How Diverse Cells Position Themselves in an Embryo: Variations on a Common Cytoskeletal Theme

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ABSTRACT

Jessica Ragas Harrell: How Diverse Cells Position Themselves in an Embryo: Variations on a Common Cytoskeletal Theme
(Under the direction of Dr. Bob Goldstein and Dr. Mohanish Deshmukh)

Understanding morphogenesis, the spatial and temporal distribution of cells during development of an organism, is a key goal in studies in developmental biology. Throughout diverse developmental systems, only a few cytoskeletal mechanisms are used to achieve the cell shape changes and movements that are required for development to proceed properly. Among these mechanisms is apical constriction, the active narrowing of the apical side of a cell to drive its movement. This mechanism is critical during development of *C. elegans*, sea urchins, *Drosophila*, and *Xenopus*, including neural tube closure in vertebrates. The organisms use a variety of patterning mechanisms to spatially regulate this common cytoskeletal movement. We have found that apical constriction is used reiteratively in cells of distinct lineages to internalize during gastrulation in *C. elegans.* This presents an opportunity to examine the diversity of patterning mechanisms that regulate cell movements within a single organism. Our results show that these cells in distinct lineages use different fate regulators and surprisingly different cell polarity regulators to control the timing of internalization during gastrulation. We conclude that while diverse organisms utilize diverse patterning mechanisms to regulate common

cytoskeletal mechanisms for cell internalization, diverse patterning mechanisms can be associated with common cytoskeletal mechanisms within a single organism.

To my biggest fan, my grandmother

ACKNOWLEDGEMENTS

Writing the acknowledgement section of my thesis seems surreal to me. Didn't I just start graduate school yesterday? Well ok, I admit, it doesn't really feel like it was yesterday. It has actually been a long road. I feel like there are countless people that I need to thank for getting me through this. From the difficult times during and after Hurricane Katrina to the difficult decision to change labs, as well as the challenges of being a working mom added to the normal stresses of being a graduate student, I have had the most wonderful support system. I'm going to try not to get too mushy and sentimental, but sometimes I just can't help myself.

UNC has been a wonderful place to do my graduate work. I'm going to begin by thanking Dr. Vytas Bankitis, the chair of the cell biology department, for believing in me. When I came to the crossroads of graduate school at the end of my third year, he made an investment in me because he was convinced that I would succeed, even though I no longer believed in myself. Dr. Sharon Milgram also offered an enormous amount of support during this time, and I appreciated all of her advice as well. Their encouragement led me to seek out a new lab, and I am especially grateful to Dr. Bob Goldstein for accepting me into his lab.

I don't think Bob knows how thankful I am that he gave me a chance to join his lab and continue graduate school. He had no idea who I was when I showed up in his lab, homeless and needing a place to continue on this journey, yet he and his

lab adopted me and made me part of their family. I also don't think that I will ever be able to thank him enough for his support of me as a graduate student-parent. He was supportive from the moment that I told him that I was adding to my family, and being the father of two adorable and energetic boys, he has offered many valuable pieces of parenting advice to me as I have navigated from infancy to the terrible twos. Bob has also been extremely supportive of my career. I am tremendously thankful for the teaching opportunity that I had this semester, and it was only possible because of his assistance. Not many students are fortunate enough to have such an accommodating advisor in graduate school and I don't take for granted how lucky I have been.

I would also like to thank the members of the Goldstein lab. I have truly enjoyed working with all of them. Their encouragement, support, and advice for one another is unrivaled, and I am so fortunate to have spent several great years with them. Dr. Jenny Tenlen has been the most wonderful resource for anything and everything – from worms to careers, and I appreciated her willingness to help me, even at 1am. Dr. Jessica Sullivan-Brown has been very encouraging during the last several months as I have juggled many different tasks. She's been a wonderful friend, and I appreciated her attendance in the cheering section of my first lecture in my genetics class. Dr. Gidi Shemer and Dr. Dan Marston are no longer in the lab, but both of them deserve thanks as well. Gidi has been a wonderful resource this semester as I taught my first genetics class, and his encouragement has been invaluable. As for Dan, I probably owe him a six-pack of beer or something for helping me through that awful pMLC protocol.

My fellow graduate students in the trenches with me were what kept me going through this process. Erin McCarthy Campbell has graduated and moved on, but she was a wonderful source of inspiration as I watched her finish her graduate career with a smile. Jacob Sawyer always knew when I was having a bad day, but as the best storyteller around, he could easily cheer me up. Adam Werts kept me laughing, even when he wasn't trying, and I appreciated the comic relief when I couldn't seem to get a simple PCR to work. Minna Roh-Johnson was a brief labmate when she rotated in the Schaller lab, and I was so happy to be working with her again in the Goldstein lab. I really admire her work ethic and drive – I think she has a long and successful career ahead of her. She has been an amazing friend through all of this, and I truly don't think I would have made it without her. She was always willing to listen and often offered alternative perspectives when I thought a failed experiment was the end of the world.

I would like to thank the members of my committee that encouraged me through this process: Dr. Mohanish Deshmukh, Dr. Ken Jacobson, Dr. Dave Reiner, and Dr. Frank Conlon. I have high regard for all of them as scientists and each of them brought a unique perspective to my project. They were challenging, yet encouraging, at every committee meeting, and I have appreciated their contributions and guidance.

I feel as though I would be remiss if I did not acknowledge Dr. Mike Schaller. As the graduate studies director of the cell biology department at the time that I applied to graduate school, he played a large part in recruiting me to UNC. While I wish that my time in his lab would have had a different outcome, I think that he

would agree that things have worked out better for both of us. I have done very well in the Goldstein lab and I have heard that he is enjoying his position in West Virginia.

There are a couple of people from my past that I would like to thank. Dr. Samir El-Dahr hired me as his technician at Tulane and was extremely generous in the opportunities and experiences he offered me. He was very encouraging during the application process to graduate school and has continued to be interested in my success.

I would not be getting my Ph.D. if it wasn't for a professor when I was an undergraduate at LSU that took the time to tell me about research and gave me the chance to work in his lab. I am extremely thankful to Dr. Patrick Limbach and all of the opportunities he gave me when I worked for him. The experiments, the paper, the meetings, and the guidance were all invaluable to my career. I appreciate that he is still just a phone call away.

Lastly, I would like to thank my family. I need to thank my parents, Mom, Bobs, and Dad, who have always believed in me and encouraged me to challenge myself. They always told me that I could do anything, and I am thankful for the confidence they instilled in me. They all made sacrifices to ensure that I received a good education, which allowed me to take advantages of many opportunities that have been instrumental in getting me where I am today. My sister Tobi has always kept me on my toes and always gave me a good laugh when I needed one. I just wish that she lived closer. I need to mention my grandparents, who played a large role in my upbringing – they have been wonderful role models. My grandmother was at every band concert, every play, and picked me up from every after-school activity

(and there were a lot of them). She has always been my biggest fan. I have countless aunts, uncles, and cousins, (I'm from south Louisiana, after all) that cheered me on during this process as well, and I feel very blessed to come from such a large and loving family. I would also like to thank my in-laws for their willingness to help whenever we needed them to, and for their interest in my success.

Finally, I need to thank the two most important men in my life. My husband Patrick has been my rock through all of this. He didn't always understand exactly what I was doing in the lab, but whenever I needed a little bit of encouragement or support, he was there to keep me going. I can hear him singing the Rocky song to me now. Without the sacrifices that he has made for me, none of this would be possible. He has given me the best gift of all, my son Peyton, who brightens my world every day and has taught me about what truly matters in life. I can't wait to see what the future holds for all of us.

PREFACE

How did I end up here? I often ask myself this very question. I didn't grow up thinking that I was going to get my Ph.D. I didn't wake up one day and decide to get one either. However, after a series of events, decisions, and influences from others, here I am.

I have always liked science. One of my first science teachers, Miss Williams, asked us to draw a picture of a scientist. I drew an old man in a white lab coat, his face covered with a beard and his eyes hidden behind goggles. Most of my classmates drew the same type of picture. Miss Williams wanted to know why we all thought you had to be a man to be a scientist. She told us about her friend that was a scientist – a young female. She wasn't old, and she certainly didn't have a beard. I suppose that was the stereotype at the time, and I'm actually not sure that it has changed much, despite the fact that half of my graduate school class getting their Ph.Ds in science are women. Nonetheless, Miss Williams's point was that any of us could be a scientist, and her class was where my love of science began.

When I started my undergraduate studies at LSU, I wanted to major in biomedical engineering. Then I thought about being a physical therapist. A nurse. A medical technician. Obviously, I liked science but I needed direction. One of the majors I was considering required me to take an analytical chemistry class, and I ended up in a class being taught by Dr. Patrick Limbach. I enjoyed the class,

and I felt that Dr. Limbach was approachable. I visited him in office hours one day when I had some questions about a homework problem I was working on. He asked me what I was majoring in. I didn't really have a clear answer and he asked me if I had thought about doing research and majoring in biochemistry. The word biochemistry actually scared me. We talked for quite awhile that afternoon and he told me all about a career in research, what people in the lab did, the types of classes that I could take, and the idea of going to graduate school. He even offered to let me do research in his lab. That day was probably a defining moment for me in my career. I certainly hadn't gone to his office to figure out what I was going to do with my life, but I felt like things were starting to take shape when I left.

The next semester, the spring of 1999, I declared biochemistry as my major and I started undergraduate research in Dr. Limbach's lab. While a member of his lab, I worked with a fantastic graduate student, Tracey Simmons, on an independent project comparing different techniques to purify DNA for analysis. I was able to attend several meetings to present my research and wrote and published a paper on my work. It was a wonderful opportunity and I'm extremely thankful that Dr. Limbach generously offered to let me start my research career in his lab.

While still an undergraduate, I was encouraged to participate in a summer research program at another university to get a feel for a different environment and a different field of research. In the summer of 2000, I became a member of the summer undergraduate research program at Rockefeller University in New York. That was a fantastic summer. I had already taken biochemistry and studied restriction digests, PCR reactions, and gels, but I had not gotten the chance to do

any of these types of experiments myself. I remember putting together that first digest and running my first gel and how exciting it was. I could actually see the bands on the gel and they were the right size! (At the time, no one told me that it doesn't always work correctly the first time!) That summer, I fell in love with my project and developmental biology. I worked in a neuroscience lab, specifically one that was working on the function of the hair cells in hearing. I was examining the expression patterns of two very similar genes in developing zebrafish embryos. I took some beautiful pictures of the hair cells and neuromast cells in these embryos. I enjoyed the biological application of the research that I was doing that summer, and this experience really influenced my next step.

I graduated from LSU in May 2001, but I didn't think I was quite ready for graduate school. I really liked biology and model organisms but most of my research background had been in chemistry, except for the short summer at Rockefeller. I wanted to get more biological research experience before starting graduate school.

I worked at Tulane Medical School as a technician for two years. The first year I worked in a yeast lab examining telomere chromatin structure. The second year I worked for Dr. Samir El-Dahr in a developmental biology lab analyzing the role of p53 and cell adhesion molecules in kidney development. Dr. El-Dahr was very encouraging and willing to give me many opportunities to learn new techniques. I considered staying in his lab to do my graduate work, but ultimately decided that I really liked the environment and research atmosphere at UNC. For a New Orleans girl with strong family ties and deep roots, this was not an easy decision. Leaving for

a summer project was easy – I knew I'd be back but this move away to another state would be long-term, perhaps even permanent. Luckily, I had a loving partner, my husband Patrick, that fully supported this move.

In the fall of 2003, I started my graduate program in the Cell and Developmental Biology Department at the University of North Carolina in Chapel Hill. I rotated through three labs my first year and ultimately decided to join the laboratory of Dr. Mike Schaller. I wanted to understand the role of Focal Adhesion Kinase (FAK) in breast cancer, since many breast cancer cells have more FAK than normal cells. However, for a number of reasons, some of which were out of my control, things did not go well in the Schaller lab. I pondered my life quite a bit in my third year of graduate school. Did I want to stay in graduate school and change labs? Did I want to find something completely different to do? If so, what? I don't make decisions very quickly and this was a major decision that I certainly didn't take lightly.

I was in the process of preparing for my proposal exam at the beginning of my third year when Hurricane Katrina struck the gulf coast in August 2005. This was one of the worst periods of my life. My mother and stepfather evacuated to North Carolina to stay with us and we spent that week trying to assess the damage, figure out where everyone was, what they were doing, and what was next. It was several agonizing days before we knew that everyone was safe. Meanwhile, I was supposed to be writing up my thesis proposal, and that didn't seem to be going very far. It was hard to get away from the sensational news coverage and the ringing phone. Thankfully, my department chair and committee were understanding and

allowed me to delay my proposal exam for a couple of weeks. While a very difficult time, I decided that this was not the best time for me to make a decision about my future. I didn't want what was going on at home to cloud my judgment about my career.

After several months of considering my options, I finally determined in July 2006 that I needed to make a decision about my future. The results of the FAK and cancer project that I had been working on had just been published by another lab, and I was going to have to start over no matter what. With the influence and guidance of several wonderful professors, including those from my past, I decided that it was in my best interest to change labs. This was not a decision I made lightly. I didn't like giving up on something and I certainly didn't want to hurt anyone in the process. But I knew the reality was that I would not finish graduate school in the lab that I started in. There were just too many confounding factors. I had come to UNC to get my Ph.D. and I still wanted to get my degree.

As I pondered where and how to start over, I thought a lot about what I needed in my next lab. I knew that I needed to be in a lab with a lot of good energy, a good learning environment, and I needed to be doing something that I was excited about. I asked for and received much advice as I took this next step. I was fairly certain that I wanted to teach when I finished graduate school, and one suggestion I received was to choose a lab with a good teaching model organism. There were several to choose from, but I was particularly intrigued by *C. elegans*. I had heard a *C. elegans* talk by Dr. Geraldine Seydoux at a symposium the year before and was blown away. I had no idea that worms were so cool. There were two worm labs on

campus and I started reading a little more about each of them. One of Dr. Bob Goldstein's papers caught my attention, and I was impressed by the simplistic yet elegant approach he used to answer a question about gut cell specification in *C. elegans* embryos. Since then, I have used this paper a number of times in journal clubs that I have led in a variety of classes – it still remains a favorite of mine.

I contacted Bob, explained who I was, gave him a little background on my situation, and asked him if I could meet with him. I waited several agonizing days to hear back from him and I was ecstatic when he agreed to talk with me. It really wasn't until much later that I learned he had no intention of taking me into his lab. Actually, it was best that I didn't know this at the time. We met in his office and he told me about the different projects in the lab. I was especially intrigued by the gastrulation projects and only became more excited about the possibility of working there. Bob was kind of vague at the end of that meeting about whether he had any space available, but he suggested that I might want to meet with the different members of the lab and hear more about their individual projects.

