

UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

The role of the ATP-binding cassette (ABC) transporter ABCC5a in hindgut invagination of sea urchin embryos

Permalink

<https://escholarship.org/uc/item/6596v0x6>

Author

Shipp, Lauren Elizabeth

Publication Date

2015

Supplemental Material

<https://escholarship.org/uc/item/6596v0x6#supplemental>

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

**The role of the ATP-binding cassette (ABC) transporter ABCC5a
in hindgut invagination of sea urchin embryos**

A dissertation submitted in partial satisfaction of the
requirements for the degree

Doctor of Philosophy
in
Marine Biology

by
Lauren Elizabeth Shipp

Committee in charge:

Professor Amro Hamdoun, Chair
Professor Nicholas Holland
Professor William McGinnis
Professor Martin Tresguerres
Professor Victor Vacquier

2015

Copyright

Lauren Elizabeth Shipp, 2015

All rights reserved.

The Dissertation of Lauren Elizabeth Shipp is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2015

DEDICATION

For my parents, Bonnie and Paul Shipp, who have always believed
I was capable of anything.

And for my husband, Reggie Robertson, whose love and support
makes my capabilities a reality.

EPIGRAPH

People should do what they are capable of.

Heidi Solomon on This American Life

TABLE OF CONTENTS

SIGNATURE PAGE	iii
DEDICATION	iv
EPIGRAPH	v
TABLE OF CONTENTS	vi
LIST OF ABBREVIATIONS	x
LIST OF SUPPLEMENTAL FILES	xii
LIST OF FIGURES AND TABLES	xiii
ACKNOWLEDGEMENTS	xiv
VITA	xx
ABSTRACT OF THE DISSERTATION	xxiv
CHAPTER 1. INTRODUCTION AND BACKGROUND	1
1.1. Introduction	2
1.2. Background.....	7
1.2.1. ABCC5/MRP5 transporters	7
1.2.1.1. Expression	7
1.2.1.2. Localization.....	9
1.2.1.3. Gene splicing and paralogs	10
1.2.1.4. Structure	10
1.2.1.5. Substrates and inhibitors	12
1.2.1.5.1. Exogenous substrates.....	12
1.2.1.5.2. Endogenous substrates	13
1.2.1.5.2.1. Cyclic nucleotides.....	13
1.2.1.5.2.2. Heme	15
1.2.1.5.2.3. Hyaluronan	16
1.2.1.5.2.4. N-lactoyl amino acids	16
1.2.1.5.3. Inhibitors	17
1.2.2. Sea urchin development.....	17
1.3. References.....	21

CHAPTER 2. ATP-BINDING CASSETTE (ABC) TRANSPORTER EXPRESSION AND LOCALIZATION IN SEA URCHIN DEVELOPMENT	30
2.1. Abstract.....	31
2.2. Introduction	32
2.3. Results	35
2.3.1. ABC transporter genes expressed in early development.	35
2.3.2. Temporal patterns of ABC transporter gene expression.	38
2.3.2.1. Temporal expression patterns of paralogs	40
2.3.3. Quantification of ABC transporter mRNAs.	41
2.3.4. Spatial patterns of ABC transporter gene expression.	42
2.3.5. Cellular localization of ABCB1a and ABCC5a proteins.	44
2.4. Discussion.....	48
2.4.1. Xenobiotic transport	48
2.4.2. Mitochondrial transport.....	49
2.4.3. Lysosomal transport.....	50
2.4.4. Multifunctional transport.....	50
2.4.5. Potassium channel conductance regulators.....	51
2.4.6. White transporter homologs	52
2.4.7. Transporters with unresolved classification	52
2.4.8. Differential regulation of transporters	53
2.4.9. Conclusions.....	54
2.5. Materials and methods	56
2.5.1. Animals	56
2.5.2. RNA isolation.....	56
2.5.3. cDNA synthesis.....	57
2.5.4. Quantitative real-time PCR (qPCR).....	57
2.5.5. Primer design and testing.....	58
2.5.6. Confirmation of amplicon specificity to targeted genes	58
2.5.7. qPCR Analyses	59
2.5.8. Transcript copy number calculations.....	60
2.5.9. ABC transporter protein expression	61
2.5.10. Whole mount <i>in situ</i> hybridization (WMISH).....	62

