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Twists and turns—How we stepped into and had fun in the "boring" lipid field

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Compared to proteins and RNAs, functional specificities associated with structural variations in fatty acids and lipids have been greatly underexplored. This review describes how our lab naively started to work on lipids 14 years ago, and how we have gradually overcome obstacles to address some interesting biological questions by combining genetics with biochemical methods on the nematode *Caenorhabditis elegans*. Our studies have revealed lipid variants and their metabolic pathways, in specific tissues, impact development and behaviors by regulating specific signaling events. The review also discusses the general research approach, style of lab management, and funding mechanisms that have facilitated the frequent research direction changes in the lab, including the journey into the lipid field.

monomethyl branched-chain fatty acid, mmBCFA, glucosylceramide, TORC1, IP3, foraging behavior, SUN and KASH domain, genetic redundancy

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1 The "bad" habit of shifting research directions

It is not uncommon for laboratories that use genetic model organisms to often move to or add research directions to address questions in fields outside their expertise. Over a long period of time, these labs would have studied problems in a fairly broad range of biological fields. There are perhaps two major reasons that drive such research dynamics in these labs. First, genetic screens and analyses of mutations following specific developmental/behavioral problems often lead investigators to address mechanism questions in different fields (e.g., signal transduction pathway, gene expression regulation, vesicle trafficking.). In addition, genetic screens often reveal mutants with somewhat surprising phenotypes that raise interesting questions and open new research directions. Second, many investigators understand that model organisms, especially non-vertebrate organisms such as worms and flies, are best used to understand basic mechanisms underlying fundamental biological processes. In other words, using these model organisms to address new problems in under-explored research areas may provide better opportunities to identify new paradigms that would apply to mammals or humans. In addition, these organisms are not advantageous compared to cell culture systems when biochemical analyses are the dominant approaches used to answer detailed mechanistic questions.

These two reasons are surely the ones to blame for the unusually broad scope of research in my lab over the past 24 years. In the early 1990s, we were mostly focused on, and having great time, dissecting the Ras signaling pathway in controlling *Caenorhabditis elegans* development, riding on a large number of suppressor/enhancer mutations from several modifier screens (e.g., [1–6]). However, thinking

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that the pathway would soon be worked out at the level we could handle, in the mid-1990s we started to shift attention to cellular events that occurred downstream of the signaling transduction events. In fact, while my first National Institute of Health (NIH) R01 grant, started in 1992, focused on genetic screens and analyses of factors regulating the Ras signaling pathway, the renewal application submitted in 1996 already proposed to study vulval morphogenesis. Several of the new research directions that attracted much of the lab's efforts for many years were spin-offs of interesting mutants isolated during the studies of cell signaling and morphogenesis for vulval development. In some cases, it was very much by serendipity, but embraced by students or postdoctoral fellows who had the noses to sense important and unanswered questions. For example, Chris Malone, a new graduate student in 1994, analyzed a mutation "incorrectly" isolated in the lab as a suppressor of activated Ras. After he determined that this was really an allele of the *unc-83* gene with a nuclear migration defect, he decided to work on nuclear positioning for his Ph.D. thesis (collaboration with Robert Horvitz lab at Massachusetts Institute of Technology). His effort led to the publication of a groundbreaking paper that defined the SUN protein family at the nuclear envelope and their roles on nuclear migration and anchorage [7]. Later, Dan Starr's work on two other related genes led to his proposal of KASH domain proteins and the universal pairing between SUN and KASH proteins at the nuclear envelope for various cellular functions [8,9]. Both Dan and Chris continued to work on these nuclear envelope complexes in their own labs. Following these pioneer studies, we were able to play a leading role in functional studies on these protein complexes including uncovering their roles in several important cellular and development processes in mice [10–16]; the majority of the mouse genetics were done at the Institute of Developmental Biology and Molecular Medicine of Fudan University, collaborating with Tian Xu (Howard Hughes Medical Institute (HHMI), Yale), Yuan Zhuang (Duke) and others.

Similarly, our extensive efforts on microRNA-related research were also initiated by an unexpected discovery. Lei Ding, a graduate student, position cloned a novel gene defined by two mutations from a morphology-based genetic screen aimed for factors acting downstream of a transcription factor. To understand the mechanism of its function, Lei decided to take a hardcore biochemical approach and discovered the tight interaction of this protein with Argonaute proteins and miRNAs. The combination of genetics and biochemistry led to a publication that was the first to report the essential role of a GW182 family protein in the miRNA-induced silencing complex (miRISC) [17]. He also showed that it was GW182 that brings miRISCs to specific cellular locations for target inhibition. Several researchers in the lab have since carried out intelligent systematic analyses of miRNA-target interaction networks and functions by pioneering cross-linked immunoprecipitation (CLIP) against GW128 proteins and doing combinatory genetics (e.g., [18–21]). We also enjoyed collaboration with Victor Ambros, who discovered miRNA, on two papers on this subject [22,23].