I returned to Bob's lab the next day and spent time with each graduate student and post-doc, still not really knowing where this was headed. It was quite an exhausting day and I felt like my head was going to explode from information overload by the time it was over. Bob mentioned that he wanted me to come back the following day and tell him more about what I had been working on in the Schaller lab. Unfortunately, I wasn't going to be able to, so I offered to tell him about it right then and there. I pulled out the dry erase markers, started drawing on the board, and gave a chalk talk about my project. Luckily, I enjoy explaining things to people,

so I found this part to be pretty easy. Again, I left his office without really knowing if there was space available for me. We agreed to meet the next week. In the meantime, Bob did a background check, called my references, (I'm quite certain he googled me), and I read a few papers on *C. elegans* gastrulation. I showed up in Bob's lab the next week, and I was really hoping he was going to let me stay permanently. Looking back, I was really optimistic that Bob was going to let me set up shop in his lab because I didn't have any other plans for that day. Fortunately for me, he decided to give me a chance in his lab, and this was yet another defining moment on my way to my Ph.D.

The first chapter of this thesis is a portion of a published review on apical constriction that many lab members contributed to. This chapter represents my contribution to the review. The second chapter represents my project in the Goldstein lab. Having already spent a number of years in graduate school, I chose a straight-forward project to begin when I joined the Goldstein lab. Much work had been done in our lab and by Dr. Jeremy Nance that characterized the internalization of the endodermal precursor cells, the beginning of gastrulation in *C. elegans.* However, there are many more cells that need to internalize – cells that contribute to the germline, muscle cells, pharynx, etc. Failure of any of these cells to internalize would be a tragic ending for these embryos too. Not much attention had been given to these other cells, so my goal was to understand how these cells were internalizing and enlighten the rest of the world about gastrulation in *C. elegans.*

Many people had the misconception that the endodermal precursor cells were the only cells that internalized in this organism. Those other important cells

were often forgotten about and I decided that it was time that people gave them the recognition that they deserved. I remember telling Dr. John Wallingford, a respected scientist studying *Xenopus* morphogenesis, about my project when he visited UNC, and even he hadn't realized that there were other gastrulating cells in *C. elegans*. He told me that he thought my project was "bitchin'" and I walked around on cloud nine for weeks afterwards.

In theory, my project was straightforward, but that didn't make it easy. One of the first experiments that I attempted was immunostaining staged embryos for phosphorylated myosin regulatory light chain. I think I tried that experiment two or three times a week for the first 18 months I was in the lab. There were so many places where things could go wrong in that protocol and I managed to fail at every single one of them at some point. Dan Marston, a post-doc in the Goldstein lab, had developed the protocol in our lab, and finally we sat side-by-side one day doing the protocol and trading experimental slides at specific places in the protocol to narrow down the trouble spots. This helped tremendously and I stopped cursing him for developing the protocol in the first place.

Dan's help exemplified why I loved the Goldstein lab so much. I worked with some of the smartest people I have ever met, but for me the best part was how generous and supportive all of them were. Being a part of this lab was like being part of a family – we helped each other troubleshoot, we prepared each other for important talks, we gave feedback on papers and proposals, and we celebrated each other's triumphs.

Bob has many Bobisms, and one of my favorites is, “It’s your career!” He feels like we are in graduate school to be trained for what we want to do next, and that next step is different for each person. I think Bob may be one of the few PIs that thinks this way. Many feel as though we are here to do research and only research, and I can understand and respect where they are coming from too. But I was lucky to have Bob as an advisor because he has been very understanding and supportive of my desire to teach. He has allowed to me to determine what would be best for my career and has allowed me to pursue my teaching interests, even when it distracted from my research. I have managed to acquire quite a bit of teaching experience and I have loved every minute of it. I am excited about what the future holds, and I can’t wait to share everything I’ve learned with the next generation of students.

There were many defining moments for me on my way to getting my Ph.D. Some of these moments had a positive impact, others sent me reeling in the other direction. I have learned a great deal about myself in this process. There were many, many times where I doubted myself and thought that I wouldn’t make it. I’ve cried a lot, but now as I stand at the finish line, I think I’ve taught myself that with hard work and determination I can do anything

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LIST OF ABBREVIATIONS AND SYMBOLS

AB	AB lineage
C	C lineage
C. briggsae	*Caenorhabditis elegans*
C. elegans	*Caenorhabditis elegans*
DIC	Differential Interference Contrast
D	D lineage
E	endoderm
MS	mesoderm
GEF	Guanine nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GTPase	Guanine Triphosphate hydrolase
min	minute
n	number
N2	wild-type *C. elegans* strain
NMY-2	Non-muscle Myosin-2
p	p-value
P_4	germ line precursor
PAR	Partitioning Defective
PH	pleckstrin homology
PLC	Phospholipase C
p-RMLC	phosphorylated regulatory myosin light chain
SD	Standard Deviation

μm	micrometer
WT	wild-type
ZF1	Zinc Finger 1
Z2	primordial germ cell
Z3	primordial germ cell
<	less than
>	greater than
δ	delta
±	plus and minus

CHAPTER 1

INTRODUCTION

This chapter represents my contribution to a review on apical constriction that has been accepted for publication in Developmental Biology (Sawyer et. al. 2010).

Organisms use a variety of mechanisms to achieve the morphogenetic movements that shape the final animal. One conserved mechanism that is used repeatedly in a variety of organisms to drive cell rearrangements and movements is apical constriction, the narrowing of the apical surface of a cell. In 1902, Rhumbler first proposed the importance of the constriction of the apical sides of cells to drive the bending of cell sheets in a variety of developmental systems (Rhumbler, 1902). Later, an epithelial sheet of cells was modeled using brass bars and rubber bands. The addition of rubber bands increased tension on one side and resulted in a concave shape, much like the bending of a sheet of cells (Lewis, 1947). This model showed that increased tension on one side could result in bending, supporting the hypothesis first proposed by Rhumbler.

Many years after Rhumbler and Lewis's models, there are many known examples of the use of apical constriction during morphogenesis, including during *Drosophila* gastrulation and dorsal closure, wound healing in *Xenopus*, and vertebrate neural tube formation. (For a complete review on these topics, see

(Sawyer et al., 2010). In addition, many historical and fundamental experiments on the mechanisms driving cell shape changes have been completed in sea urchins, *Xenopus*, and *C. elegans* gastrulation and those will be focused on here.

1.1 SEA URCHIN GASTRULATION: MULTIPLE MECHANISMS MAY DRIVE TISSUE BENDING

Perhaps surprisingly, given the large repertoire of classes of morphogenetic movements available to embryos, many organisms have evolved a role for apical constriction in gastrulation (Stern, 2004). In gastrulating sea urchin embryos, cells on the vegetal surface of the embryo become columnar, forming the vegetal plate. The surface of this plate bends inward, a process termed primary invagination (Figures. 1.1 and 1.2). Primary invagination is accompanied by a number of other movements; here we discuss only the primary invagination, which has been proposed to be driven by apical constriction (for review see Davidson et al., 1995; Kominami and Takata, 2004).

The cells that undergo primary invagination form the archenteron, or future gut. Computer modeling suggests that apical constriction of cells in the vegetal plate could feasibly drive primary invagination, so long as the extracellular matrix can be deformed easily—about as easily as the cells can be deformed (Davidson et al., 1995). In principle then, changes of individual cell shapes can drive tissue bending, although other mechanisms for bending a cell sheet are possible (Davidson et al., 1995). Forces generated within the vegetal plate are sufficient to drive tissue bending, as invagination can occur normally in a dissected vegetal plate (Moore and Burt, 1939; Ettensohn, 1984). The cells proposed to undergo apical constriction have bands of actin microfilaments associated with apical adherens junctions and also spanning across the inside of each cell's apical surface, as might be expected in cells undergoing apical constriction. But microfilaments are also enriched apically

in cells that do not undergo such shape changes. Hence the presence of such an apical microfilament network does not necessarily indicate that it will bend a cell sheet (Ettensohn, 1984).

In certain species of sea urchin, a ring of cells along the edges of the vegetal plate has been recognized to undergo more pronounced apical constriction, as judged by scanning electron micrographs (Nakajima and Burke, 1996; Kimberly and Hardin, 1998, Fig. 2). Cells in this ring have been referred to as bottle cells, a term coined by Ruffini (1907) for amphibian embryonic cells that are shaped like bottles, with dramatically constricted apical sides and enlarged basolateral areas. Bottle cells in sea urchin embryos have a greater enrichment of apical arrays of F-actin than do other cells in the vegetal plate (Nakajima and Burke, 1996). Laser ablation of bottle cells interferes with normal invagination, whereas laser ablation of neighboring cells does not (Kimberly and Hardin, 1998), consistent with the notion that apical constriction may drive primary invagination. RhoA is required for the initiation of primary invagination (Beane et al., 2006), as it is for apical constriction and resulting tissue bending in other systems discussed below. How are specific cells driven to apically constrict during primary invagination? This is not yet clear, although calcium signaling (Nakajima and Burke, 1996), Wnt/Frizzled signaling (Croce et al., 2006), a transcriptional gene regulatory network (Davidson et al., 2002; Wu et al., 2008), and FGF signaling (Rottinger et al., 2008) have all been implicated in regulating primary invagination. The links between these regulators and RhoA activity have yet to be explored.

One key result is at odds with the model that actomyosin- dependent apical constriction is the key driver of primary invagination: cytochalasin treatment, which should depolymerize F-actin networks, fails to fully disrupt primary invagination in sea urchins (Lane et al., 1993). This result suggests the possibility that other mechanisms may provide force, either alone or redundantly with actin-based mechanisms. Interestingly, among the mechanisms proposed to drive apical constriction and tissue bending in sea urchins during primary invagination is one in which vegetal plate cells secrete extracellular matrix components into a multi-layered structure, in a calcium regulated manner (Lane et al., 1993). In this model, later-deposited matrix, secreted into a layer between the cells and the earlier layers of matrix, swells as it hydrates, driving bending of the matrix and hence the attached epithelial sheet. This is similar to the way in which the thermal expansion of a layer of metal in a thermostat's bimetallic strip can bend the entire strip. In Lane et al.'s model, the proposed source of force is extracellular, driving cell shape changes by bending of the matrix, rather than mediated by intrinsic cell shape changes, an interesting departure from traditional models. As an experimental model, sea urchin primary invagination leaves a variety of possible mechanisms for tissue shape change and some valuable tools for dissecting the contributions to forces made by each.

1.2 BOTTLE CELLS IN XENOPUS GASTRULATION: ROLES FOR MICROFILAMENTS AND MICROTUBULES

The amphibian archenteron also includes bottle cells at the site where invagination begins (Holtfreter, 1943). Early embryologists believed that amphibian bottle cells functioned in gastrulation because of the cells' unique shapes (Figure 1.2). Rhumbler (1902) suggested the possibility that these cells were actively migrating toward the interior of the embryo. Experiments by Holtfreter (1944) were consistent with this hypothesis, as isolated bottle cells could stretch in a polarized manner on a glass substrate, similar to migrating cells. While no live imaging evidence exists for the active migration of these bottle cells *in vivo*, vital dye tracings demonstrate that these cells do migrate to the interior of the embryo in *Ambystoma mexicanum* (Lundmark, 1986). In addition, cell tracing experiments in which labeled bottle cells from *Xenopus* laevis were grafted into unlabeled host embryos demonstrate that bottle cells spread out and form the anterior of the archenteron (Hardin and Keller, 1988).

X. laevis bottle cells (Figure 1.3) are a potentially valuable model for studying mechanisms of cell shape change in morphogenesis, as the cells are large and readily treated with inhibitors. These cells can be manipulated in culture much as sea urchin cells can be, and the potential exists to identify key molecular players using genetic screens in the model frog *Xenopus tropicalis*. Blastopore initiation begins and proceeds on schedule in explants that include the bottle cells (Hardin and Keller, 1988; (Lee and Harland, 2007). When bottle cells are removed from *X. laevis* embryos, a truncated archenteron still forms, and involution of the mesoderm

cells still occurs, but archenteron length is compromised (Keller, 1981). Therefore, bottle cells appear to initiate blastopore formation and to contribute to the full extension of the archenteron in *X. laevis*.

A number of distinct mechanisms control cell shape in *X. laevis* bottle cells. *In vivo*, the apical surfaces of these cells shrink while the apicobasal sides lengthen. Isolated, cultured bottle cells contract uniformly around the entire cell surface, suggesting that contraction is an intrinsic behavior but that the apicobasal elongation seen *in vivo* depends on contact with surrounding cells (Hardin and Keller, 1988). This likely reflects a cellular mechanism that distinguishes the basolateral and apical sides of bottle cells, or surfaces contacting other cells and free surfaces, perhaps similar to a mechanism that has been outlined in *C. elegans*, discussed below. F-actin and activated myosin accumulate at the apical surfaces of bottle cells just before the apical surfaces narrow, consistent with a role for F-actin and myosin in apical constriction (Lee and Harland, 2007). Furthermore, pharmacological inhibitors of F-actin or myosin demonstrate that they are both required for bottle cell formation. Interestingly, treatment with a microtuble depolymerizing drug, nocodazole, prevents full apical constriction of bottle cells and invagination without affecting apicobasal cell lengthening, and without apparent effects on F-actin or activated myosin distribution (Lee and Harland, 2007). This result suggests that microtubules may have an as yet undefined role in apical constriction in *Xenopus* bottle cells.

1.3 *C. ELEGANS* GASTRULATION: CELL MANIPULATIONS AND GENETICS MEET TO IDENTIFY KEY REGULATORS

Unlike gastrulation in sea urchins or *Xenopus*, where entire cell sheets are internalized, gastrulation in *C. elegans* involves the internalization of many cells or groups of cells at distinct times. *C. elegans* gastrulation begins at the 26-cell stage when two endodermal precursor cells move from the perimeter to the inside of the embryo (Figure 1.4). This event is followed later by internalization of mesoderm and germline precursors (Sulston et al., 1983; Nance and Priess, 2002). Internalization of the endodermal precursors has been most thoroughly studied and is the focus of our discussion here.

Cell movements associated with *C. elegans* gastrulation can occur *in vitro*, allowing mechanisms to be explored by cell manipulation experiments as in sea urchins and *Xenopus* (Lee and Goldstein, 2003). One revealing finding from such studies is that very few cells are required for the movements of *C. elegans* gastrulation to occur: even a line of embryonic cells in culture arranged in single file will fold at the time of gastrulation (Lee and Goldstein, 2003). This makes clear that mechanisms requiring large numbers of cells to work in concert, such as multicellular purse string mechanisms, are not essential for cell movements in *C. elegans* gastrulation. Some of the strengths of this system lie in the ability to combine such manipulations with live cell microscopy and genetics, and to study mechanisms of morphogenesis at the level of individual cells, in a developmental system where spatial patterning is so thoroughly studied.