2.6. Acknowledgements.....	65
2.7. Supplementary material	66
2.8. References.....	69
2.8. Chapter acknowledgments	74
CHAPTER 3. ABCC5 IS REQUIRED FOR cAMP-MEDIATED HINDGUT INVAGINATION IN SEA URCHIN EMBRYOS	75
3.1. Abstract.....	76
3.2. Introduction	78
3.3. Results	82
3.3.1. C5a is a 210 kDa protein similar to human ABCC5/MRP5	82
3.3.2. C5a strongly effluxes FDA but not other chemicals in the fluorone or bodipy classes.....	83
3.3.3. C5a expression is highest during and immediately following gastrulation.....	85
3.3.4. C5a is expressed in the pigment cell precursors.....	86
3.3.5. C5a expression is required for gut formation, but not pigment cell formation	89
3.3.6. C5a expression is required for orientation of the hindgut.....	91
3.3.7. Live cell confocal time-lapse imaging of C5a-morphants	93
3.3.8. C5a-mediated prolapse is rescued by cAMP, which is derived from sAC in pigment cells.....	96
3.4. Discussion.....	99
3.4.1. Substrates of C5a.....	100
3.4.2. C5a is expressed in motile pigment cells but is necessary for gastrulation.....	101
3.4.3. Contributions of veg-lineage cells to the gut and timing of hindgut morphogenesis.....	103
3.4.4. How does C5a in pigment cells affect the position of the hindgut? ..	104
3.4.5. Evolutionary implications of C5a-mediated gut morphogenesis.....	105
3.5. Materials and methods	108
3.5.1. Animals and reagents	108
3.5.2. Gene expression analyses	108
3.5.3. Microinjection of mRNAs and MASOs.....	110

3.5.4. Western blot	110
3.5.5. Transporter efflux activity assays	111
3.5.6. Immunohistochemistry (IHC)	111
3.5.7. Quantification of C5a-knockdown phenotypes and rescue	112
3.5.8. Microscopy	113
3.5.9. Statistics	113
3.6. Acknowledgements	115
3.7. Author contributions	115
3.8. Funding	115
3.9. Supplementary material	116
3.9.1. Supplementary methods	116
3.9.1.1. Sp-C5a antibody generation and validation	116
3.9.1.1.1. Antibody adsorption for immunohistochemistry	117
3.9.1.2. Time-lapse imaging of C5a-knockdown embryos	120
3.10. References	121
3.11. Chapter acknowledgments	128
CHAPTER 4. EPILOGUE	129
4.1. Looking forward	130
4.1.1. Further studies of C5a	133
4.1.1.1. Evolution of ABCC5 transporters and gene duplication	133
4.1.1.2. Regulation and trafficking of C5a	135
4.1.1.2.1. Gene regulation	135
4.1.1.2.2. Post-translational processing	136
4.1.1.2.3. Membrane trafficking	137
4.1.1.2.4. Membrane dynamics	137
4.1.2. Hindgut movement and the GRN	138
4.2. Conclusions	141
4.3. References	142
4.4. Chapter acknowledgments	146

LIST OF ABBREVIATIONS

ABC, ATP-binding cassette

B1a, *Sp*-ABCB1a

BCECF-AM, 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-
acetoxymethyl ester

Bra, Brachyury

b-VER, bodipy-verapamil

b-VIN, bodipy-vinblastine

C5a, *Sp*-ABCC5a (homolog of ABCC5/MRP5)

cAMP, cyclic adenosine monophosphate

cGMP, cyclic guanosine monophosphate

CL, cytoplasmic loop

CMFDA, 5-Chloromethylfluorescein diacetate

DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester

D/N, Delta/Notch

ECM, extracellular matrix

EL, extracellular loop

EM, electron microscopy

FDA, fluorescein diacetate

FSW, filtered seawater

GAG, glycosaminoglycan

gcm, glial cells missing

GRN, gene regulatory network

hpf, hours post-fertilization

KH7, 2-(1H-benzimidazol-2-ylthio)-2-[(5-bromo-2-

hydroxyphenyl)methylene]hydrazide, propanoic acid

LBP, ligand binding pocket (a.k.a. drug binding pocket, ligand binding domain)