In contrast to the above examples, some of the "off track" studies in the lab were the consequence of meticulous thinking and bold moves. For example, after writing a short review on "synthetic multivulva genes" [24], postdoctoral fellow David Fay thought that the so called "genetic redundancy by structurally unrelated genes" is so widely present, it is perhaps the biggest obstacle for applying genetics to uncover gene functions. He abandoned his two years of effort on vulval morphogenesis and designed a new screen that could be used to identify developmental functions associated with any gene that displays no obvious loss-of-function phenotype in development. His 2002 paper described a successful genetic screen for mutations that cause "synthetic" phenotypes in combination with a mutation in tumor suppressor Rb/lin-35, which also identified factors acting with Rb for specific functions [25]. A few years later, Yo Suzuki followed the idea and uncovered many developmental functions associated with tumor suppressor PTEN/*daf-18*, albeit using a different screen strategy [26]. Both went on to pursue their own independent research careers doing things related to genetic redundancy. Cui Mingxue, another postdoctoral fellow, made a breakthrough in understanding the functions of Rb and other "synthetic multivulva genes": they repress undesired expression of a growth factor in the epidermis [27] (collaboration with Iva Greenwald and Paul Sternberg). Later, he also showed that Rb critically regulates starvation-induced stress responses [28].

More recently, Ben Weaver and Rebecca Zabinsky thought that the "genetic redundancy" concept should apply very well to miRNA-mediated gene silencing, since studies from the labs of Bob Horvitz, Victor Ambros, and others have demonstrated that individually mutating the majority of miRNAs, or even miRNA families, do not cause obvious developmental defects [29,30], and the extent of down-regulation of individual target mRNAs by a specific miRNA is usually limited [31,32]. Therefore, miRNAs commonly affect specific physiological functions by not only silencing genes through a complex miRNA-target interaction network, but also by sharing functions with other expression regulatory mechanisms. Each individual mode of regulation contributes to the robustness of gene expression dynamics. With this idea in mind, they carried out a genome-wide genetic modifier screen to search for factors that collaborate with miRNAs to regulate gene expression dynamics needed for robust development. This screen, using mutations in the two GW182 genes, uncovered a large number of interactors. Their efforts on one of them, the CED-3 caspase that was extensively studies for its role in apoptosis [33], uncovered unexpected but widely used non-apoptotic functions of the CED-3 pathway in repressing gene expression during development [34]. The collaborative role of CED-3 and miRNAs in regulating

the LIN-28 pluripotency/developmental timing pathway was demonstrated at both genetic and biochemical levels. This discovery led to another "synthetic phenotype" screen to systematically investigate the non-apoptotic roles of CED-3 in repressing gene expression for a broad range of cellular processes. These findings drove Ben into a new research direction, which will serve him well once he becomes a PI.

2 A bold but naïve move to explore the functional consequences of obscure fatty acid variants

Perhaps the most dramatic change of research direction in our laboratory was the decision in the early 2000s to work on lipids, which had no relationship with any of the *C. elegans* projects in the lab. From 1996–2001, my lab hosted a small human genetic project brought in by a friend who had just finished his Ph.D. and MD training from Harvard. I hired a non-conventional postdoctoral fellow, Marina Kniazeva, to work with this ophthalmologist-to-be to map several familiar eye disease genes [35–37]. One major outcome of this collaborative effort (Merck also stepped in to determine the DNA lesion) eventually lead to the cloning of an autosomal dominant macular dystrophy gene *ELOVL-4* that encodes a very long chain fatty acid elongation enzyme [38]. Wondering why changing the level of certain very long-chain fatty acids (VLCFA) would lead to this degeneration, Marina searched the literature only to find that essentially nothing was known about functional specificities associated with VLCFAs. In fact, we realized that functional specificities associated with the vast majority of fatty acid (FA) variants were essentially unexplored, even though sporadic studies have linked FA imbalance to changes in cellular processes and human health problems (e.g., [39–45]. There are nearly 100 different types of fatty acids, varying in (i) their lengths from as short as six carbons to more than 30 carbons, (ii) degree of saturation, (iii) whether the number of carbons in the main chain is even or odd, and (iv) whether they contain one or more branched chains (Figure 1). Even though some of these differences were understood on some level (e.g. degree of saturation impacts the fluidity of lipid bilayers by changing physical properties of the lipid), our understanding of the physiological functions that resulted from these differences (e.g., the difference between omega-3 vs. omega-6 FAs) was actually still very limited (Figure 1). Evolution does not select these structural variations without needed functional consequences. The lack of studies on this general subject made it particularly attractive to Marina and I—we were pleased to see this huge knowledge gap in our literature surfing. Marina decided that she would use *C. elegans* genetics, like the rest of the researchers in the lab, to study functional specificities associated with certain FA variants, leading to the eventual mechanistic studies. I liked the idea. Already recognizing