Apical constriction plays a key role in *C. elegans* gastrulation. Just before endodermal precursor cells internalize, the cell surface that faces the perimeter of the embryo on each of these cells (the apical surface) flattens, and myosin II becomes enriched at this surface(Nance and Priess, 2002). Although the apical surfaces become smaller until they disappear at the time of cell internalization, these cells do not become noticeably bottle-shaped. Contraction of apical cell surfaces was revealed by tracking the movements of fluorescent, microscopic beads placed on the surfaces of the endodermal precursor cells (Lee and Goldstein, 2003). The observed surface movements exclude the possibility that shrinking of the apical surface reflects only a flow of apical surface to lateral positions—a possibility that is difficult to exclude in many systems. Myosin has been implicated in driving apical constriction because pharmacological inhibitors of myosin activity prevent the endodermal precursors from internalizing (Lee and Goldstein, 2003). In addition, apical myosin becomes activated near the time that gastrulation begins: apically localized myosin regulatory light chain is phosphorylated at a residue that in other systems unkinks myosin heavy chains, allowing myosin complexes to bundle into bipolar filaments, which can bind to and walk on actin filaments (Lee et al., 2006; Somlyo and Somlyo, 2003). These results suggest that local activation of myosin shrinks the apical actin mesh. Actin architecture is likely to be important as well. Indeed, the Arp2/3 actin-nucleating complex has been reported to localize to the cell cortex in gastrulating embryos, and depletion of this complex results in failure of endodermal precursor cells to internalize on schedule (Severson et al., 2002; Roh-Johnson and Goldstein, 2009).

Do neighboring cells contribute to internalization of the endoderm precursors? When neighboring cells were removed and reassociated with endodermal precursor cells in various orientations, the neighboring cells still moved in a direction consistent with the hypothesis that apical constriction in endodermal precursors drives the movement of the neighboring cells, suggesting that neighboring cell polarity is not important for the bulk of their movement (Lee and Goldstein, 2003). However, short, actin-rich extensions form on three of the six neighboring cells of the ring that closes beneath the endoderm precursors, and Arp2/3-depleted embryos that fail to gastrulate also fail to produce these extensions, raising the possibility that the extensions might contribute to completion of endodermal internalization *in vivo* (Nance and Priess, 2002; Roh-Johnson and Goldstein, 2009).

C. elegans genetics has identified multiple regulatory inputs that are important for gastrulation, including inputs that specify which cells should enrich myosin to one side, inputs that specify to which side of a cell this enrichment should occur, as well as a signaling input that directs activation of myosin. Cell fate specification genes including genes encoding endodermal GATA factors are necessary for early cell internalization, and embryos with ectopically specified endoderm have ectopic early cell internalization, suggesting that endoderm fate is both necessary and sufficient for early cell internalization (Lee et al., 2006). One aspect of endodermal cell fate is a gap phase uniquely introduced to the cell cycle of endodermal progenitors one cell cycle after the endoderm precursor cell is born, which is near the time of cell internalization (Sulston et al., 1983; Edgar and McGhee, 1988). This pause is required for internalization, possibly because it delays a reorganization of the

actomyosin cytoskeleton that normally accompanies cell division (Lee et al.,2006; Oegema and Hyman, 2006).

For the endodermal precursor cells to accumulate myosin near their apical surfaces, an apical surface must be established. PAR proteins function in anteroposterior polarization of the embryo first, and are known to become apicobasally polarized later, starting at the four cell stage (see Goldstein and Macara, 2007 for review. To test whether PAR proteins function in apicobasal polarization, Nance and colleagues devised a clever method for degrading the polarity proteins PAR-3 or PAR-6 specifically in somatic cells, adding a motif from another protein that becomes degraded in somatic cells. They demonstrated in this way that PAR-3 and PAR-6 are required for apical flattening, apical myosin enrichment, and timely cell internalization (Nance et al., 2003). Elegant cell manipulation experiments revealed that these PAR proteins' localization depends on where cells contact each other: only contact-free membranes accumulate apical PAR proteins, establishing an apical domain at the contact-free surface (Nance et al., 2003). Myosin later accumulates at apical domains, and this is dependent on apical PAR proteins (Nance et al., 2003). Once myosin becomes enriched apically, it becomes activated downstream of a Wnt–Frizzled–Dishevelled signaling pathway that causes regulatory light chain phosphorylation, through an unidentified kinase (Lee et al., 2006).

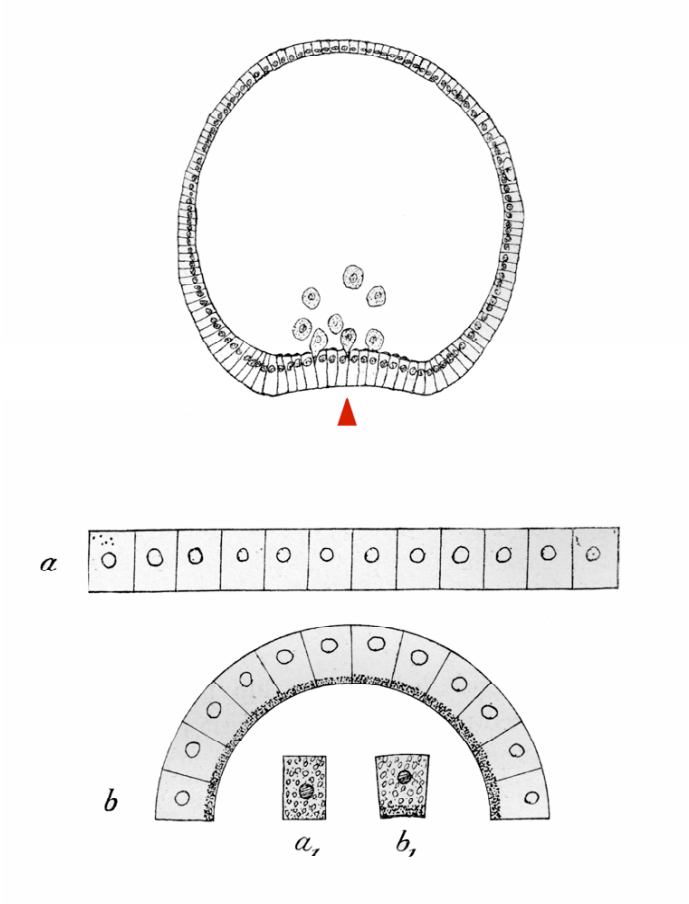
These results paint the outlines of a potentially generalizable mechanism for cell internalization by apical constriction: Among cells that polarize PAR proteins apicobasally, the cells with the right cell fate specification machinery enrich myosin

where the apical PAR proteins become localized—at contact-free surfaces. Activation of myosin can then result in shrinking the myosin-enriched, contact-free surfaces of any such cells, pulling neighboring cells across the free surfaces and, as a result, displacing the apically constricting cells toward the interior. The ability to shrink any exterior surface of specific cells could, in theory, make it possible for a cell to internalize regardless of which specific surfaces initially contact other cells.

How then do certain PAR proteins become enriched only apically in response to cell contacts? Anderson et al. (2008) screened for genes required for cell contact-dependent PAR protein localization and identified a key intermediate, a RhoGAP domain-containing protein, PAC-1. PAC-1 localizes to the cell cortex at cell–cell contact zones, where it has been proposed to inactivate CDC-42 at these zones, potentially restricting the active form of CDC-42 to contact-free cell surfaces. Active CDC-42 interacts with a semi-CRIB domain in PAR-6,and through this interaction is thought to establish apical localization of PAR-6 and PAR-6 complex members in these cells. PAC-1 localization to contact zones is therefore the earliest known step in recognizing contact zones as unique, spatial information that is critical to PAR protein and myosin localization. How PAC-1 becomes localized to contact zones is an interesting topic for future study.

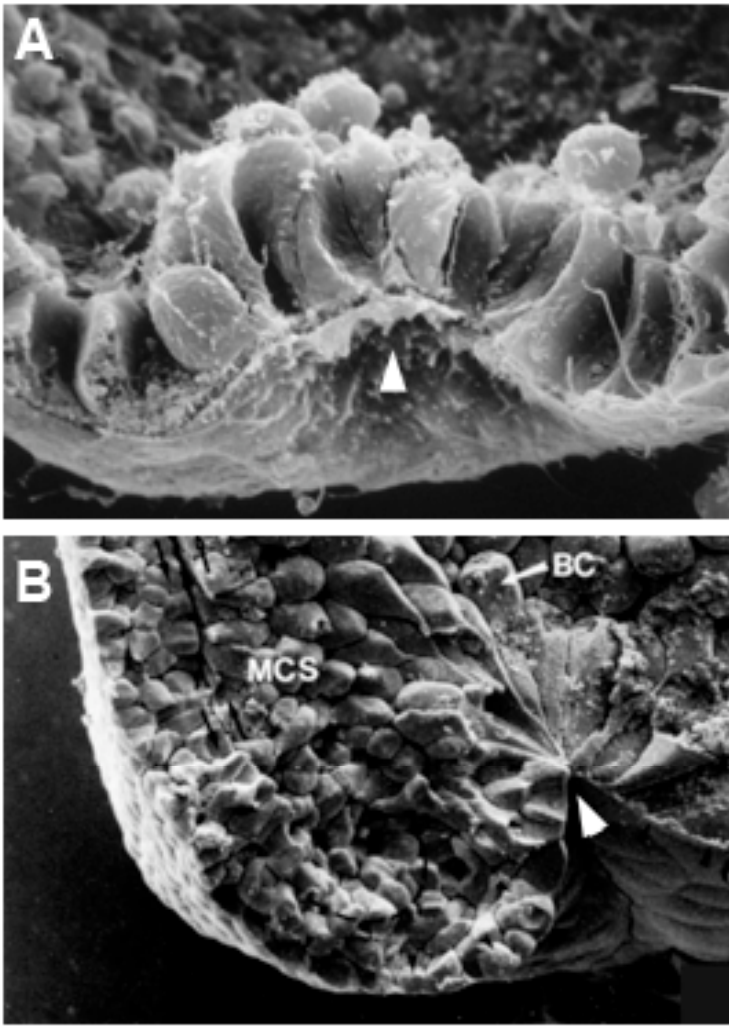
While much work has been done to characterize the patterning mechanisms that lead to apical constriction in the endodermal precursor cells, very little information existed previously about the internalization of the other cells that must gastrulate in *C. elegans.* We address this issue in the following chapter.

Figure 1.1. Rhumbler's 1902 drawings of cell shape changes driving morphogenesis (Rhumbler, 1902).



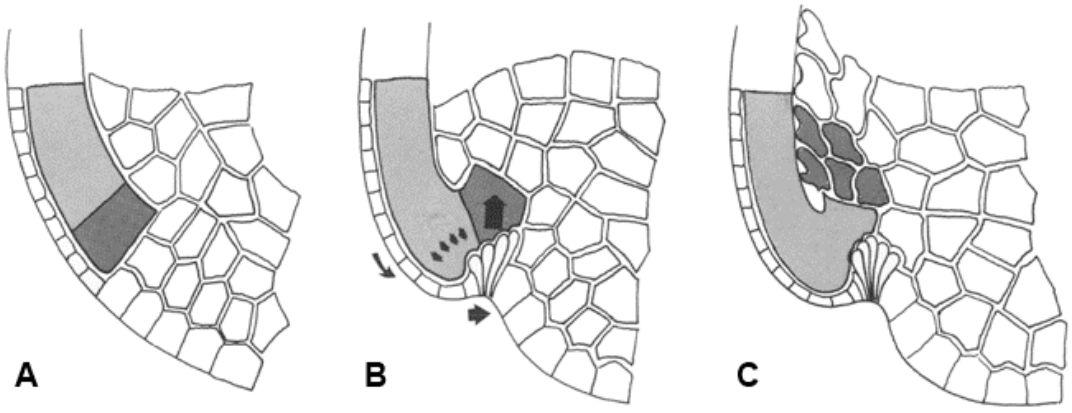
Top: A sea urchin embryo undergoing primary invagination. The vegetal-most part of the embryo bends inward (arrowhead). Bottom: "Theoretical gastrulation scheme, to show that invagination (b) of a cell plate (a) necessarily must take place if each cell changes from form a1 (due to higher pressure on the pigmented side) to the form b1. The invagination effect is significant even though the change in cell form from a1 to b1 is very small" (translation of figure legend in Rhumbler, 1902). We have inverted some parts of this figure to match the orientation of tissue bending between drawings.

Figure 1.2. Scanning electron micrographs of apically constricting cells in sea urchins and *X. laevis* (Kimberly and Hardin, 1998; Keller, 1981).



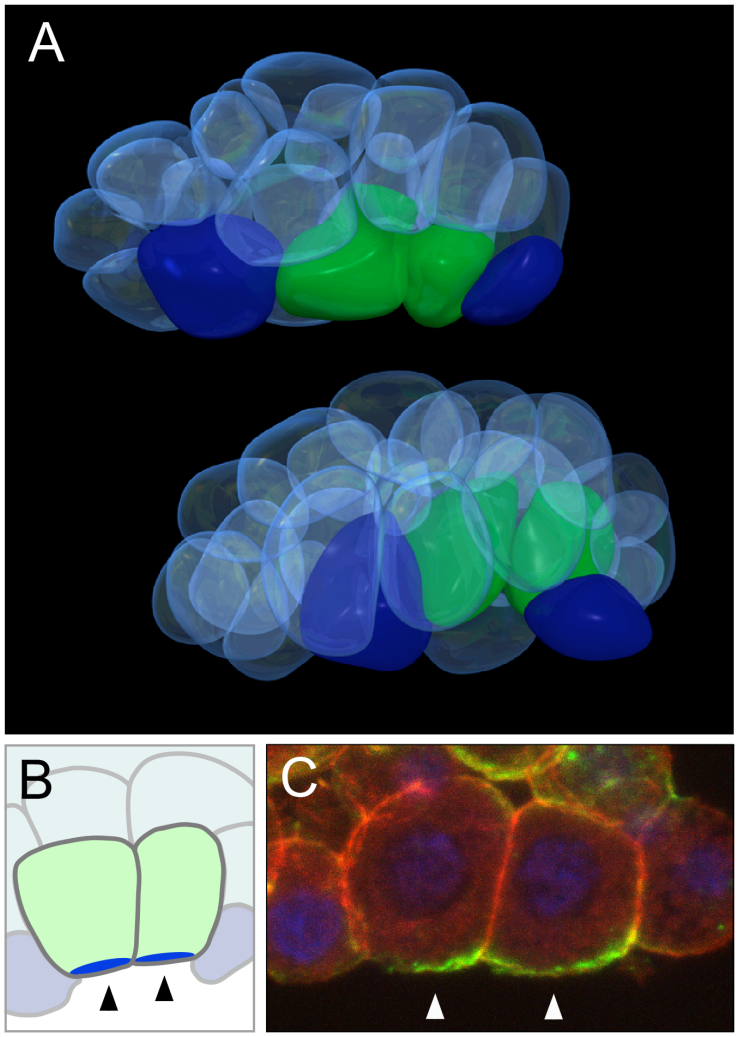
A) Sea urchin vegetal plate (Kimberly and Hardin, 1998), B) *X. laevis* midsagittal section at early gastrula showing bottle cells (BC) and involuted mesodermal cell stream (MCS) (Keller, 1981). Arrowheads mark bends in epithelia at proposed sites of apical constriction.