MASO, morpholino antisense oligonucleotide

MDR, Multidrug Resistance

MIP, maximum intensity projection

MRP, Multidrug Resistance Protein (ABCB, ABCC, and ABCG transporters)

MSD, membrane spanning domain

NBD, nucleotide binding domain

NSM, non-skeletogenic mesoderm (a.k.a. SMC, secondary mesenchyme cell)

P-gp, P-glycoprotein (ABCB1/ABCB1a)

PKA, protein kinase A (a.k.a. CAPK, cAMP-dependent protein kinase)

sAC, soluble adenylyl cyclase

SM, skeletogenic mesoderm (a.k.a. PMC, primary mesenchyme cell)

Sp-, *Strongylocentrotus purpuratus*

Su(H), suppressor of hairless

tmAC, transmembrane adenylyl cyclase

TMH, transmembrane helix

LIST OF SUPPLEMENTAL FILES

Movie S1: Maximum intensity projections (MIPs) of C5a-knockdown embryos

Movie S2: MIPs of a control embryo (membrane labeled only) and a C5a-knockdown embryo (membrane and nuclei labeled) shown side-by-side

Movie S3: Cross-sections of the C5a-knockdown embryo from Movie S2

LIST OF FIGURES AND TABLES

Figure 1.1.	Development of the sea urchin from the 60-cell stage (~10 hpf) to the pluteus larva stage (~72 hpf).....	18
Table 2.1.	ABC Transporter genes detected in sea urchin development and functions of their homologs.	36
Figure 2.1.	Developmental stages surveyed and relative ABC transporter gene expression.....	39
Figure 2.2.	Relative ABC transporter gene expression during sea urchin development.....	40
Figure 2.3.	Number of ABC transporter transcripts per egg/embryo. Number of mRNA copies per egg/embryo is shown	43
Figure 2.4.	Spatial expression of ABC transporter genes	45
Figure 2.5.	Localization of ABCB1a and ABCC5a proteins.....	46
Figure 2.6.	Model of endogenous ABCB1a and ABCC5a protein localization	47
Table S2.1.	Quantitative ABC transporter gene expression in sea urchin development.....	67
Table S2.2.	Primers used for qPCR, <i>in situ</i> hybridization, RACE, and cloning	68
Figure 3.1.	C5a is a 210 kDA membrane protein that is not a broad chemical transporter	83
Figure 3.2.	Expression of C5a peaks after gastrulation and is controlled by Delta/Notch signaling from the SM.....	86
Figure 3.3.	C5a is expressed in pigment cells that migrate away from the archenteron during early gastrulation.....	88
Figure 3.4.	Gut prolapse is the major morphology in C5a-knockdown embryos	91
Figure 3.5.	Long-term imaging of C5a-knockdown embryos reveals defects in hindgut formation	94
Figure 3.6.	C5a-knockdown mediated prolapse is rescued by cAMP, which is derived from sAC in pigment cells.....	97
Figure 3.7.	A model of C5a-mediated gut invagination	100
Figure S3.1.	Detecting C5a with affinity-purified and adsorbed anti-C5a, and demonstrating knockdown with two MASOs	118
Figure S3.2.	Two MASOs block translation of <i>C5a</i> and cause hindgut prolapse	119
Figure S3.3.	Chambers used to contain swimming embryos for long-term imaging.....	120
Figure 4.1.	Spatio-temporal mapping of transporters	132

ACKNOWLEDGEMENTS

Thank you to the incredible mentors who made each phase of my career possible. To my graduate advisor, Dr. Amro Hamdoun, thank you for taking the time to usher me into the world of marine cell biology a solid year before I even began at Scripps – this was a good indicator of your commitment to mentoring and had a huge impact on my ability to organize project ideas and procure funding. Your inquisitiveness, love for biology, and desire to make a positive societal impact have shaped your lab for the better. As one of your founding lab members, I have been privileged to help establish this system. Thank you for giving me the freedom to pursue my project, and for supporting my career advancement. It has been quite a journey, and I am a more thoughtful and intellectual researcher because of your influence.

