The near demise and subsequent revival of classical genetics for investigating *Caenorhabditis elegans* embryogenesis: RNAi meets next-generation DNA sequencing

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ABSTRACT **Molecular genetic investigation of the early *Caenorhabditis elegans* embryo has contributed substantially to the discovery and general understanding of the genes, pathways, and mechanisms that regulate and execute developmental and cell biological processes. Initially, worm geneticists relied exclusively on a classical genetics approach, isolating mutants with interesting phenotypes after mutagenesis and then determining the identity of the affected genes. Subsequently, the discovery of RNA interference (RNAi) led to a much greater reliance on a reverse genetics approach: reducing the function of known genes with RNAi and then observing the phenotypic consequences. Now the advent of next-generation DNA sequencing technologies and the ensuing ease and affordability of whole-genome sequencing are reviving the use of classical genetics to investigate early *C. elegans* embryogenesis.**

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Starting ~40 years ago, the nematode *Caenorhabditis elegans* rapidly grew in prominence as a model organism for inferring gene requirements based upon the phenotypes observed after reducing or otherwise altering gene function. For the first 20 some years of this period, *C. elegans* geneticists—led by Sydney Brenner, the founder of this now large and diverse lineage of researchers (Brenner, 1974)—used a forward or classical genetics approach: screening for mutant phenotypes after mutagenesis of nematode populations and then determining the identity of the altered gene by mapping and positional cloning of the affected loci (Jorgensen and Mango, 2002). Most genetic analyses initially focused on viable mutants, with the early goal being to investigate nervous system function. However, developmental studies of organogenesis (especially vulval development) and apoptosis received perhaps the most attention during these early years, and studies of aging also became prominent. Nevertheless, neurobiology has always been a key focus of *C. elegans* research and today is perhaps the largest single body of work derived from this user-friendly little worm (adults are ~1 mm in length and hermaphrodites have 302 neurons).

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All genetic studies in *C. elegans* benefit greatly from three prominent virtues it has as a model organism: its remarkably rapid and hermaphroditic life cycle of ~3.5 d from fertilization to self-fertile adulthood, its optical transparency, and its nearly invariant and very simple cell lineage, which enables one to define phenotypes with single-cell, and subcellular, resolution (with 558 cells present in a hatched hermaphrodite larva and 959 somatic cell nuclei in a mature adult hermaphrodite; Sulston and Horvitz, 1977; Sulston *et al.*, 1983).

Whereas genetic studies of developmental pathways that act during larval development grew rapidly in number and scope during the early years of this roundworm's laboratory life, studies of early *C. elegans* embryogenesis lagged behind. Although John Sulston published his monumental embryonic cell lineage of *C. elegans* in 1983 (Sulston *et al.*, 1983), genetic studies of embryogenesis remained relatively limited in scope. Three features of the worm embryo contributed to this early lack of effort. First, the essentially invariant embryonic cell lineage led to a simplistic assumption that developmental mechanisms in the early embryo would be highly mosaic and not relevant to the cell signaling perspective that dominated developmental biology research during these early years. Second, most *C. elegans* researchers viewed the embryo as a mass of undistinguishable cells and feared that it would be difficult to make sense out of mutant phenotypes (in contrast to the handy cuticle patterns that greatly facilitated the identification of embryonic cell fate patterning mutants in the fruit fly, *Drosophila melanogaster*). Finally, the genetic screens required to identify embryonic-lethal mutants were challenging: convenient genetic tools, such as stable

recombination-defective chromosomal balancers that greatly facilitated screens for lethal embryonic cell fate patterning mutants in *Drosophila*, were lacking. Nevertheless, Ann Rose and David Baillie identified many lethal *C. elegans* mutations (Johnsen and Baillie, 1991; Johnsen *et al.*, 2000; Clark and Baillie, 1992; Stewart *et al.*, 1998). However, most of these are larval lethal, and screening specifically for maternal-effect, embryonic-lethal mutants required singling out individual worms over multiple generations to score embryonic viability.