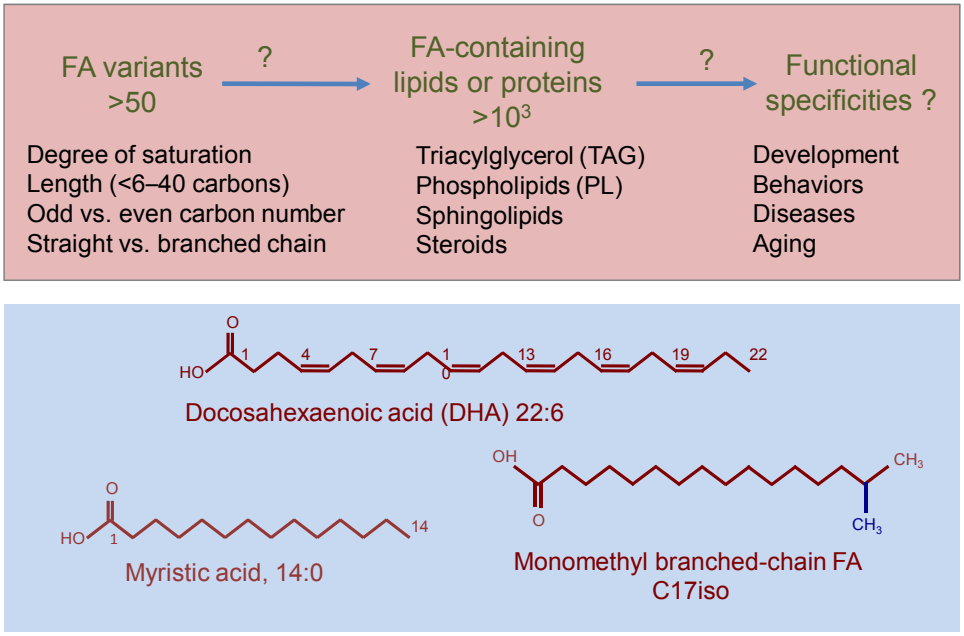

Figure 1 (color online) Functional specificities associated with structural variants remain largely unclear. Top, schematic illustration of variations in FA structures and outstanding functional questions associated with these variations. "?" above the arrows denotes potential regulations involved the processes. Specifically, incorporation of specific FAs into certain classes of high-order lipids may be modulated by changes in the levels or activities of certain enzymes involved in specific cells/tissues during development, which would in turn alter the composition of lipids in cell membranes (Figure 3 illustrates an example). Functional specificities associated with most of the FA variants are unclear. Bottom, three examples of fatty acid structural variants.

her exceptional talent, I simply left her alone for such a journey and tried to recruit more people to join her. Thinking back today, we were actually incredibly naïve about working on lipids at the time, but knowing too much would probably have served as a giant stop sign for this bold move.

Unlike proteins and non-coding RNAs, lipid variants have no linear relation to the genome, which severely limits the power of straightforward genetics to connect the structural variations to physiological functions. The strength of the lab had been the ability to do sophisticated genetic analysis, which would help us to deal with the difficulty of doing "lipid genetics", but we had to also be able to apply biochemical and genomic tools to characterize lipid and gene expression profiles. For example, we were later forced to establish our own mass spectrometry for analyzing lipid profiles, after failing to form collaborations with several lipid experts.

Assuming that each of the elongation enzymes would likely have a certain degree of specificity for different fatty acids, Marina first applied RNA interference to eight genes that were homologous to the mammalian elongation enzymes and examined the FA composition and developmental defects. She then analyzed the interesting phenotype associated with the *elo-2* gene more in depth [46]. Her effort on *elo-2* received collaborative help from Jenny Watts at Washington State University, who pioneered studies on FA biosynthesis and functions in *C. elegans* [47].

Among the eight *elo-* genes, *elo-5* was the most attractive one because knocking it down generated the most specific change in FA composition, deficiency in monomethyl branched-chain fatty acids (mmBCFAs), and prominent developmental defects [48]. These observations would potentially allow us to uncover physiological functions specifically associated with one type of fatty acid. Excitingly, Marina soon found that feeding worms with a chemical synthesized mmBCFA (C17iso) could totally overcome the defect that resulted from *elo-5(RNAi)* or an *elo-5(-)* deletion mutation, meaning that lack of this specific fatty acid was the main cause of the developmental defects.

What did we know about mmBCFAs in animals? From the literature we recognized: (i) mmBCFAs are abundantly present in mammals including humans, as we not only eat a lot of them in dairy products and meat but also synthesize them through a *de novo* pathway that had not yet been revealed in metazoans [49–55]; and (ii) the functions of mmBCFAs in animals had been essentially unexplored. These two facts further enhanced our interest in using mmBCFA as a "model system" in *C. elegans* to study the functions associated with special FA variants and the mechanisms underlying the functions.