To the Hamdoun Lab's most experienced post-doc, Dr. Victor Vacquier, you have been a source of encouragement, honesty, clarity, support, and endless information throughout my graduate career. You have always helped steer me and my project in the right direction; after seeing my early data, it was you that pointed to C5a and said, "That's your thesis!". Indeed you were right, and I thank you for that and for too many other insights to name.

I thank my remaining committee members for being engaged and supportive, and for the many constructively critical discussions. Dr. Nicholas Holland, thank you also for your generous help with electron microscopy and editing. Dr. Martin Tresguerres, thank you for lending your expertise on sAC and

for inhibitors. Dr. William McGinnis, thank you for your expertise in developmental biology and your willingness to discuss my ideas and progress. All of your input on my research and career pursuits has been immeasurably helpful. I also thank Drs. Linda Holland, Michael Latz, and Peter Franks for valuable mentoring and perspective throughout my time at SIO.

To my previous employer, Dr. Jen-Chywan (Wally) Wang, thank you for taking a chance on a new graduate with little molecular biology experience. (I'm still not sure why you did that, but I'm really glad you did.) Your parting message to me – a post-it on which you wrote the Chinese symbol for crisis and the words “crisis = danger + opportunity” – remained on my office wall throughout grad school and guided me through some opportunity-full moments. I learned an incredible amount in three years with you, much of which was gleaned from the example you set, with great dedication, efficiency, and kindness towards your lab. I am thankful that you brought Drs. Keith Yamamoto and Alex So in to my research sphere. Alex, you likely thought I was kidding when I said I had not pipetted before, but nonetheless you imparted on me valuable technical skills. I am grateful for that and for all the training and encouragement I received throughout my years in the Wang Lab.

To my undergraduate mentor, Dr. Sheila Patek, you have been a role model since I first met you in 2005, and your ever-growing accomplishments continue to inspire. You were the first to consider that I might have potential in scientific research, and without your confidence in me, I likely would not have

continued. Thank you for your encouragement, the opportunities you opened, and the solid intellectual and technical foundation you helped lay for my research career.

For helping me gain rewarding teaching and curriculum development experience, I greatly appreciate the support of Dr. Amro Hamdoun, Dr. Bianca Brahamsha, Dr. Valerie Hohman, Dr. R. Jeffery Chang, and Patricia Winter. Thank you also to the Scripps Graduate Department, especially Maureen McCormack, for helping me jump through a few hoops to get some of this experience.

Thank you to my SIO family for being the best group of people anyone could hope to work with. Dr. Tufan Gökırmak, your infectious energy and humor has brightened many days at SIO. Gary Moy, your patience, technical prowess, and calming presence have made lab life easier in so many ways. It is because of you we even had a C5a antibody, and I am very thankful for all your work to optimize the antibody and Western blots. Jose Espinoza, thanks for your clutch last-minute rowing skills and for being the wise old soul that tries to keep us all sane. To my former undergraduate researcher Rose Hill, thank you for all your hard work throughout the C5a study. Your intelligence and commitment helped in many ways, and you have made me a proud mentor. To other past and present members of the Hamdoun Lab, thank you for your constructive discussions and companionship in the lab; and to our wonderful undergrads, thanks especially for your enthusiasm and lab maintenance. Members of my cohort, especially Jill

Harris, Noelle Bowlin, Christian Briseno, and Mindi Summers: you are inspiring and wonderful humans who taught me a lot, and I'm proud to call you my friends and colleagues. Finally, Joseph Campanale, we have lived and learned side by side for six years, and I could not imagine graduate school without you (nor would I want to, considering how injecting tens of thousands of embryos would go without your patience and superior rowing abilities). Thank you for being a dear friend, my science sibling, and for teaching me about echinoderms with your trademark fanatical enthusiasm.