Figure 1.3. Schematic diagrams of bottle cell formation (Hardin and Keller, 1988).



A) Prior to gastrulation, the prospective anterior mesoderm (darker shading) and posterior mesoderm (lighter shading) comprise the deep marginal zone. B) The bottle cells have undergone apical constriction. Arrows indicate movements hypothesized to result. C) This causes reorientation of the vegetal edge of the marginal zone (anterior mesoderm) such that it is now leading the movement into the blastocoel (Hardin and Keller, 1988). All images approximate midsagittal views.

Figure 1.4. *C. elegans* gastrulation.



A) Illustrations of embryos just before (top) and during (bottom) endodermal internalization. Green, endodermal progenitors. Two neighboring cells are marked in purple. Renderings by J. Iwasa based on confocal sections of phalloidin-stained embryos (Lee et al., 2006). B) Diagram showing where apical constriction occurs (arrowheads). C) Myosin is activated in the apical cortex of the internalizing cells. Phospho-regulatory myosin light chain staining is in green (Lee et al., 2006).

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CHAPTER 2

INTERNALIZATION OF MULTIPLE CELLS DURING *C. ELEGANS* GASTRULATION DEPENDS ON COMMON CYTOSKELETAL MECHANISMS BUT DIVERSE CELL POLARITY AND CELL FATE REGULATORS

Understanding the links between developmental patterning and force-producing cytoskeletal mechanisms is a central goal in studies of morphogenesis. Gastrulation is the first morphogenetic event in the development of diverse organisms. Gastrulation involves the internalization of surface cells, often driven by the contraction of actomyosin networks that are deployed with spatial precision, in specific cells and in specific positions within each cell. These cytoskeletal mechanisms in diverse organisms rely on a multitude of cell polarity and cell fate regulators. *C. elegans* gastrulation presents an opportunity to examine the diversity of mechanisms used within a single organism by dozens of cells that become internalized at distinct times. Nearly half of the cells of the *C. elegans* embryo are born on the embryo's surface and move to the interior. Here, we present an identification of all of the cells that become internalized in *C. elegans* gastrulation. To gain mechanistic insights into how diverse cells internalize, we have manipulated cell polarity, cell fate and cytoskeletal regulators and determined the effects on cell internalization. Our results demonstrate that cells of distinct lineages use common

actomyosin-based mechanisms to gastrulate, but different cell fate regulators, and, surprisingly, different cell polarity regulators. Apical PAR proteins that function in apical myosin localization and internaliztion of endodermal cell precursors do not function in cell internalization in other lineages. We conclude that much as diverse organisms employ diverse patterning mechanisms to regulate common cytoskeletal mechanisms for cell internalization, diverse patterning mechanisms can be associated with common cytoskeletal mechanisms within a single organism.

2.1 INTRODUCTION

Morphogenesis is the process by which cells in an embryo become an organism with ordered shape and structure. One key goal in studying morphogenesis is to understand the links between patterning mechanisms controlled by cell fate and cell polarization and the cytoskeletal mechanisms that drive the embryonic shape changes (Wieschaus, 1995). Such links are being identified in diverse developmental systems. One theme emerging is that diverse animals employ a handful of common cytoskeletal mechanisms, and these are regulated by diverse and often organism-specific patterning mechanisms. For example, contraction of an apical actomyosin network internalizes cells in diverse systems. In *Drosophila* gastrulation, an actomyosin network is spatially regulated by an apically-localized guanine nucleotide exchange factor for Rho (RhoGEF). Recruitment of this RhoGEF to the apical sides of cells depends on the secreted protein Fog and the transmembrane protein T48 (Barrett et al., 1997; Nikolaidou and Barrett, 2004; Rogers et al., 2004; Kolsch et al., 2007). Fog and T48 homologs are not known in vertebrates, where the F-actin binding protein Shroom3 acts instead as an apical determinant. Shroom3 recruits a myosin activator, Rho kinase, to the apical sides of cells, and also affects directly or indirectly the apical localization of myosin and F-actin (Hildebrand and Soriano, 1999; Haigo et al., 2003; Hildebrand, 2005; Nishimura and Takeichi, 2008). *C. elegans* uses yet another mechanism for apico-basal polarization of similar actomyosin based forces. Here, a putative GTPase activating protein for Cdc42 localizes basolaterally in response to cell contacts, where it prevents the localization of apical PAR proteins that are important for apical myosin localization (Nance et al., 2003; Anderson et al., 2008). These cases illustrate that different apico-basal patterning mechanisms can control apical constriction in different systems, and similar data exist for cell fate: a diversity of cell

fate regulators control the apical constriction machinery in specific cells in diverse animal systems (see Sawyer et al., 2010 for review).

The extent to which such diversity exists within each organism is less clear. There are cases where different cells internalize by morphologically distinct processes in a single animal system, for example between gastrulation and tracheal tube formation in *Drosophila*. In these cases, different patterning mechanisms are indeed known to act upstream of common cytoskeletal mechanisms (Leptin and Grunewald, 1990; Brodu and Casanova, 2006). But the extent to which such diversity of spatial patterning mechanisms might regulate repeated, superficially similar morphogenetic processes within a single organism is not well known. When many cells move in the same direction and by superficially similar patterns, do they use the same cytoskeletal mechanisms to do so, and are these mechanisms associated with common upstream spatial patterning mechanisms? Answering this will be a key component to understanding the links between patterning mechanisms and the cytoskeletal mechanisms that drive morphogenesis. Answering this will also provide insight into which specific mechanisms discovered in model systems will be more or less likely to be informative to understanding morphogenesis-related birth defects in humans. We consider this to be important because morphogenesis-related birth defects like neural tube closure defects are common human birth defects (Copp and Greene, 2010)

Gastrulation is the earliest morphogenetic process in many animal embryos. During gastrulation, cells that will establish the internal germ layers – the endoderm, the mesoderm and the germ line – move from the outside of the embryo to the interior, leaving just the ectoderm at the surface. *C. elegans* gastrulation is a valuable model for studying the cellular and molecular mechanisms involved in cell internalization in part because gastrulation begins early in development, soon after cell fates are acquired. The relatively small numbers of cells involved suggests that

an understanding of the regulation of gastrulation at the level of individual cells is possible. Many cells internalize at distinct times during *C. elegans* gastrulation, and nearly all of these cells do so from the ventral surface of the embryo (Sulston et al., 1983; Nance and Priess, 2002). As we will show here, nearly all of these cells internalize as sister cell pairs. Therefore, *C. elegans* gastrulation also presents an unusual opportunity to study the links between patterning and cytoskeletal mechanisms for a multitude of cells that are internalized at distinct times but in superficially similar ways.

C. elegans gastrulation begins with the internalization of two endoderm precursor cells, a process that is driven at least in part by actomyosin-dependent apical constriction (Nance and Priess, 2002; Lee and Goldstein, 2003; Nance et al., 2003; Lee et al., 2006; Anderson et al., 2008; Roh-Johnson and Goldstein, 2009). Cell fate, cell polarity and cytoskeletal mechanisms comprise three known requirements for timely internalization of the endoderm (Figure 2.1). First, endodermal cell fate must be specified properly for the endodermal precursors to internalize on schedule. Mutant embryos defective in endodermal cell fate specification fail to internalize the cells that would normally become endodermal (Zhu et al., 1997; Maduro et al., 2005; Lee, 2006). In addition, in mutants that produce ectopic endoderm, the ectopic endodermal precursors internalize soon after the normal endodermal precursors do (Lee, 2006). Second, apicobasal polarity is regulated by apically-localized PAR proteins. These PAR proteins are required for apical flattening and enrichment of a nonmuscle myosin II protein (NMY-2) on the apical surface of each endodermal precursor cell (Nance, 2003). Third, these endodermal precursor cells must undergo an actomyosin-driven apical constriction, which is dependent on a Wnt-Frizzled-Disheveled signaling pathway (Lee, 2006). Phosphorylation of the regulatory light chains of myosin at a conserved site is thought to activate the contraction of the actomyosin network at the apical cortex in

the endodermal precursor cells, resulting in the movement of neighboring cells under the endodermal precursors and hence the internalization of the endoderm (Lee, 2006).

In this study, we have dissected the cell fate, cell polarity and cytoskeletal mechanisms used by multiple cells during *C. elegans* gastrulation. Before we began to study mechanisms, some descriptive work was necessary. Six founder cells are traditionally recognized in *C. elegans*: AB, MS, E, C, D, and P_4 (Sulston et al., 1983). Time intervals have been reported during which progeny of each of these founder cells become internalized (Sulston et al., 1983; Nance, 2002), but there has not been a complete identification of all the cells that gastrulate. Therefore, we started by identifying these cells. Together with previous work, this revealed that sixty-four cells gastrulate in *C. elegans* – the two endodermal precursors and then sixty-two additional cells that contribute to the nervous system, the mesoderm and the germ line. We then addressed the mechanisms by which these cells gastrulate using gene disruption, live imaging, and protein localization experiments to identify cell fate regulators, cell polarity mechanisms, and cytoskeletal mechanisms required for timely internalization. We report that actomyosin-based cytoskeletal mechanisms function to internalize diverse cells, but that cell internalization is under the control of different cell fate and cell polarity mechanisms in different cells.

2.2 MATERIALS AND METHODS

2.2.1 Strains and Worm Maintenance

Nematodes were cultured and handled as described previously (Brenner, 1974). Unless indicated, experiments were performed with the wild-type N2 (Bristol) strain. The following mutant and reporter strains were used: JJ1473 *unc-119 (ed3) III; zuIs45* [*nmy-2*::NMY2::GFP;*unc-119(+)*]; referred to here as NMY-2::GFP, OD70 *ltIs44* [*pie-1*::PH domain of PLCδ::mcherry] (PH::mCherry) (Kachur et al., 2008), LP54 (PH::mCherry; NMY-2::GFP), *unc-32 (e189) par-3(it71); zuIs0* (*par-3*::PAR-3;ZF1-GFP) (PAR-3-ZF1) (Nance et al., 2003), par-6(zu222) unc-101(m1); zuIs54(par6::PAR6-ZF1-GFP) (PAR-6 ZF1**)** (Nance et al., 2003)**,** MS632 unc-119(ed4) III; irIs39 [ceh-51::NLS::GFP] (CEH-51::GFP) (Broitman-Maduro et al., 2009), MS1293 tbx-35(tm1789) II; ceh-51 (tm2123) V; irEx572, (Brotiman-Maduro et al., 2009); SS149 *mes-1 (bn7)* (Capowski, 1991*)*; Strains were maintainted at 20°C for embryonic experiments, except *mes-1*(bn7), which was maintained at 15°C and shifted to 25°C 1-2 days before use in experiments. The *C. briggsae* strains used were AF16 (Fodor, 1983) and RW20025 CbHis72::mCherry (a gift from the Waterston lab).

2.2.2 DIC and Confocal Time-Lapse Microscopy

Embryos were mounted and DIC images were acquired as described previously (Roh-Johnson and Goldstein, 2009). Time-lapse images were acquired at 1μm optical sections every 1 minute and analyzed with Metamorph software (Molecular Devices). Internalization of cells was scored by examination of whether each cell was beneath the surface of the embryo, fully covered by other cells, before division. Spinning disk confocal images were acquired and processed as described

previously (Roh-Johnson and Goldstein, 2009). Lateral surfaces of PH::mCherry; NMY-2::GFP or NMY-2::GFP only embryos were filmed under DIC conditions until cells of interest were born. To analyze NMY-2::GFP only embryos, images were acquired every 30 seconds over several planes once cells of interest that would internalize were born under both DIC and fluorescence conditions. One plane was chosen for analysis. To analyze PH::mCherry; NMY-2::GFP dynamics, one to three planes 0.5μm apart were acquired every 5 seconds in each channel. One plane was chosen for analysis, and these films were analyzed with Metamorph software. Kymographs were generated in Metamorph from lines drawn perpendicular to MS lineage cells and germ line precursor cells. The embryonic cell lineage was drawn by a custom-written program (available on request) using timing data from wormbase.org.

2.2.3 Cell Fate Transformation

tbx-35;ceh-51 embryos were filmed and assessed for lethality and mCherry expression several hours later. Only those embryos that were both dead and lost the mCherry expression were analyzed for MS descendant cell internalization defects. *mes-1* strain was maintained at 15°C. Plate was placed at 25°C overnight and gravid adults cut open the next day and filmed. Only embryos where P_4 and D divided at the same time were included in analysis

2.2.4 Polarity Regulators

par-3(ZF1) and *par-6(ZF1)* embryos were filmed using DIC microscopy and the timing of MS cell and D cell divisions as well as the timing of the internalization of sister cells pairs in the MS and D lineages were recorded. p values from Student's t tests (unpaired) were obtained by comparing the timing of internalization of sister

pairs of cells between N2, *par-3(ZF1)*, and *par-6(ZF1)* by using the Bonferroni method of multiple comparisons. Because 16 comparisons were made, values were considered significantly different if $p<0.003$. Of the six pairs of MS descendant cells that internalize after the fourth MS cell division and the two pairs of D cells that internalize, the timing of only one pair of cells in a *par-6(ZF1)* mutant was considered statistically different than WT (MSpppa/MSpppp $p=0.002$).

2.2.5 Laser Delay

Cells were irradiated as previously described (Lee, 2006) with minor changes. Cells were targeted for delay at a sublethal dose by irradiation with a 1 minute duration of 3 nanosecond pulses at 20Hz. The laser was targeted to the nucleus of the P_4 cell, approximately 55 minutes after it was born. Experiments were only included in analysis if the P_4 cell failed to divide before internalizing.

2.2.6 Immunostaining

Immunostaining with anti-Phospho-Ser19-MLC (1/250, Abcam) was done as previously described (Lee and Marston, 2006) with minor changes. Embryos were allowed to develop to the desired stage based on timing from the 4-cell stage. CEH-51::GFP was detected with GFP antibodies (1/100 Molecular Probes). PAR-3 immunostaining was done using a freeze-crack method as previously described (Tenlen et al., 2008), with minor changes. Incubations with anti-PAR-3 (1/25, Developmental Studies Hybridoma Bank) were at 4°C overnight, and incubations with anti-GFP (1/1000, Abcam) were at 37°C for 1 hour. Embryos were imaged with a Zeiss LSM510 confocal microscope and LSM software, and images were processed with Metamorph software. The number of MS descendent cells was counted based on positive GFP staining of nuclei in CEH-51::GFP embryos. Anti-

PAR-3 fluorescence intensity was measured by recording linescans across the MS descendant cell apical and basolateral membranes using Metamorph software. The basolateral and apical membranes were identified by determining the localization borders of an MS-cell specific marker, CEH-51::GFP in the same embryos. CEH-51::GFP levels were calculated as averages of three-pixel-wide linescans in each embryo, and apical and basolateral GFP pixel intensity fall-offs were used to align anti-PAR-3 intensity measurements between embryos.