In 2004, we had done a basic characterization of mmBCFAs in *C. elegans*, which included: identifying some key components of the mmBCFA *de novo* synthesis pathway, showing their essential roles in postembryonic development, investigating the impact of mmBCFA deficiency on gene expression and FA metabolism, and identifying genes potentially involved in regulating mmBCFA homeostasis [48]. In particular, we observed that depleting mmBCFAs in the embryo caused the worms to arrest their postembryonic growth and development and such arrest can be reversed by dietary supplementation of mmBCFAs. We decided to submit this long paper to the newly established open-access journal PLoS Biology, for which I had joined a group to voice the support. If "impact factor" would be considered the only measuring stick for the success of this new journal, we had certainly done them a terrible disservice, as the two-year post publication citation of this paper was zero. However, shortly after its publication, someone posted a high praise of the impact of the work on "Faculty of 1000" and gave this study the "Exceptional" ranking. The lack of citation sat well with us because it confirmed the novelty of our study and the lack of competition. The interest on this subject has certainly grown in the past decade as both the citation and views of this paper has steadily increased.

3 Impacts of monomethyl branched-chain fatty acids on cell signaling and postembryonic growth control—the TORC1 connection

The reversibility of the developmental arrest raised the possibility that these FA variants, or more complex lipids containing them, may play a "regulatory" role in growth and development. This speculation became a plausible hypothesis after Marina published the second paper on mmBCFAs in 2008 with the following observations [56]. First, using several markers, we observed that the L1 arrest phenotype was fully penetrant and within a few hours after hatching, halting most, if not all, postembryonic divisions and differentiation. Second, the L1 arrest was similar to starvation-induced L1 arrest (L1 diapause) that was known to be regulated by the insulin/insulin-like growth factor signaling (IIS) pathway [57–59]. Third, the growth response of the arrested animals to a gradient of supplemental mmBCFA concentrations was nonlinear; the dosage response was nearly all-or-none. In addition, the amount of mmBCFA supplement sufficient to reverse the L1 arrest was also sufficient to provide normal reproductive growth. Fourth, mmBCFAs repressed cyclin-dependent kinase inhibitor CKI-1 that was known to inhibit cell division and growth during postembryonic development [58,60]. Finally, mmBCFAs appeared to function in a mechanism that was in parallel to the IIS pathway that mediates the food-sensing pathway. Although none of these observations provided definitive evidence for a regulatory role of mmBCFAs, they were collectively sufficient for the lab to commit to a serious chase for such a role. This 2008 paper also described a simple feedback loop that involves transcription factors SREBP and CBP in regulating mmBCFA homeostasis [56].

There are two possible explanations for why mmBCFA-

deficient worms robustly halt their postembryonic development. One simple possibility is that mmBCFAs have structural functions that are absolutely essential for the execution of cell division, growth and differentiation. Alternatively, mmBCFAs or lipids containing them act to facilitate or promote a regulatory system that "turns off" postembryonic development when it senses the deficiency of these lipids. If the later hypothesis would be correct, we might be able to generate a genetic mutation that would constitutively activate the regulator or sensor (flipping the switch on) and force the worms to continue to develop without these special FAs. Experimentally, we could just mutagenize *elo-5(-)* mutants growing on food without mmBCFA supplement and screen for rare suppressors that permit worms to grow past L1 stage.

Four researchers in the lab, two graduate students and two postdoctoral fellows, made sequential runs at this technically challenging suppressor search with variations in screen designs. From independent screens, Emylie Seamen and Jen Blanchette isolated three promising suppressors that permitted *elo-5(-)* mutants to grow for one generation without altering mmBCFA biosynthesis or absorption [61]. Subsequent mapping and cloning showed that all three of them are alleles of the same gene encoding a P-type ATPase/flippase (TAT-2) that likely acts to translocate specific lipids in the lipid bilayer or between different organelles [62,63]. Their further analysis suggested that the role of mmBCFAs on postembryonic growth may be mediated by mmBCFA-containing sphingolipids and that TAT-2 antagonizes this function in intestinal cells, possibly by moving the lipids to different locations [61]. Mutating TAT-2 would increase the local concentration of these lipids at the intestinal membrane and permit the worms to continue to develop under the condition that mmBCFA *de novo* synthesis is shut down. Rencheng Wang performed another screen and isolated several effective suppressors as well. Interestingly, none were alleles of *tat-2* and all seemed to partially recover mmBCFA levels, which was interesting to investigate for a different reason. He mapped and cloned two genes and focused his further study on *prx-5* that encodes an ortholog of the human receptor for the type-1 peroxisomal targeting signal protein. His study indicated that compromising peroxisomal protein import likely reduced peroxisome-involved degradation of mmBCFAs, at least in part through a β-oxidation-independent mechanism [64].