A turning point for study of the embryo came when Jim Priess devised a clever approach for finding embryonic lethal mutants (Jorgensen and Mango, 2002). He wanted to identify essential maternally expressed genes that act early in embryogenesis to establish cell fate patterning programs. To this end, Priess nicely exploited the availability of viable egg laying–defective *C. elegans* mutants. By using an egg laying–defective background for mutagenesis and screening, he could easily see worms homozygous for a loss-of-function maternal-effect mutation fill up with dead embryos (instead of "bagging out" with hatched larvae) and recover the mutation from heterozygous siblings. This approach led to an impressive series of landmark papers that established the early *C. elegans* embryo as an important model for genetic studies of developmental mechanisms, beginning with the discovery that Notch signaling acts at the four-cell stage of embryogenesis to distinguish the fates of two sister blastomeres (Priess *et al.*, 1987; Kemphues *et al.*, 1988; Bowerman *et al.*, 1992; Mello *et al.*, 1992, 1994; Mango *et al.*, 1994; Draper *et al.*, 1996; Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). Other researchers, most prominently Tony Hyman, then began to use the early embryo for genetic studies not only of development, but also of cell division and other cell biological processes (Oegema and Hyman, 2005).

Subsequent modifications to the egg laying–defective screen made it possible to isolate not only nonconditional mutations but also temperature-sensitive mutations in essential genes (Encalada *et al.*, 2000; Golden *et al.*, 2000; Pang *et al.*, 2004). Indeed, *C. elegans* is unique as an animal model in which one can feasibly isolate large numbers of temperature-sensitive mutations in essential genes (O'Rourke *et al.*, 2011). Because many essential genes have multiple requirements throughout the life of an organism, conditional alleles are particularly valuable for bypassing early requirements at a permissive temperature, followed by shifts to the restrictive temperate to study later requirements and thus more fully dissect gene function. In addition, growth at semipermissive temperatures sensitizes conditional mutants for use in modifier screens (Labbe *et al.*, 2006; O'Rourke *et al.*, 2007; Dorfman *et al.*, 2009).

One remarkable and unexpected contribution of early *C. elegans* embryo genetics research was the initial observation that led to the discovery of RNA interference (RNAi). In an effort to positionally clone the *par-1* gene, Ken Kemphues resorted to microinjecting antisense RNAs from gene candidates in the genetic interval to which *par-1* mapped. This effort identified one gene that reproduced the Par-1 mutant phenotype when antisense RNA corresponding to its coding sequences was microinjected into the syncytial ovary of adult wild-type worms. Surprisingly, Kemphues found that injecting sense RNA, intended as a negative control, also resulted in a Par-1 mutant phenotype. Kemphues admirably went ahead and published this confounding data as part of the procedure used to positionally clone the *par-1* gene (see Table 1 in Guo and Kemphues, 1995). Andy Fire and Craig Mello astutely noted this result and correctly deduced that the ability of either strand to cause a Par-1 mutant phenotype upon microinjection resulted from small amounts of contaminating double-stranded RNA (Fire *et al.*, 1998). Thus this puzzling result from the Kemphues lab led to the landmark discovery of Fire and Mello that short double-stranded RNAs mediate RNAi, with base pair complementarity ultimately resulting in degradation of the targeted mRNA.

RNAi has revolutionized genetics research, making it possible to reduce gene function in many systems previously not amenable to such analysis (e.g., cells grown in culture). The impact on *C. elegans* genetics also has been transformative. Soon after the discovery of RNAi, several ambitious genome-wide screens for embryonic lethality were undertaken in which researchers used RNAi to inactivate one by one each predicted gene in the fully sequenced worm genome (Maeda *et al.*, 2001; Kamath and Ahringer, 2003; Sönnichsen *et al.*, 2005). The methods used to introduce double-stranded RNAs included microinjection into the ovary, the soaking of worms in a concentrated solution of RNA, and, most remarkably, the feeding to worms of bacterial cultures expressing double-stranded RNAs from plasmid-borne coding sequence inserts (Timmons and Fire, 1998). RNAi in *C. elegans* is particularly effective at reducing maternal gene expression in the ovary, and these genome-wide screens have identified most of the essential genes required for embryonic viability. Rather than the laborious and time-consuming approach of mutagenizing worm populations and screening for mutant phenotypes, followed by the even more time-consuming positional cloning of the affected genes, researchers can now simply reduce the function of any gene using RNAi and in short order examine the phenotypic consequences. Indeed, an unfortunate consequence of the RNAi revolution has been the substantial abandonment of chemical mutagenesis and phenotypic screening for identifying essential *C. elegans* gene requirements during embryogenesis. In conducting such screens one has to wade through many mutants that represent additional alleles of genes in which mutations already are known. Moreover, in all too many cases, by the time a gene mutation was identified and its requirements analyzed, other groups already had published papers describing basic gene requirements using RNAi to reduce gene function.