2.3 RESULTS

2.3.1 Identifying each of the cells that gastrulate

We began by tracing cell lineages and identifying each cell that could be seen internalizing during gastrulation from twenty-four multiplane DIC recordings and four spinning disk confocal recordings of embryos expressing a plasma membrane marker, PH::mCherry (Kachur et al., 2008;). Specific lineages were followed in each embryo depending on orientation of the embryo. We mapped each of these cell identities onto the *C. elegans* embryonic cell lineage (Figure 2.2A). We define gastrulating cells here as precursor cells that internalize during development, i.e., before completing embryonic cell divisions, distinguishing this from the later ingression of individual postmitotic cells at the end of embryogenesis. The identities of many of the gastrulating cells had been reported before (Sulston 1983; Nance 2002), but a full lineage could not be drawn from this data because ambiguity had remained as to exactly which cells internalized at which time in the MS and C lineages, and we found many cells in the AB lineage internalizing that had not been recognized previously.

We identified ten new AB lineage cells that internalized during gastrulation, including much of the AB-derived portion of the nervous system. AB-derived nervous system had not been reported to internalize during gastrulation previously. The ten cells identified included the first cell of the embryo to internalize after the endoderm precursors internalize, ABalapp. This cell produces only neuronal progeny -- ten ring ganglion cells and a lateral neuron (Suslton et al., 1983). The nine other newly-identified AB lineage cells that internalized included a set of five neuronal precursors, ABalpppa (which makes eight ring ganglion cells), ABalpppp (seven ring ganglion cells), ABprpaap (seven ring ganglion cells and one ventral ganglion cell),

ABplppap (four ventral cord cells and five retrovesicular ganglion cells), ABprpapppa (one ventral ganglion cell and one retrovesicular ganglion cell), and four other cells that function as precursors to other internal structures: ABalpaapp (four anterior buccal cavity cells, including two hypodermal cells just inside the mouth), ABprpapppp (left and right intestino-rectal valve cells), ABaraapap (pharynx), and ABaraappp (pharynx). Previous work had identified in the AB lineage only pharyngeal and buccal cavity precursors that gastrulate (Sulston et al., 1983; Nance and Priess, 2002). Our findings above reveal that much of the AB-derived nervous system becomes internalized as part of gastrulation, instead of internalizing postmitotically in single cell ingression events.

Although *C. elegans* gastrulation involves many cells moving in at distinct times, we found nearly complete invariance with respect to which cells move in at which division round. Cells of the MS lineage internalize after 4 or 5 divisions of the MS founder cell (Nance et al., 2002). In all embryos where the MS cell lineage was examined, we found that the specific MS descendents that internalized after the fourth MS lineage cell division was the same in all embryos, and the specific descendants that remained on the surface until after the fifth MS lineage cell divisions was the same (n=8). These cells that remained on the surface until after this fifth MS cell division internalized in 8/9 wild-type embryos, but there was one embryo where 6/8 remaining MS cells did not internalize within 90 minutes of their last division. This was a rare case of variation in the lineage of wild-type embryos. The internalization of the germ line precursors Z2 and Z3 (n=8) and the four D lineage cells (n=7) was invariant as well (Figure 2.2A). In the C lineage, we found one case with variability in the Capp lineage: In two out of three embryos in which the C lineage was traced, Cappa and Cappp internalized, and in the other embryo, their mother cell, Capp, internalized instead. All other C lineage cells that internalized (Figure 2.2A) did so at the same division round in each case

We found that nearly all of the gastrulating cells, including the newly-identified AB lineage cells comprise a continuous stripe along the ventral side of the embryo running from the anterior pole to the posterior pole (Figure 2.2B and Supplemental Movie 1).

2.3.2 Different Lineages Use Distinct Fate Regulators to Control Internalization

Cell fates of internalizing cells are determined before the cells move to the interior of the embryo (Priess et al., 1987; Strome, 2005; Maduro, 2006) Fate regulators might determine the timing of cell internalization as in the endodermal lineage (Lee et al., 2006). Consistent with this hypothesis, gastrulating cells with similar fates often have similar internalization times (Nance and Priess, 2002). This question was addressed before for the AB lineage using two embryos from a mutant in which AB lineage cells are transformed into MS lineage cells (Nance and Priess, 2002). In these mutant embryos, a small number of AB lineage cells internalized that were not known to do so in wild-type, but the interpretation of this result is complicated by our finding here that more AB lineage cells internalize than were known at the time of the earlier experiments. We have revisited this question using mutations in key cell fate regulators to alter cell fates, examining the consequences of these mutations on the timing of cell internalization. We focused on two lineages with well-studied fate specification regulators: the MS lineage, which produces mostly mesodermal cells, and the P_4 lineage, which produces the germ line. Our results below show that mutations in key fate regulators result in aberrant cell internalization patterns, patterns that are roughly consistent with the cell fate transformations that occur in each case.

Two transcription factors play important roles in the initial establishment of MS lineage fate: TBX-35, a T-box protein, and CEH-51, an NK-2 homeodomain

protein (Broitman-Maduro et al., 2009). Double mutant embryos with loss of function mutations in both of these genes show an incomplete MS to C lineage transformation, generally failing to make MS-derived tissue, and with the MS lineage often developing C lineage markers (Broitman-Maduro, 2009). We recorded four *tbx-35*;*ceh-51* double mutant embryos and analyzed gastrulation timing in each cell of the MS lineage of each embryo and compared that to a WT embryo (Figures 2.3A-J, 2.4). In some embryos, at least one cell internalized one cycle early, after only three rounds of MS divisions, as occurred rarely in the wild-type C lineage (Figures 2.2,2.4). Some cells that would normally internalize after five rounds of MS lineage divisions in wild-type embryos internalized instead one division round earlier, after four rounds of division (Figure 2.4), as occurs in nearly all cases in the C lineage (Figure 2.2). Because internalization occurred at aberrant stages in the double mutant embryos, we conclude that MS cell fate specification regulators are required for the normally invariant temporal pattern of MS cell internalization.

MES-1 is a receptor tyrosine kinase-like protein that plays a key role in the specification of the germ line (Strome et al., 1995; Berkowitz and Strome, 2000; Capowski et al., 1991). In embryos produced by *mes-1* loss of function mutant worms raised at 25ºC, germ line ribonucleoprotein particles termed P granules do not become partitioned properly to the germ line blastomeres, and the primordial germ cell P_4 can adopt the fate of its sister cell, D, a muscle precursor cell (Strome, 1995). The cell division that establishes P_4 and D is normally an asymmetric division, and the larger D cell divides next before the smaller P_4 cell does (Sulston, 1983). Cell size and cell division order are equalized to various degrees to individual *mes-1* mutant embryos (Strome, 1995). We examined recordings of twelve embryos from *mes-1* hermaphrodites that had been placed at 25ºC for 24-48 hours before filming. In five of these embryos, P_4 and D cells divided synchronously, suggesting that the fate transformation of P_4 was likely to be more complete than in the other embryos,

in which the division delay between D and P_4 varied. Therefore, these five embryos were used for further analysis of cell internalization timing. In wild-type embryos, P_4 divides once, and its two daughter cells internalize, and D divides twice before the resulting four cells internalize (Figure 2.5A-D). In three of the five *mes-1* mutant embryos examined, P_4 divided twice before its four descendants internalized, as the D lineage normally behaves (Figures 2.5E-H, 2.6). In the other two cases, some of the four descendants internalized at this same stage and others did not (Figure 2.6).

We wanted to determine if the cell fate mutant *mes-1* would affect internalization of other lineages besides the germ line. *mes-1* is known to affect the endodermal progenitor cell division orientation (Bei et al., 2002). For this reason, we did not quantify endodermal cells' internalization. We did quantify the internalization of the MS descendant cells that we could follow in these embryos. In four *mes-1* embryos, 44/44 MS descendant cells internalized during the same cell cycle as the MS descendant cells in WT embryos. We conclude that the germ line cell fate regulator MES-1 is required for the normally invariant temporal pattern of P_4 cell internalization. These results together with previous results discussed above (Nance and Priess, 2002; Lee et al., 2006) demonstrate that factors responsible for cell fate specification in specific lineages also affect internalization timing in only these lineages.

2.3.3 Apical PAR Proteins that Function in Endodermal Precursor Polarization and Internalization are Not Required for Timely Internalization of Mesodermal Lineages

Nance et al. (2003) found that the apical PAR proteins PAR-3 and PAR-6 are required for apical localization of NMY-2, a myosin heavy chain protein, in endodermal precursors and for internalization of these endoderm precursors at the 28 cell stage. They also showed minor defects in internalization timing in two MS

descendants that were followed in the absence of PAR-3. However, PAR-3 has been reported to disappear below detectable levels by around the 50 cell stage, earlier than MS cell internalization (Etemad-Moghadam et al., 1995). Therefore, we have re-examined this issue, determining whether PAR-3 localizes apically in MS lineage cells only briefly, specifically as they internalize. We have also examined cell internalization times in the absence of PAR-3 or PAR-6 in two mesodermal cell lineages.

We examined PAR-3 distribution by immunostaining, using a second marker, CEH-51::GFP, to specifically identify MS descendants at a stage when 16 MS descendants are present (the MS16 stage). We compared apical to basal localization of PAR-3 at the stage of MS cell internalization, and, for comparison, at the stage when 4 MS descendants are present (the MS4 stage), when PAR-3 is known to be apically enriched (Etemad-Moghadam et al., 1995). PAR-3 appeared apically enriched as expected at the MS4 stage, but not in MS descendants at the MS16 stage (Figure 2.7A-B). We quantified fluorescence intensity at the apical and basal cortexes and for a one micron-wide strip on either side of each cortex (Figure 2.7C). The quantification confirmed our conclusion: No apical peak of PAR-3 was apparent in MS16 cells, and apical PAR-3 levels were statistically indistinguishable from basal PAR-3 levels in MS16 cells ($p=0.88$) and significantly lower than apical level at the MS4 stage ($p<0.005$). We conclude that there is no detectable apical enrichment of PAR-3 in the MS lineage cells at the time that these cells internalize.

Although PAR-3 is not apically enriched at this time, it is possible that apical PAR proteins establish polarity in earlier MS lineage cells, and that this is required for later cell internalization. Therefore, we tested whether PAR-3 and PAR-6 are required for timely internalization of MS lineage cells, as well as D lineage cells. To test roles for these proteins, we used *par*(ZF1) strains, in which PAR-3 or PAR-6

become degraded specifically in somatic cells (Nance, 2003). DIC recordings were generated from these strains, and the timing of cell internalization was examined for sister cell pairs in the MS and D lineages. The results are shown on lineage drawings of wild-type, *par-3*(ZF1), and *par*-6(ZF1) embryos (Figure 2.7D). We found that the six pairs of MS descendents that internalize after the fourth MS cell division in wild-type embryos internalized during the same cell cycle in *par-3(ZF1)* and *par-6(ZF1)* embryos. The difference in timing of internalization of these MS cells between wild-type and *par-3(ZF1)* and *par-6(ZF1)* embryos was small and statistically significant in only one pair of these cells when we performed statistical analysis (see Materials and Methods). We found similar results for the two pairs of sister cells in the D lineage. In all *par-3(ZF1)* and 6/7 *par-6 (ZF1)* embryos, the cells in the D lineage internalized as 4 cells, with timing that was statistically indistinguishable from D lineage internalization in WT embryos (see Materials and Methods, Figure 2.7D). We also examined the internalization of the four pairs of MS descendant cells that normally internalize after the fifth MS cell division. In 5/9 *par-3(ZF1)* embryos and 5/10 *par-6(ZF1)* embryos, the four pairs of MS lineage cells that normally internalize after the fifth MS cell division do so in these embryos as well. However, we did see variation in the internalization of these particular cells, which is consistent with slight variation of internalization of these cells in 1/9 N2 embryos (discussed above). We found that some of these eight MS lineage cells internalized only after another round of MS cell divisions the in the *par-3(ZF1)* and *par-6(ZF1)* mutants. In other cases, no division or internalization of these cells was observed during the duration of our films. Because PAR-3 and PAR-6 do not appear to be required for timely internalization of the majority of the MS and D descendants, we conclude that PAR-3 and PAR-6 are not playing a major role in the polarization of these cells.

2.3.4 Internalizing Cells Accumulate Myosin at Their Apical Surface, And Apical Myosin Becomes Activated

Although the data above suggest that cell fate and even cell polarity mechanisms that control gastrulation vary from lineage to lineage, we do not know if cytoskeletal mechanisms vary as well. To address this, first, we looked for accumulation of myosin at the apical sides of internalizing cells in live embryos expressing myosin marker, NMY-2::GFP, in places where cells of the MS lineage, the D lineage, and the germ line precursor cells were internalizing. We found that NMY2::GFP accumulated in or near the apical sides of internalizing cells in each case: In 9 embryos examined, 43/43 internalizing MS lineage cells, 8/8 internalizing germ line precursor cells, 12/13 internalizing D lineage cells, and 11/11 internalizing cells whose lineal origin was not traced showed myosin accumulation. These results are consistent with an embryo shown by Nance and Priess (2002) where one pair of MS lineage cells could be seen to accumulate myosin apically. We found that no other cells visible to us at these stages accumulated myosin similarly, and the only other places where we saw clear myosin accumulation were at the cytokinetic rings of dividing cells, as expected (Figure 2.8 A-D).

To determine if the myosin accumulation we observed occurred within each internalizing cells, or for example in neighboring cells that could feasibly extend processes over these cells, we examined cell internalization in embryos expressing both NMY-2::GFP and an mCherry labeled plasma membrane marker PH::mCherry. We imaged the surface of live embryos at five second intervals by spinning disk confocal microscopy and found that the myosin accumulation occurred within the internalizing cells, rather than their neighbors, in 50/50 MS descendant cells in 9 embryos, 5/6 D lineage cells in 2 embryos, 8/8 germ line precursor cells in 4 embryos, and 5/5 unidentified internalizing cells in 3 embryos (Figure 2.8E,F). We

confirmed this accumulation within internalizing cells by kymograph analysis of NMY-2::GFP and PH::mCherry (Figure 2.8G,H).

To determine if this myosin was activated in internalizing cells, we examined a conserved marker for myosin activation. Myosin II complexes comprise two heavy chains, two essential light chains and two regulatory light chains. The phosphorylation of the two regulatory light chains at serine 19 (p-RMLC) is required for the formation of active myosin filaments that bind to actin filaments to drive contraction (Somlyo, 2003). The conserved residue is phosphorylated in endodermal precursor cells for a short period of the cell cycle, during cell internalization (Lee, 2006). We immunostained embryos expressing CEH-51::GFP, a marker for MS lineage fate (Maduro 2009) at the stage during which MS lineage cell internalization begins (16MS lineage cells), using cell morphology to determine if cells examined were internalizing. We found 34 cells in 10 embryos where the morphology of those cells suggested that they were internalizing, and 28 of these cells were enriched for apical p-RMLC (Figure 2.8I), indicating that activated apical myosin was associated with cell internalization in these cells.