Since neither TAT-2 nor PRX-5 was part of the hypothetical regulatory system, Zhu Huanhu, a new postdoctoral fellow, continued the suppressor screen effort in 2009. Among the suppressors he isolated, one appeared to be the one we had chased all along: this mutation permitted the *elo-5(-)* worms to grow for indefinite generations without recovering mmBCFA biosynthesis. These suppressed worms were very unhealthy and grew slowly, which was totally expected given the likely diverse roles of these lipids and the gene defined by the suppressor. He position cloned the suppressor gene and showed it encodes a homolog of the NPRL3 family protein, which instantly implicated a connection to the TORC1 signaling pathway because previous genetic work in yeast had indicated a repressive role of the NPR2/NPR3 complex on TOR [65]. Huanhu then made three transgenes, following the published schemes used in mammal cell cultures [66–68], to show that activation of TORC1 allowed mmBCFA deficient animals to bypass L1 arrest (Figure 2) [69]. In addition, the suppression by the *nprl-3(-)* mutation can be reversed by reducing TORC1 activity. We concluded that mmBCFAs promote TORC1 activity that controls postembryonic development.

Ironically, the lab had suspected the role of TOR-related pathways in mmBCFA-function several years prior; a postdoc even wrote an NIH fellowship on testing TORC1 functions. However, those early efforts, mostly relying on making transgenes and applying RNAi, did not reveal the connection. Indeed, the suppression effect by this *nprl-3(ku340)* mutation is far more robust than any of the hyper-TORC1 transgenes Huanhu made [69], which validated the strategy

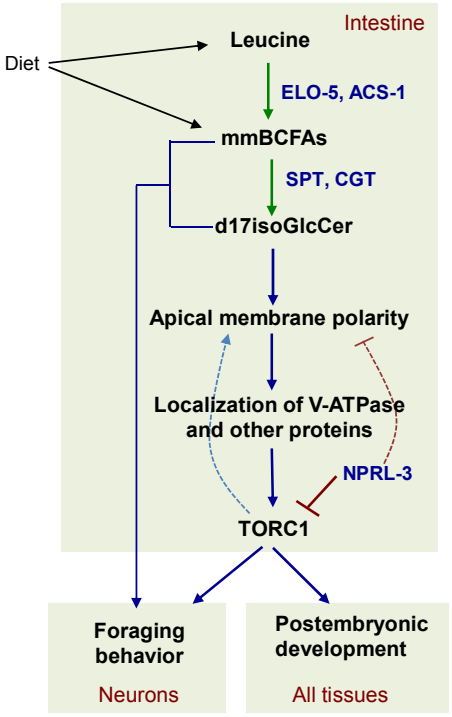

Figure 2 (color online) A simplified model of how mmBCFA biosynthesis impacts TORC1 signaling, postembryonic growth and development, and foraging behavior in *C. elegans*. Each green arrow represents multiple enzymatic reaction steps in the biosynthesis pathway [56,69,76,94]. Each blue arrow may not mean direct actions in regulation. Loss of NPRL-3 activates TORC1 and also partially restores apical polarity in the absence of mmBCFAs. The latter effect could be due to a potential role of NPRL-3 on apical polarity or a potential role of TORC1 in promoting apical polarity (see text for discussion). mmBCFAs: monomethyl branched-chain fatty acids. GlcCer: glucosylceramide. SPT and CGT denote palmitoyltrasferases and ceramide glucosyltransferases, respectively. Only a few selected proteins among all acting in this circuit are indicated in this simplified scheme.

of doing chemical mutagenesis and the modifier screen.

In Huanhu's 2013 paper, we also described a brute force effort to identify d17iso-glucosylceramide as the high-order lipid that mediated the role of mmBCFAs on TORC1 signaling, following the suggestion from our earlier study on TAT-2 [61]. Mass spec and other biochemical methods were effective arsenals in this study. For example, to prove mmBCFA-containing sphingosine is an intermediate in the pathway, Huanhu and another postdoctoral fellow Shen Huali, an analytical chemist with experience in mass spectrometry, had to purify this special lipid from a special bacterial strain using mass spectrometry as the readout. Additionally, they also had this lipid chemically synthesized. Huali also analyzed the lipid profile of *elo-5(-);nprl-3(-)* mutants to make sure that d17iso-glucosylceramide was not present in the suppressor strain, which helped to solidify the proposal that these lipids, mmBCFAs and d17iso-glucosylceramide, are not absolutely essential for development, but only when TORC1 is activated by the *nprl-3* mutation (Figure 2).

