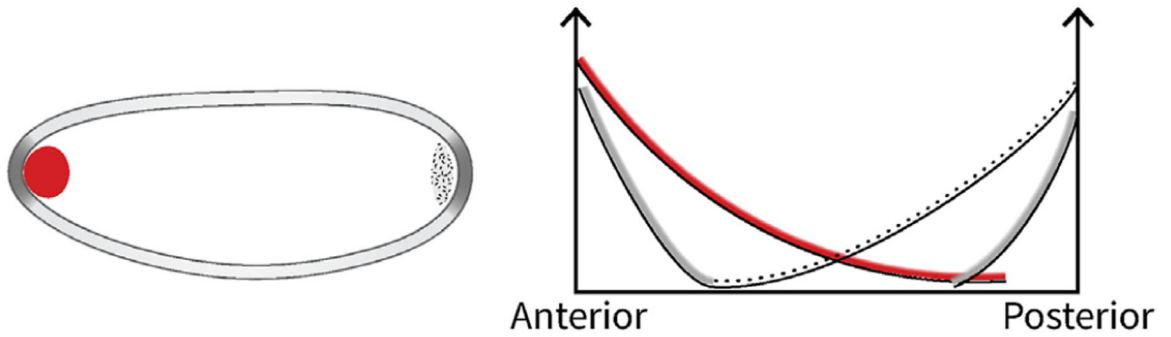


Fig. 24. Localized signals and gradients of the anteroposterior axis.

The products of the *bicoid*- and *nanos* gene are prelocalised as mRNA at the anterior and posterior tip of the egg of *Drosophila* (left). The proteins are expressed in gradients (right) distributing along the anteroposterior axis. At the two ends of the embryo, short range gradients emanate that are triggered by extracellular cues of the terminal system.