2.3.5 Is Internalization as Sister Cell Pairs Most Efficient?

In our experiments tracking myosin dynamics above (Figure 2.8A-I), and in our initial films (Figure 2.2), we observed that the vast majority of gastrulating cells internalize as sister cell pairs, i.e., with pairs of sister cells internalizing synchronously, and often only one pair at a time, consistent with Nance and Priess (2002). There is evidence that cell internalization is temporarily prevented as cells divide (Lee et al., 2006), and this may contribute to this synchrony. Alternatively, it is possible that there is a yet-undiscovered property of the internalization mechanism that makes internalization of cell pairs more efficient than internalization of larger

numbers of cells, or of single cells. Junkersdorf and Schierenberg (1992) have shown previously that a single E cell can internalize alone after preventing its division by laser irradiation, and we have found that some cells in the AB lineage internalize alone during normal development (Figure 2.9D). However, these results leave open the interesting possibility that internalization of pairs of cells may be most efficient.

To investigate this possibility, we began by exploring a finding of Zhao et al. (2008), who showed that the P_4 cell in a transgenic strain of *C. briggsae*, a relative of *C. elegans*, divided an average of 23 minutes later than in *C. elegans*. We reasoned that this might result in P_4 in this species internalizing alone, before it divides. We filmed P_4 division and internalization of this cell or its daughter cells in a wild-type strain of *C. briggsae* and in a transgenic *C. briggsae* strain expressing mCherry-histone. However, we found no significant difference using the student's t test in the P_4 cell cycle lengths between the two species (76±5 min in *C. elegans* N2 strain, n=7*;* 77±9 min in *C. briggsae* AF16, n = 7; 71±4 min in *C. briggsae* mCherry histone RW20025, n = 4; $p>0.05$ for each C. briggsae strain compared to *C. elegans*). P_4 divided into two cells and internalized as a pair of cells in all cases in both species (Figure 2.9A,B). We conclude that the difference reported by Zhao et al. (2008) is characteristic of their transgenic strain or imaging techniques and not of *C. briggsae* more generally.

To investigate this issue in *C. elegans*, we used a laser to delay cell division, and then followed cell internalization to determine if a single, laser-delayed cell could internalize efficiently. We found that P_4 internalized as a single cell in 7/8 cases, and that it took no longer to do so than its two daughter cells do in untreated embryos (Figure 2.9C,E). We conclude that although *C. elegans* gastrulation involves internalization of sister cell pairs in many lineages, pairs of cells are not required for efficient internalization.

2.4 DISCUSSION

C. elegans gastrulation offers a valuable model system to examine the connections between patterning and morphogenesis. In *C. elegans,* gastrulation involves the sequential internalization of cells with distinct fates. Therefore, we can ask how the patterning mechanisms affect the cytoskeletal mechanisms during different developmental time points in the same organism. We have found that changing the cell fate of specific cells in the *C. elegans* embryo alters the timing of their internalization. In addition, the PAR proteins that are responsible for establishing apicobasal polarity in the endodermal cells do not appear to act alone, if at all, in polarizing the mesodermal or germ line cells. However, all of the internalizing cells that we examined accumulate myosin II at their apical surface, suggesting that the same cytoskeletal mechanism is being used reiteratively in cells with different patterning mechanisms. Therefore, we propose *C. elegans* uses common cytoskeletal mechanisms to internalize cells of distinct fates that are regulated by diverse cell polarity and cell fate regulators (Figure 2.10).

Gastrulation in *C. elegans* has visual similarities to gastrulation in *Drosophila melanogaster*, specifically in ventral furrow formation, in which a continuous stripe of cells internalizes along much of the ventral side of the embryo (Figure 2.2B) (Costa, 1993). This similarity was not inherently obvious from previously published data and images of gastrulating *C. elegans* embryos, but through the gastrulation analysis and movie presented here, a similar stripe of cell internalization is more visually clear. In contrast to *Drosophila*, however, where a secreted factor, fog, is required for proper ventral furrow formation (Costa et al., 1994), it remains unknown if any requirement exists for a secreted factor in *C. elegans* gastrulation.

The gastrulating cells in *C. elegans*, which already have determined fates, internalize sequentially in a line first designated by the position of the internalizing

endodermal cells. There is very little variance in the order and timing of this process between embryos. Of the sixty-four cells that gastrulate, these include precursors of much of the ring ganglion in the head, sometimes referred to as the *C. elegans* brain (Thomas and Lockery, 1999). The internalization of a concentration of nervous system precursors might reasonably be referred to as *C. elegans* neurulation, although we found that most of these AB-derived cells internalized as a ventral patch of cells that form portions of a strikingly continuous, ventral stripe of cells (Fig 2.2B). We therefore refer instead to all of these cells as gastrulating cells.

How closely is cell fate tied to gastrulation? Cell fates are acquired prior to cell movement to the interior of the embryo, but it was unknown if there was a requirement for proper cell fate for timely internalization in the MS and germline cells. We have confirmed that cell fate does affect the timing of internalization, leading to the conclusion that cell fate regulators must be acting upstream of internalization mechanisms.

What about other patterning mechanisms such as cell polarization? The endodermal cells' apicobasal polarity is regulated by the PAR proteins and these polarity proteins are required for timely internalization of the endoderm cells (Nance, 2003). It is possible that the PAR proteins may be activated in cells of different fates and therefore they may be polarizing the other cells that internalize too. Alternatively, PAR proteins may be just one set of polarity regulators and other cells with different fates use either completely different polarity regulators or a combination of redundant polarity regulators.

The work demonstrated here unveils that the majority of MS and D lineage descendants in *par-3(ZF1)* and *par-6(ZF1)* mutants internalize during the same cell cycle and with a timing similar to MS and D descendants in wild-type embryos. This suggests that PAR proteins are not the sole polarity regulators in these lineages. As Wieschaus (1995) suggested, once cell fates are determined, it is essential that cells

achieve morphological changes as well. Multiple redundant pathways for patterning may exist in order to ensure that these changes occur. Paralogs of proteins involved in polarity complexes in epithelial cells in *Drosophila* exist in *C. elegans* (Assemat et al., 2008). It will be interesting to determine if these paralogs from the Crb/Pals/Patj or Scrib/Dlg/Lgl complexes are playing a role in the internalization of MS and D fates in *C. elegans*, either in concert or independent of PAR proteins.

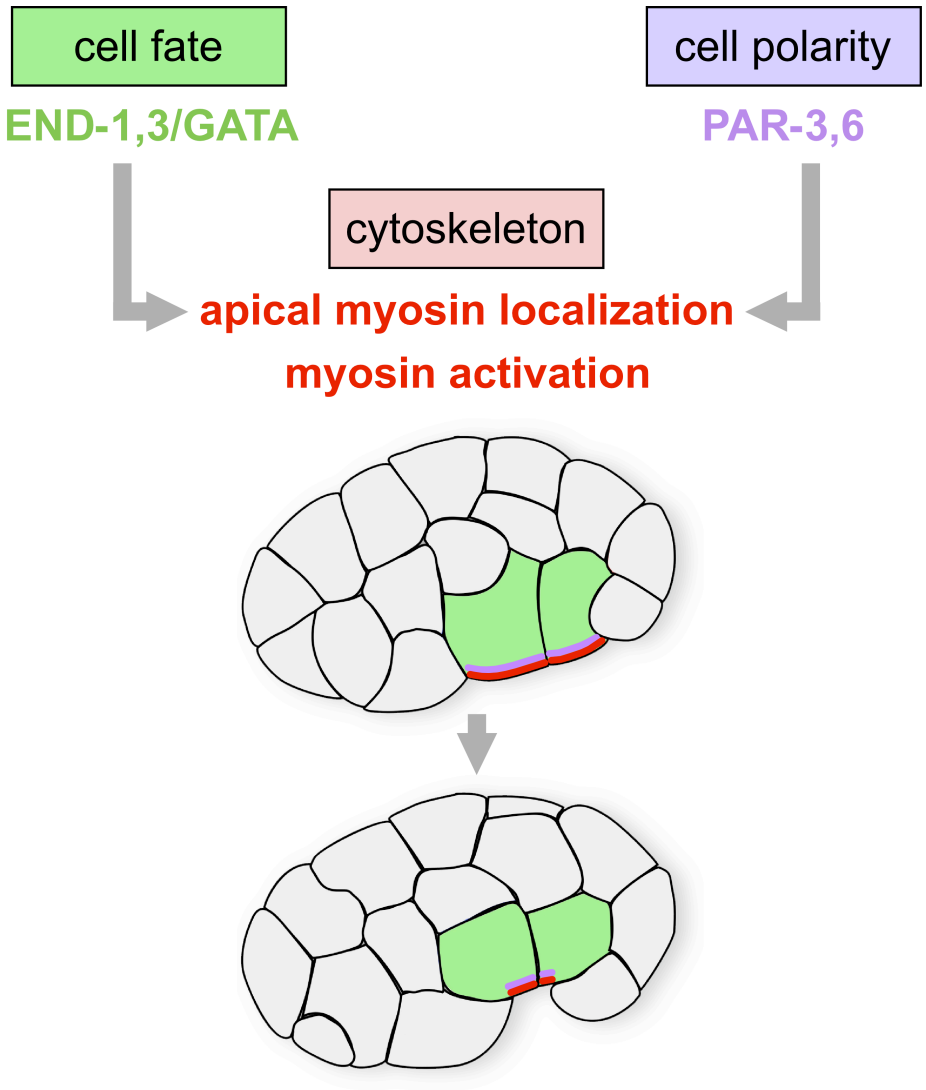
Is patterning in *C. elegans* acting upstream of a different cytoskeletal mechanism, or is the same cytoskeletal mechanism reiterated in the embryo a number of times? Apical constriction is used a number of times in the *Drosophila* embryo, although different patterning genes are driving the cytoskeletal changes in the individual processes. For example, trachea formation in *Drosophila* is regulated by the fate gene Trachealess through the EGFR signaling pathway and the RhoGAP Crossveinless to affect apical constriction and tube formation (Brodu and Casanova, 2006). On the other hand, mesodermal cells invaginate using apical constriction as well, but downstream of different patterning genes, namely Twist and Snail (Leptin and Grunewald, 1990). Something similar may be true in *C. elegans* since mesoderm and germ line cells, regulated by different patterning genes, accumulate myosin at their apical surface during internalization. Activation of myosin at the apical surface in mesoderm strongly suggests that apical constriction is regulating the internalization of non-endodermal cells.

These results enhance our understanding of gastrulation in *C. elegans*. While much had been known about the internalization mechanism of the endodermal precursor cells, very little had been known about the internalization of other cells. The apical constriction mechanism driving internalization during gastrulation appears to be conserved among different lineages but upstream control of this mechanism lies with different set of cell fate and polarity regulators. A question that remains is what lies between the patterning genes and the cytoskeletal mechanism? Wnt

signaling is implicated in the activation of apical constriction in the endodermal cells, but its role in the phosphorylation and activation of myosin II in other lineages is not clear. It will be interesting to determine exactly how signaling pathways translate the cytoskeletal mechanisms downstream of fate regulators such as MES-1 and the mesodermal transcription factors TBX-35 and CEH-51. Perhaps Rho Kinase is involved in the phosphorylation and activation of myosin, or it may be a yet unidentified kinase.

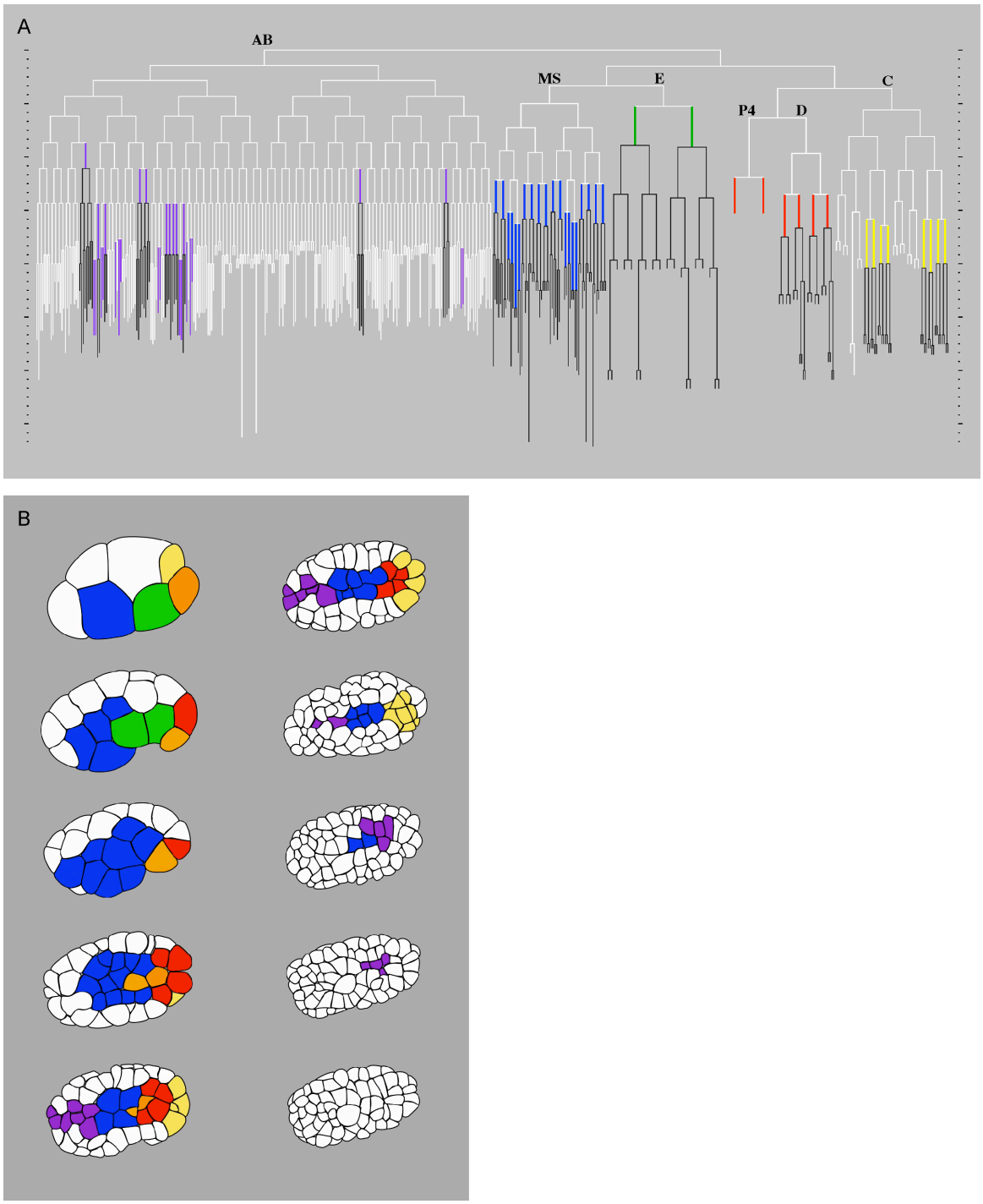
Although the upstream patterning regulators are not the same in different types of cells, we propose that the cellular process that results is conserved, apical constriction. Understanding how multiple inputs leads to a constriction in *C. elegans* has broad implications for understanding this conserved cytoskeletal mechanism in more complex systems. For example, apical constriction has been implicated in neural tube closure in mammals, and insights to the developmental pathways leading to this common cytoskeletal mechanism may be valuable in our understanding of serious congential defects like these. Understanding the links between patterning mechanisms in *C. elegans* will give us insight into how different developmental pathways control cell shape changes and rearrangements in other systems.