An obvious question for further study was how these lipids actually promote the activity of TORC1. TORC1 is known as a "hub" that senses the levels of multiple nutrients and metabolites, including amino acids, growth factors and energy, for diverse physiological functions [70]. Some "sensing" tasks are mediated by other signaling pathways. For example, the impacts of glucose, growth factors and energy level on TORC1 are mediated by IIS, TSC/Rhb and AMPK pathways [70,71]. Mechanisms of TORC1 activation by amino acids are less clear and appear to be independent of these known signaling pathways [70,71]. Moreover, different mechanisms may be involved in the activation of TORC1 by different amino acids [72,73]. Far less has been learned about the roles of lipids on TORC1. Because branched-chain amino acids (leucine, isoleucine and valine) are precursors of mmBCFA biosynthesis [48,74,75], there is potentially an interesting connection between studies on these two types of nutrients. Additionally, leucine happens to be the precursor of *C. elegans* mmBCFA and the most extensively analyzed amino acid for TORC1 activation.

Aileen Sewell and Zhu Huanhu carried out a multi-assay screen to identify potential factors that mediate the impact of d17iso-glucosylceramide on TORC1 [76]. They identified a number of genes that have known functions in regulating membrane polarity. They found that mmBCFA deficiency disrupted apical membrane polarity in the intestine, which confirmed the previous findings from Verena Gobel's lab that sphingolipid biosynthesis is required for *C. elegans* intestinal apical membrane polarity [20,77]. Further analysis led to the conclusion that clathrin/AP-1 dependent intestinal apical membrane polarity regulates apical localization of the V-ATPase and other proteins that, in turn, affect intestinal TORC1 activation (Figure 2) [76]. One surprising observation was that apical polarity of mmBCFA deficient animals was dramatically improved by the addition of the *nprl-3(-)* mutation and the apical localization defect of the V-ATPase was also significantly suppressed. Additionally, the *nprl-3* mutation could also partially suppress the developmental defects caused by knocking down clathrin and AP-1. These data may suggest that NPRL-3 has a role in regulating apical membrane polarity, which would point to a novel mechanism by which the NPRL-2/3 complex represses TORC1, as such a mechanism is distinct from the reported role of the NPRL2/3 complex as a GAP protein to regulate RagA/B in mammalian cell lines and yeast [78,79]. Alternatively, the suppression of the apical polarity defects by *nprl-3(-)* could be due to a role of TORC1 in promoting apical polarity (Figure 2). Such a role of TORC1 would not be essential in wild type since disrupting TORC1 does not cause apical polarity defects [76]. It might be important to note that our finding was based on analysis of a specific tissue (intestine) for specific physiological roles (development) in animals, unlike the conditions in the mammalian cell and yeast studies. The functional relationships between membrane polarity, TORC1 and NPRL-2/3 need to be tested in other organisms. Huanhu will surely make more advances in this area in his own new lab in Shanghai.

These findings suggested that the TORC1 signaling system not only sensed the level of certain nutrients through various vehicles, it also sensed the integrity of metabolic pathways and their regulated specific membrane dynamics. These studies on mmBCFAs and glucosylceramides also presented an outstanding example for how lipid variants impact specific signal transduction pathways. However, the mechanisms by which the actual difference in the FA structure, the little methyl side chain in the case of mmBCFA, affects the membrane properties remains a fascinating question. In 2012, Marina and Huali published a seminal paper that tackled such a question while uncovering the role of mmBCFAs on another specific cell signaling event, IP3 signaling in the zygote [80].

4 Impact of mmBCFAs on cell signaling and embryogenesis—the IP3 connection

A free FA needs to be "activated" by an acyl-coA synthetase (ACS) to become a fatty acyl-CoA before engaging in further metabolic reactions such as elongation, degradation, incorporation into a high-order lipid, or lipidation of proteins [81,82]. The ACS enzymes belong to a large protein family, with more than 20 members in mammals and alternative splicing could potentially generate even more variants, which may suggest that ACSs play important roles in facilitating specific functions associated FA variants [81,83,84]. First, specific ACSs may only use specific types of FAs as substrates. Second, if the esterification of FAs by ACSs is coupled with further reactions, an ACS

could potentially also specify the fate of particular FAs. Finally, different ACS genes may also be differentially expressed in different tissue and at different times during development. Several ACS enzymes have been studied extensively in mammals, connecting to specific physiological functions (e.g, [84]).

An mmBCFA specific ACS in *C. elegans*, ACS-1, was discovered by Marina in her search for the feedback regulatory loop for mmBCFA homeostasis [48,56]. ACS-1 is required for the biosynthesis of C17iso and the ability of free, dietary mmBCFAs to rescue the growth of *elo-5(-)* animals. However, Marina observed a fully penetrant early embryonic lethality associated with the *acs-1(-)* mutant, but only did so when she fed the mutant with certain levels of dietary C17iso [80]. More specifically, all the membrane dynamics of the zygote and early embryo, including exocytosis and cytokinesis, were disrupted. Two questions were immediately raised about the mechanism underlying the spectacular phenotypes: what caused the membrane defects and what did the mmBCFAs do to the lipids in the absence of ACS-1?