Although RNAi has proven to be an extremely powerful genetic tool, it has limitations. The effect of RNAi can vary in reproducibility, depending on culture conditions and the carefulness of the investigators. Moreover, many genes that act later in embryogenesis and during larval development are less susceptible to RNAi, and in some cases RNAi only partially reduces gene function. Even some maternally expressed genes are largely resistant to RNAi, for unknown reasons. Furthermore, for genes with multiple essential requirements in narrow time windows, RNAi is not well suited for careful dissection of gene requirements. In addition, when the function of multiple genes needs to be reduced, RNAi often is not as effective as it is when single genes are targeted. Finally, domain-specific mutations can reveal requirements that might be missed when gene function is more generally reduced using RNAi (Canman *et al.*, 2008). Indeed, as researchers have begun to exhaust their ability to gain insight from analysis of essential genes using RNAi, the need and demand have returned for extremely useful mutant alleles of essential genes, especially uniquely powerful temperature-sensitive alleles.

Although RNAi has reduced the effort required to screen for new alleles of essential *C. elegans* genes, a newer kid on the block—next-generation DNA sequencing—is reviving interest in such screens due to the powerful high-throughput approaches now available for positional cloning (Sarin *et al.*, 2008; Doitsidou *et al.*, 2010; Zuryn *et al.*, 2010). With Illumina (San Diego, CA) DNA sequencing machines, one can in a few days sequence entire worm genomes for a few hundred dollars per strain (and the costs continue to decline rapidly). With a little more effort (outcrossing a mutant into a polymorphic worm strain and then isolating F2 descendants in which the mutation of interest is

again homozygous), one can use whole-genome sequencing to rapidly map mutations to megabase or smaller genetic intervals (via genome-wide single-nucleotide polymorphism [SNP] mapping) and at the same time identify the mutation responsible for the mutant phenotype. For example, Oliver Hobert found that sequencing entire mutant genomes in 17 different mutants isolated after chemical mutagenesis revealed ~500–1000 mutations per mutant genome, with roughly 50–100 missense mutations and two to five stop codons in coding sequences per mutant (Sarin *et al*., 2010). It was intriguing that backcrossing mutagenized strains to nonmutagenized wild-type worms did not substantially reduce the mutational load, presumably due to the surprisingly rapid and spontaneous accumulation of mutations that occurs within individual laboratory strains. Furthermore, by applying whole-genome sequencing to genome-wide SNP mapping of one mutant, using a few slightly different strategies, the Hobert lab positioned the mutation responsible for the phenotype to a roughly 2-megabase region, with only three protein-changing variants in that interval (Doitsidou *et al*., 2010). Similarly, my lab found that for three different temperature-sensitive mutations known to be located within 1- to 3-megabase intervals, only one or two missense mutations in protein-coding genes were found within each interval (unpublished data). As nearly (but not) all temperature-sensitive mutations in essential genes are missense (O'Rourke *et al*., 2011), it is somewhat easier to pinpoint the causative mutation in conditional mutants. More rarely, some conditional mutations prove to be stop codons or affect splice junctions. These exciting whole-genome sequencing approaches are reviving interest in screens for embryonic-lethal mutants and likely will also lead to analysis of many embryonic-lethal mutant categories that in the past were either discarded or ignored.

The evolution of genetic analysis in *C. elegans*, particularly for the early embryo, has taken some remarkable twists and turns over the decades. The ability to combine new technologies as they develop continues to make this elegant little roundworm a more and more remarkable model system for the always-exciting adventure of gene discovery.

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