Figure 2.1 Known requirements for endodermal cell internalization



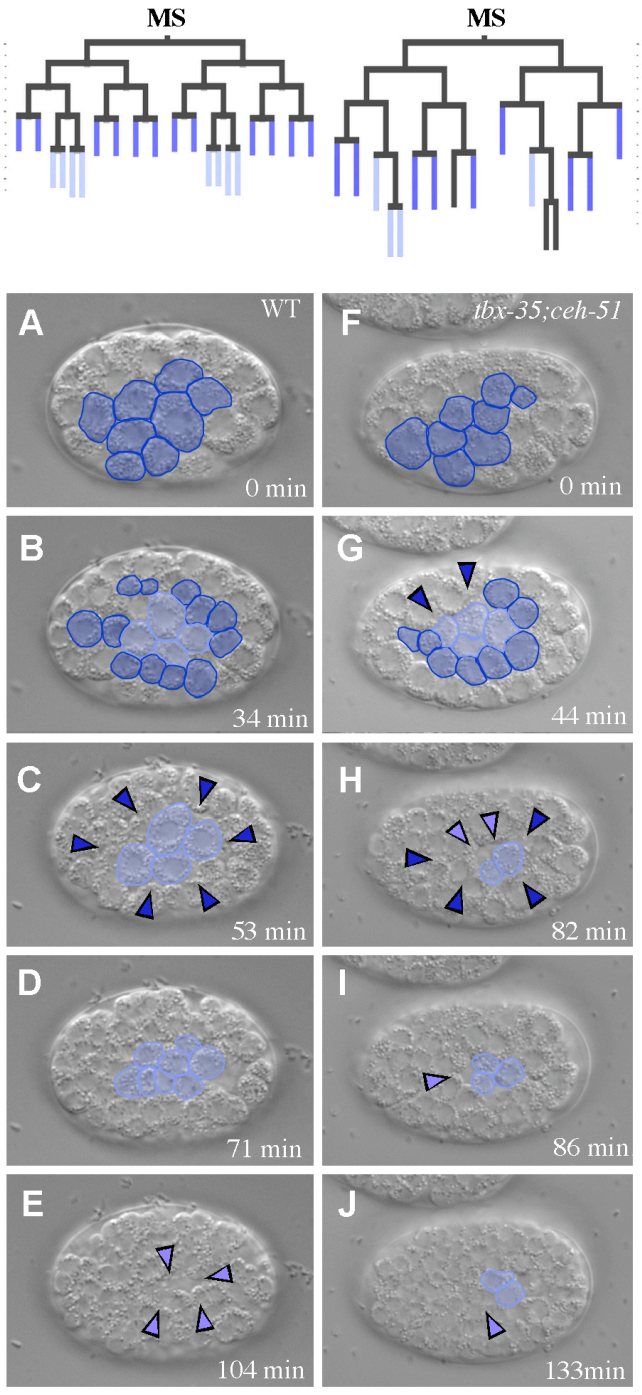
There are three known requirements for endodermal cell internalization. Cell fate and cell polarity regulators are acting upstream of apical myosin localization and activation to drive apical constriction and internalization of these cells.

Figure 2.2 Gastrulation in *C. elegans*



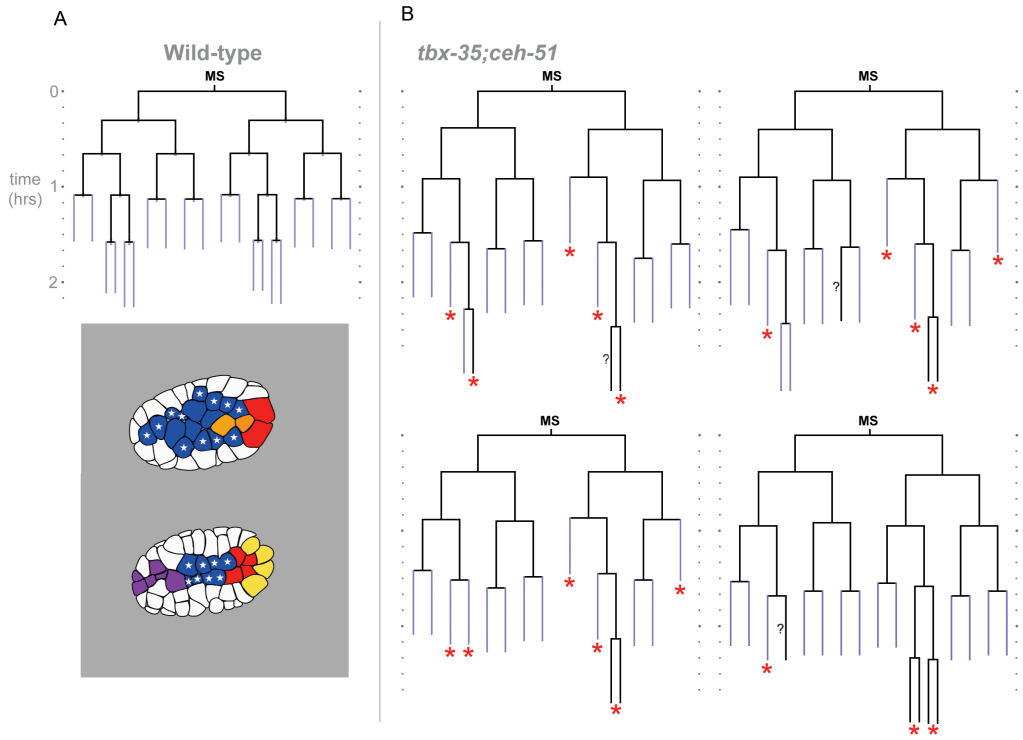
(A) The *C. elegans* lineage mapped to represent gastrulation. Distinct colors represent the specific cells of each lineage that internalize: Purple = AB lineage cells; Blue = MS lineage cells; Green = E lineage cells; Orange = Germ line precursor cells; Red = D lineage cells; Yellow = C lineage cells. The black lines indicate descendants that are born in the interior of the embryo. (B) The stages of *C. elegans* gastrulation based on an embryo. All of the descedents of E, MS, P_4, D, and C are colored from the time they are born. The AB cells are only colored during the cell cycle in which they internalize. The colors of specific cells match the lineage represented in (A).

Figure 2.3 MS descendants use distinct fate regulators to control internalization



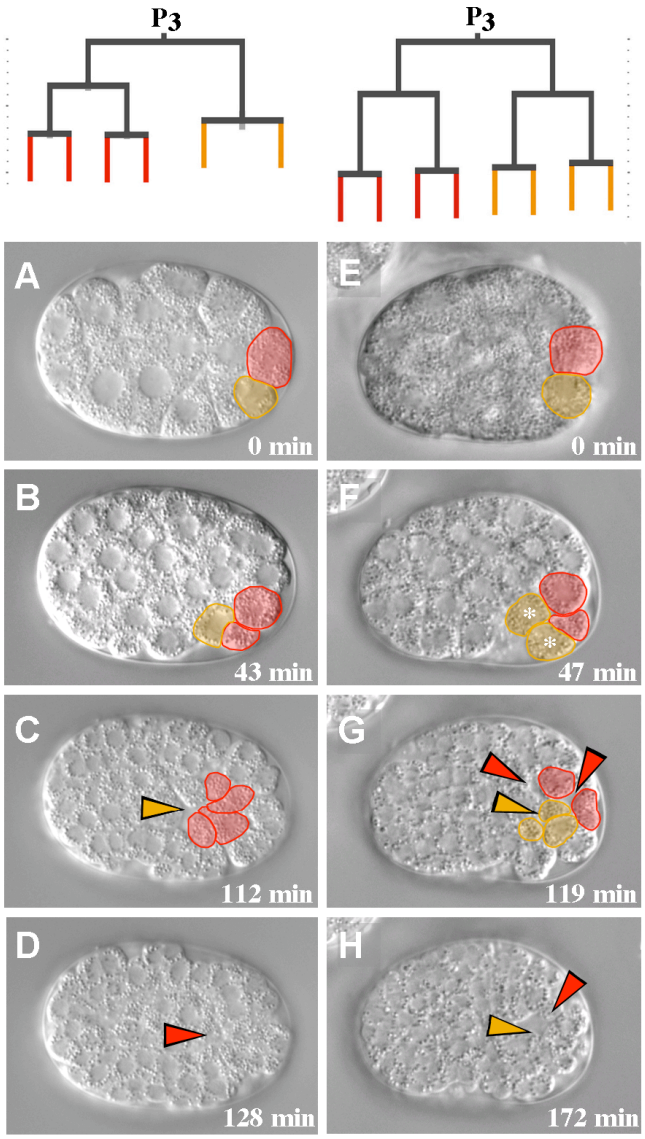
Images from a movie of N2 (A-E) and *tbx-35;ceh-51* mutant embryos (F-J). Dark blue represents the MS descendant cells that internalize after the fourth MS cell division and the light blue represents the MS descendant cells that divide on the surface before internalizing after the fifth MS cell division in WT embryos (see the lineage above). Colored arrows represent internalized cells that are no longer visible on the surface of the embryo. (A) The N2 MS descendant cells after the third MS division: 8-MS cell stage. (B) The MS descendant cells after the fourth MS cell division: 16 MS descendant cells, just before MS descendants begin to internalize. (C) 12 MS descendants have internalized and 4 MS descendants remain on the surface. (D) After the fifth MS cell division when 4 MS descendants divided on the surface to give rise to 8 MS descendants. (E) The 8 remaining MS descendants internalize. (F) The *tbx-35;ceh-51* MS descendant cells after the third MS division: 8-MS cell stage. (G) The MS descendant cells after the fourth MS cell division: 16 MS descendant cells; 2 non-sister MS descendants internalized after the third MS cell division, instead of dividing on the surface as occurs in WT [dark blue arrowheads]. (H) The remaining MS descendents that normally internalize after the fourth MS division internalize as pairs [dark blue arrowheads], in addition to 2 non-sister cells that normally internalize after the fifth MS cell division [light blue arrowheads]. (I) The two MS descendants remaining on the surface in H divide, with one cell internalizing immediately [light blue arrowhead]. Its sister cell internalizes in (J). (J) Two sister MS descendent cycle cells remain on the surface.

Figure 2.4. Lineage and internalization timing in individual *tbx-35;ceh-51* mutant embryos.



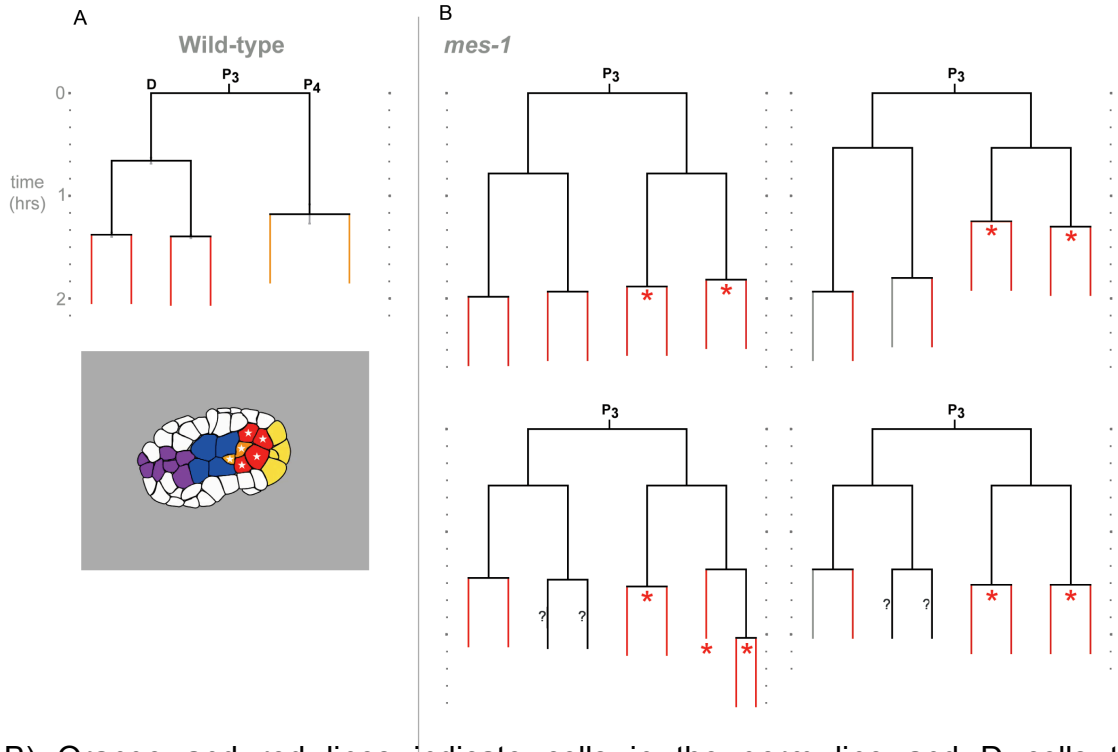
(A,B) Blue lines indicate cells in the MS lineage that internalized. (A) MS lineage in a WT embryo. White stars on the image of the embryo indicate which cells internalize in WT embryos. (B) MS lineage in four *tbx-35;ceh-51* mutant embryos. Lineage lines without blue indicate cells that did not internalize during the time the embryos were filmed. Red asterisks are abnormalities in the internalization of cells. Question marks indicate cells for which we could not determine whether they internalized.