Our previous experience with suppressor analysis served us well here in finding out that the major membrane defect in the early embryo was mainly due to the failure of IP3 signaling that regulates cellular Ca^{2+} level. Marina found that mutations that hype-activate IP3 receptor or raise IP3 levels [85–88] can suppress much of the early embryonic defects and, conversely, RNAi knockdown of the IP3 receptor produced an embryonic phenotype similar to that of *acs-1(-)* with C17iso supplementation. The role of IP3 signaling in regulating exocytosis and cytokinesis is consistent with studies in vertebrate animals [89–91]. This again linked mmBCFA metabolism to a specific signaling event, but by a very different mechanism (Figure 3).

Huali devoted a huge effort to the identification of specific changes in lipid composition using mass spec, while Marina used several genetic tools to determine that ACS-1 needs only to be expressed in the somatic sheath cells for this function. The combination of these analyses led to a model where C17iso is incorporated into phosphatidylethanolamine (PE) in the somatic gonad in an ACS-1 dependent manner, while C17iso is also incorporated into a novel phospholipid (PL) in an ACS-1-independent manner. These lipids with proper balance are transported into the oocytes and then zygotes to support proper membrane functions. In the absence of ACS-1 but with the C17iso supplement, C17iso is incorporated into this novel type of PL but not PE, creating the imbalance of PL composition or the dominant presence of this novel PL that is detrimental to the zygote. This novel PL, of which the structure had been extensively analyzed by a graduate student but not yet published, was only detected in the embryo, not in adult worms. Therefore, ACS-1, an enzyme that adds CoA to an mmBCFA, "regulates" the PL composition in the zygote by somehow channeling this FA to a subgroup of PL in the somatic gonad that

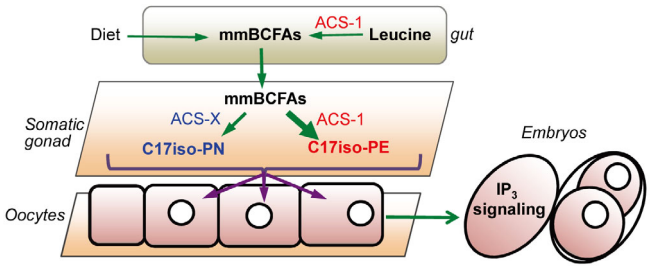

Figure 3 (color online) Model of how acyl-coA synthetase activities on mmBCFAs impact lipid composition and a specific signaling event (IP3) in a specific tissue (zygote) [80]. mmBCFAs are synthesized or obtained from diet in the intestine. They are incorporated into certain classes of phospholipids (PL) requiring specific ACS enzymes. ACS-1, acting in *de novo* synthesis of mmBCFAs in the intestine, is also required for channeling mmBCFAs into phosphatidylethanolamine (PE) and related PLs in the somatic gonad that wrap around the germ cells. However, mmBCFAs are also incorporated into a different class of PL (referred to as PN, see text) in a ACS-1-independent manner, suggesting the involvement of one or more other ACS enzymes (ACS-X) in the step. In wild type, C17iso-PE and C17iso-PN, in a proper ratio, enter oocytes and critically contribute to the proper lipid composition in the zygote. Such lipid composition, likely unique to the zygote, is essential for IP3 signaling that regulates exocytosis and membrane dynamics during early embryogenesis. When ACS-1 function is lost in the somatic gonad, C17iso-PE, but not C17iso-PN, dramatically decreases, leading to imbalanced lipid composition in the zygote and complete disruption of membrane ruffling, cytokinesis and other cellular processes. These defects can be suppressed by hyper-activating IP3 signaling.

wraps around the germline (Figure 3). How the earlier reaction (ACS action on FA) dictates the specificity of the later reaction (incorporation of FA-CoA to a PL by a transferase) is unknown and a very challenging question that is too difficult to address for multiple reasons. The results from this study may perhaps imply a more important message: the little methyl branch in mmBCFAs might not have any meaningful structural function in the PLs; the specificity of the mmBCFA-ACS-1 interaction may simply allow the animals to manipulate or alter PL compositions in a specific tissue for a specific cell signaling event.

I considered this 2012 paper, published in Genes Dev with a review [80] and a cover illustration, to be one the best papers I have ever authored and it significantly enhanced our chances to get my HHMI investigator appointment renewed one more time in 2012. Besides the scientific significance and complexity of the study, I also love the fact that it took us three years from our first journal submission to its final publication. In these three years while we revised the manuscript for multiple rounds of submission to *Science*, *Cell and Dev Cell*, before sending it to *Genes Dev*, we benefited tremendously, not only about improving our presentation and experimental support, but also in understanding the significance of our own data and working in the lipid field in general. The lessons we learned were sometimes from the suggestions by reviewers, but more often by seeing the misunderstanding by the editors and reviewers. Our initial draft, that focused too much on the

cytokinesis defects rather than the key issues I mentioned above, was quite naïve. The reviewers tended to treat it as a cell biology paper. The manuscript progressively got better as we became wiser and more realistic about working and publishing in the field. We also learned that it is generally very difficult to get editors and reviewers to fully appreciate such an interdisciplinary study in this rather "cold" lipid field. One difficulty seemed to be finding reviewers who had the diversified expertise and desire to evaluate this study, that used both extensive mass spec analysis of lipids and sophisticated genetics to address a metabolic problem with developmental phenotypes.

5 The impact of lipid metabolism on foraging behavior

Lipid metabolism has been shown in limited studies to impact animal behaviors including food responses (e.g., [92,93]). Our lab also made a significant contribution in this regard by discovering the role of the mmBCFA/d17iso-glucosylCeramide/TORC1 pathway in foraging behavior [94]. Marina noticed a few years ago that mmBCFA-deficient L1 larvae failed to locate food and much of this failure was due to defects in dwelling behavior. This defect was not associated with L1 larvae arrested due to food deprivation (L1 diapause). She then determined that mmBCFAs are required for proper expression of a transcription factor in the neurons that in turn regulates differentiation or maturation of certain sensory neurons that contribute to the defect in food seeking. Huanhu and Aileen found that this mmBCFA-dependent foraging behavior is also mediated by glucosylceramide and at least in part by TORC1 in the intestine (Figure 2) [94]. What is the physiological significance of a lipid biosynthesis pathway and TORC1 signaling in the gut having such a profound impact on food seeking responses? During starvation, when growth and development are mostly suppressed by changes in certain regulatory systems, animals must continue to develop their neuronal systems to maintain or enhance food-seeking ability [95,96]. Lipids, which are more stable than some other nutrients or metabolites under starvation, may promote neuronal functions. This study also suggested that the intestine plays a critical role in the dwelling behavior; the quality of the food is accessed by a signaling mechanism in the intestine that requires the mmBCFA biosynthesis pathway. How do the activities of these lipids and TORC1 in the intestine impact transcription factors and other factors in the neuron for this behavior is an attractive question to pursue further. Genome-wide expression analysis [94] and a systematic genetic screen (unpublished) are the first few steps to address this question.

6 Closing remarks

In today's research environment, moving to a new field where you do not have expertise and connections is expected to be difficult, especially for a small or modest size laboratory like ours. Getting your papers published in good places and earning good scores on your grants could be even bigger obstacles than actually doing the work. Our positive experience, if considered to be successful, may be attributed to two other key factors: (i) the lab has been supported since 1997 by HHMI that encourages risk taking, funds people rather than specific projects, and cares relatively less about whether you have published in certain journals; and (ii) the lab researchers are encouraged to work independently and follow their own ideas. The upside of (ii) might not seem to be as obvious as that of (i). In my opinion, the ability of students and postdoctoral fellows to take on projects in unfamiliar territories is far greater than most PIs realize. This "hands-off" style of supervision should not simply be criticized for providing a weak training environment, as has often been commented in NIH postdoctoral training grant review sessions. As you may have sensed from reading above, the vast majority of new research directions, including our studies on lipids, KASH-SUN complexes, miRNAs, and genetic redundancy, were initiated by students and postdoctoral fellows, and in a couple of cases against my initial suggestions. In fact, newcomers often prefer to work on new directions or to perform screens that have the potential to identify novel things. Furthermore, postdoctoral fellows are encouraged to take the projects they worked on to their own lab, when they obtain an independent research position.

While our research style may not be suited for or valued by too many PIs, we will continue to have fun exploring new and interesting questions, some of which will be in areas we were not familiar with. For example, two postdoctoral fellows are leading the effort in two new and underexplored directions: uncovering and characterizing cellular regulatory systems that connect changes in nucleotide levels to development and that connect lipid metabolism to germ cell development.

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Biographical Sketch

Han Min is a Professor in the Department of MCDB of the University of Colorado at Boulder and an Investigator at the Howard Hughes Medical Institute (HHMI). He obtained his BS degree in biochemistry from Peking University in 1982. Through the CUSBEA program, he enrolled in the Ph.D. program at Institute of Molecular Biology of UCLA, in 1983, where he studied functions of histones and nucleosomes in transcription under the guidance of Dr. Michael Grunstein. Between 1988 and 1991, he received postdoctoral training in Paul Sternberg's lab at Caltech where he took the developmental genetics approach to uncover the role of Ras signaling in controlling cell fate specification in the nematode *C. elegans*. His own research group, established in 1991 in Boulder, has combined animal genetics with molecular, biochemical and genomic tools to understand cellular mechanisms underlying animal development, behaviors, and metabolism. He has received several scholarships and awards during his research career and became an HHMI investigator in 1997. In the last 15 years, Dr. Han has also made significant efforts in research and education in China, including serving as adjunct professor at the Institute of Developmental Biology and Molecular Medicine at Fudan University.