Figure 2.5 Germ line descendants use distinct fate regulators to control internalization



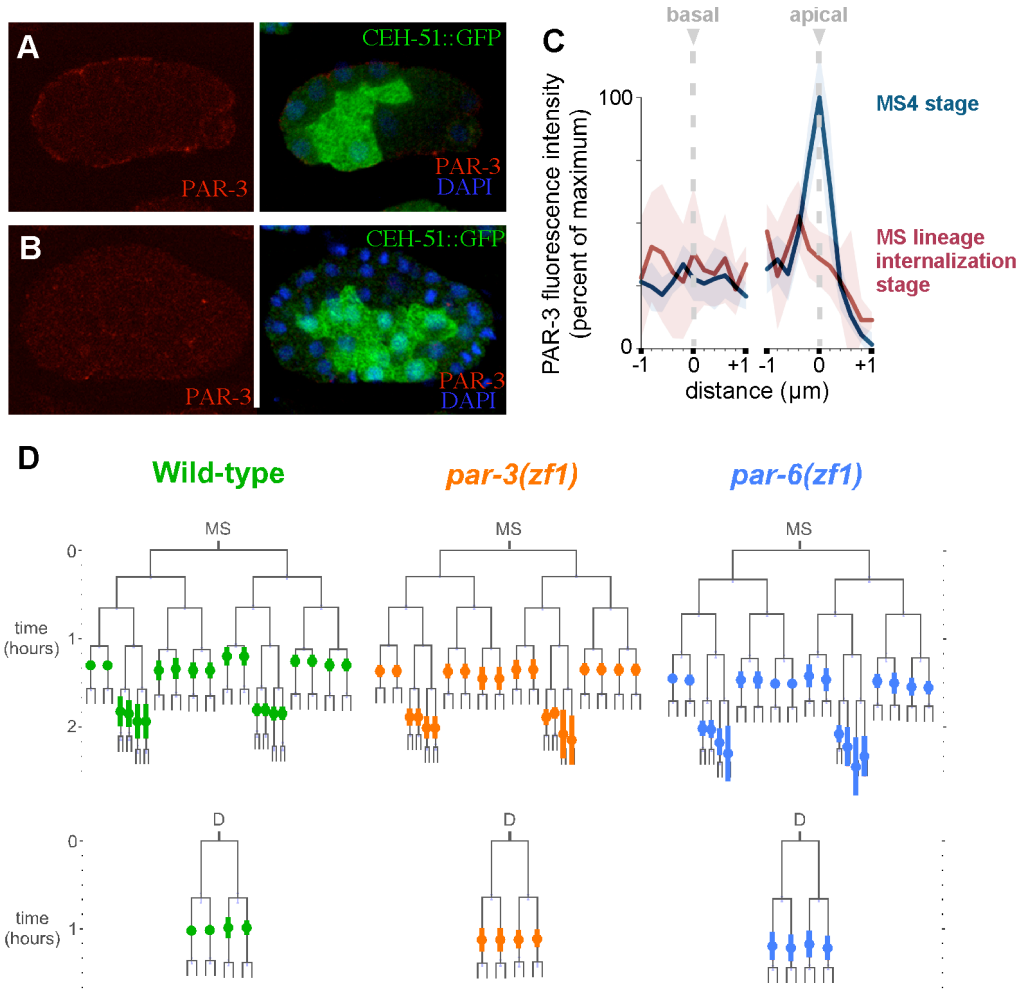
Images from a movie of an N2 embryo (A-D), and a *mes-1* embryo (E-H), with germ line and D lineage cells pseudo-colored, beginning with their birth as a result of the P3 cell division. Colored arrows represent internalized cells that are no longer visible on the surface of the embryo. (A) The P_3 cell division at time 0 is asymmetric with respect to size. The germ line precursor cell (orange) is smaller than the D cell (red). (B) The D cell divides on the surface of the embryo before P_4. (C) The germ line precursor cells internalize. (D) The D muscle cells internalize later. (E) The P_3 division at time 0 is symmetric, with the germ line precursor cell and D lineage cells being approximately the same size. (F) The P_4 cell divides at the same time as the D cell. (G) The P4 and D descendant cells divide again and begin internalizing immediately, indicated by the orange and red arrows, respectively. (H) All of the descendants of P_4 and D cells have internalized.

Figure 2.6. Lineage and internalization timing in individual *mes-1* mutant embryos.



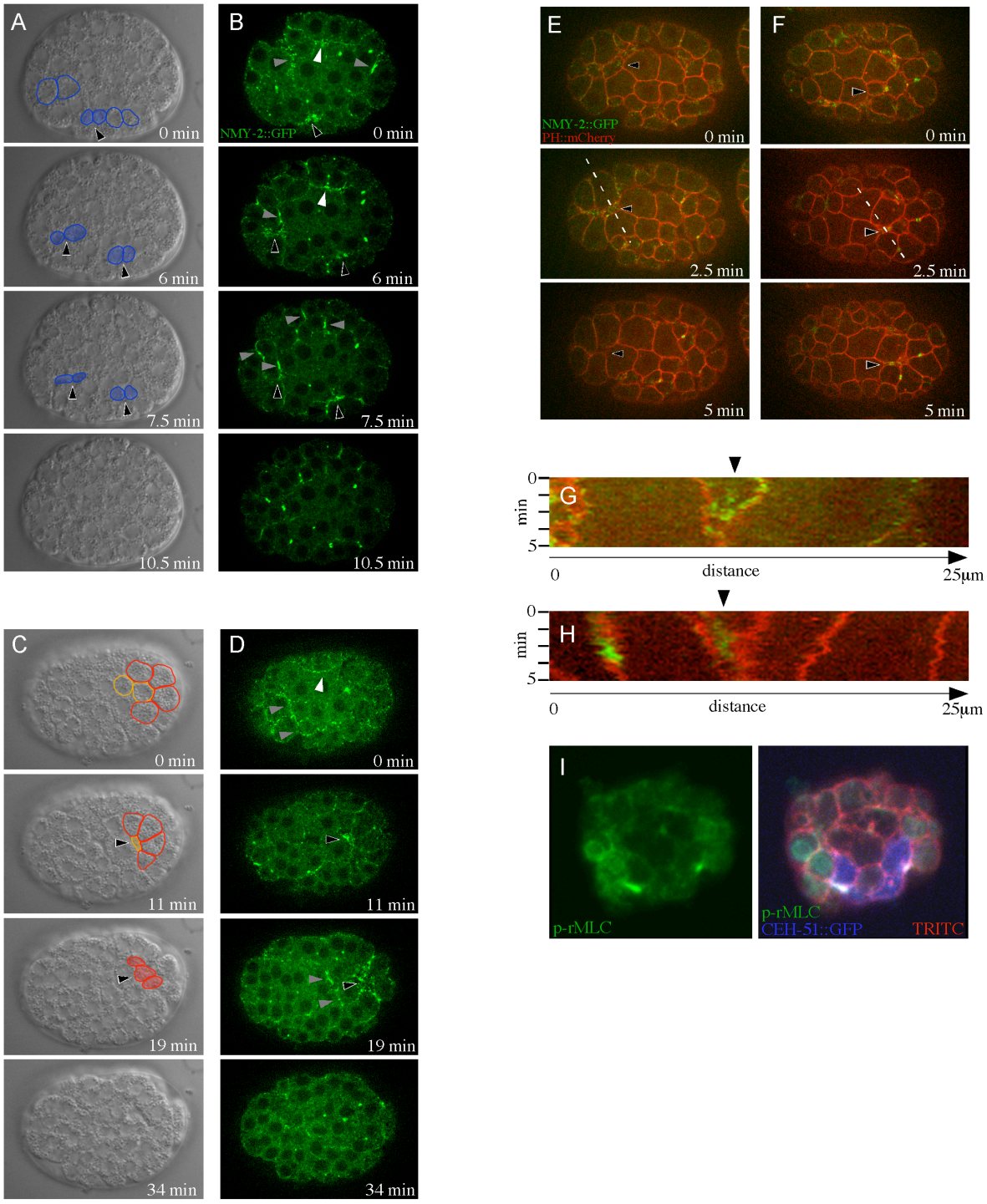
(A,B) Orange and red lines indicate cells in the germ line and D cells that internalized (respectively) (A) Germ line and D cell lineages in a WT embryo. White stars on the image of the embryo indicate which cells internalize in wild-type embryos. (B) Germ line and D lineages in four *mes-1* embryos. Gray lines indicate cells that were born to the interior. Lineage lines without colors to mark internalization indicate cells that did not internalize during the time the embryos were filmed. Red asterisks are abnormalities in the internalization of cells. Question marks indicate cells for which we could not determine whether they internalized.

Figure 2.7 PAR proteins are not required for timely internalization of MS and D lineage cells



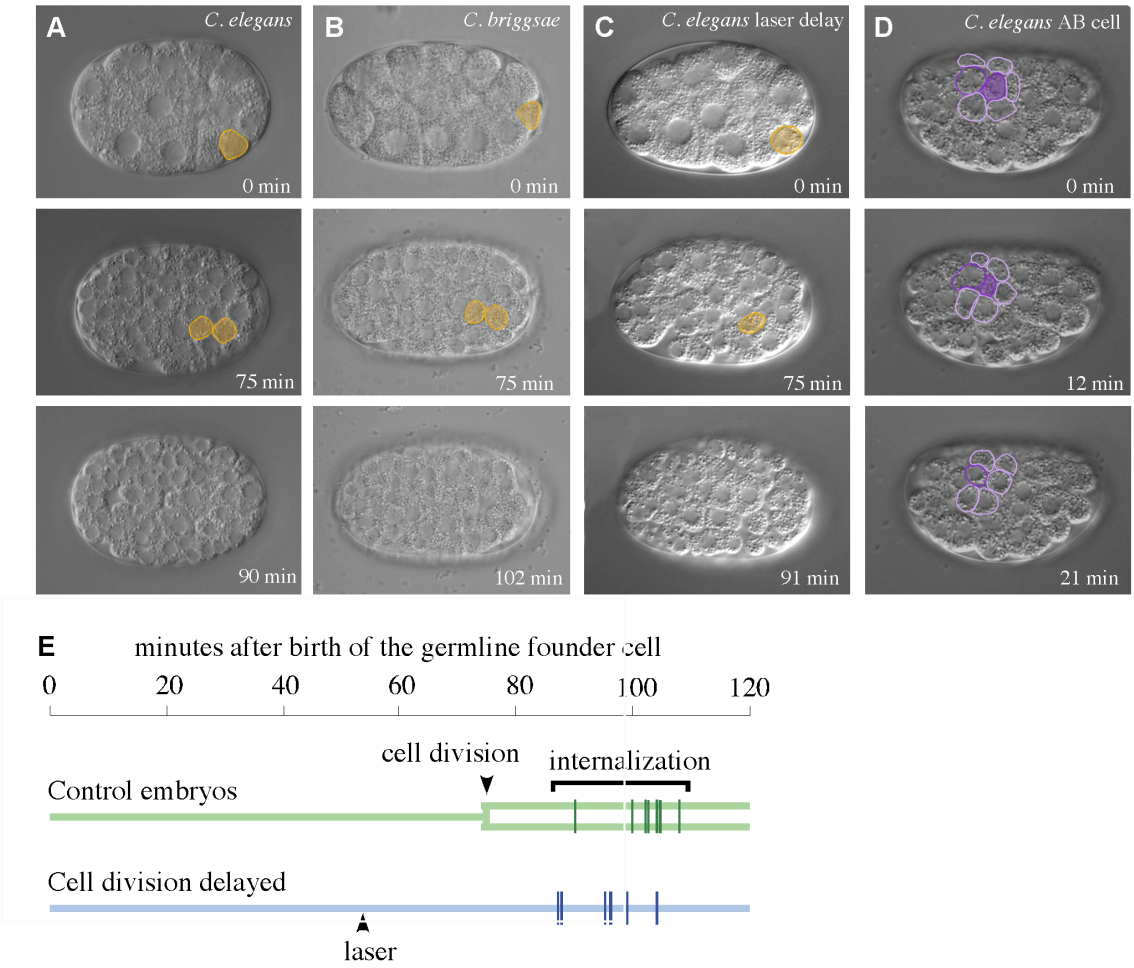
(A) CEH-51::GFP embryo at the 4 MS descendant cell stage immunostained for PAR-3 (left) and merged with GFP and DAPI (right). (B) CEH-51::GFP embryo at the 16 MS descendant cell stage immunostained for PAR-3 (left) and merged with GFP and DAPI (right). (C) anti-PAR-3 fluorescence intensity levels quantified in 4 MS (n=6) descendant stage (blue) and 16 MS (n=5) descendant cell stage (red) embryos at the basolateral membrane (left) and apical membrane (right). Shading indicates 95% confidence intervals. (D) MS and D lineages for WT (n=7), *par-3(ZF1)* (n=9)*, par-6(ZF1)* (n=10) embryos. The timing of internalization of specific cells are indicated by colored circles on the lineage, with standard deviation bars.

Figure 2.8 Internalizing mesodermal and germ line precursor cells accumulate myosin at their apical surfaces



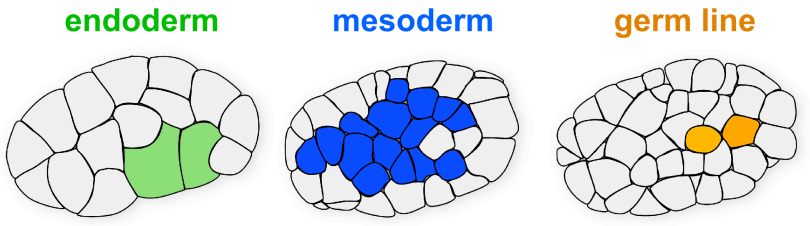
(A-D) Images from NMY-2::GFP embryos filmed simultaneously with DIC (A,C) and GFP (B,D). Black arrowheads indicate cells that are internalizing. White arrowheads are unidentified internalizing cells. Cells undergoing cytokinesis are marked by gray arrowheads. Cells that are internalizing are shaded in blue (MS lineage), orange (germ line lineage) or red (D lineage), and those descendants that will internalize in later frames are outlined in respective colors. (A,B) Black arrowheads indicate myosin accumulation in MS descendants that are internalizing at 0, 6, and 7.5 minutes. (C,D) Black arrowheads indicate myosin accumulation in internalizing germ line precursors and D descendant cells at 11 minutes and 19 minutes, respectively. (E,F) PH::mCherry;NMY-2::GFP embryos were filmed to look for myosin accumulation specifically in internalizing cells. (E) Arrowheads indicate an internalizing MS descendent cell. (F) Arrowheads indicate an internalizing germ line precursor cell. (G) Kymograph analysis of the MS descendant cell in (E). (H) Kymograph analysis of the germ line precursor cell in (F). (I) CEH-51::GFP embryos stained with p-rMLC antibody shows apical accumulation in internalizing MS descendant cells. Nuclear staining is a background signal (Lee et al., 2006). 3 planes 0.5μm apart merged for representation.

Figure 2.9 Cells generally move in as sister pairs but this is not required for efficient internalization



(A) Internalization of two germ line precursor cells during *C. elegans* gastrulation, beginning with birth of P_4 cell at time 0. (B) Internalization of two germ line precursor cells during *C. briggsae* gastrulation, beginning with birth of P_4 cell at time 0. (C) Internalization of one germ line cell after laser delay of P_4's cell cycle. (D) The ABplppap cell internalizes as a single cell. (E) Internalization timing of Z2 and Z3 in control embryos (n = 7) and P_4 in laser delayed embryos (n=7). In one case, the P_4 cell remained on the surface until at least an hour after irradiation without dividing.

Figure 2.10 Internalization of distinct lineages in *C. elegans*



cell fate	END-1,3	TBX-35, CEH-51	MES-1
cell polarity	PAR-3,6	?	?
cytoskeleton	apical myosin localization myosin activation		

Model of different upstream regulation during the cell internalization of distinct cell types. At top, still images of animated movie from Figure 2.2B. Cell fate and cell polarity regulators are listed for each cell type, if known.

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