I owe thanks to two others whose influence helped get me to and through graduate school. To Mario Noguez, my gymnastics coach for 15 years, in a world where mediocrity is celebrated, you taught me that great achievements are born from hard work, toughness, resilience, dedication, and striving for excellence. You always valued education as much as athletics, and it was because of your training that I landed at UC Berkeley. Once there, I met the brilliant and incomparable Dr. Tyrone Hayes, to whom I owe thanks for inspiring my foray into environmental cell biology.

I am extremely grateful for the funding from the National Science Foundation Graduate Research Fellowship Program (NSF GRFP) and the National Defense Science and Engineering Graduate (NDSEG) Fellowship program. I am also honored to have been supported by the Achievement Rewards for College Scientists (ARCS) foundation and Philanthropic Educational

Organization (P.E.O.). The knowledge that these organizations believed in my abilities was at least as helpful as their generous financial support.

Thank you to my dear family for love, patience, acceptance, and encouragement. To my parents, Bonnie Shipp and Dr. Paul Shipp, thank you for epitomizing the value of education with your own academic achievements, and for your constant efforts to teach us. Dad, as a kid I always admired your master's and doctoral theses and enjoyed flipping through the unintelligible pages of mechanical engineering. Now I, too, can bore future generations with technical tedium! I hope you're proud of what you've spawned. Mom, I doubt anyone could have wrangled and guided a monstrous little mischief-maker better than you. (I was just testing the child education skills you acquired in graduate school.) It is because of your patience and love that my energy was channeled for good, and I am more grateful than I can say. You have both been an incredible example by living a life with integrity, hard work, persistence, and commitment to each other and our family. I have always been proud to be your daughter and could not be where I am today without you. To my wonderful siblings and siblings-in-law, Hayden Shipp, Cat Auer, Kristen Shipp-Anderson, and RJ Anderson; thank you for your humor, love, and understanding over the years. And thanks for always being insanely enthusiastic about seeing the lab and holding sea urchins. You warmhearted goofballs are all what's in my core.

Finally, to my husband (and proverbial binding partner) Reggie Robertson. From our fated 2004 meeting in the Cal training room until this day, I have been

in awe of your kindness, confidence, intelligence, and strength. I came to rely on these especially throughout graduate school. Thank you for patiently listening to my frustrations, for helping me work through decisions, for being an excellent practice audience for too many presentations, and for feeding me. Seriously, thank you so much for feeding me. You make me better, and you made this dissertation better by being more supportive than I ever deserved. You've earned this degree as much as I have.

Chapter 2, in full, is a reprint of Shipp, L.E. and Hamdoun A. ATP-binding cassette (ABC) transporter expression and localization in sea urchin development. *Developmental Dynamics* 2012, 241 (6): 1111-1124. It has been reformatted for this dissertation. The dissertation author was the primary investigator and author of this paper.

Chapter 3, in full, is in review at *Development* and was authored by Shipp L.E., Hill R.Z., Moy G, Gokirmak T, and Hamdoun A. It has been reformatted for this dissertation. The dissertation author was the primary investigator and author of this paper.

Figure 4.1 and legend are published in Gökırmak T, Shipp LE, Campanale JP, Nicklisch SC, Hamdoun A. Transport in technicolor: Mapping ATP-binding cassette transporters in sea urchin embryos. *Molecular Reproduction and Development*. 2014 Sep; 81 (9): 778-793. The dissertation author made the figure and legend.

VITA

EDUCATION

- 2006 Bachelor of Arts in Integrative Biology. Minor in English. Integrative Biology, University of California, Berkeley (UC Berkeley).
- 2012 Master of Science in Oceanography. Scripps Institution of Oceanography, University of California, San Diego (UC San Diego).
- 2015 Doctor of Philosophy in Marine Biology. Scripps Institution of Oceanography, University of California, San Diego (UC San Diego).

PUBLICATIONS

8. **Shipp LE**, Hill RZ, Moy GW, Gökırmak T, Hamdoun A. ABCC5 is required for cAMP-mediated hindgut invagination in sea urchin embryos. *Development*. In review.
7. *Gökırmak T, **Shipp LE**, Campanale JP, Nicklisch SC, Hamdoun A. Transport in technicolor: Mapping ATP-binding cassette transporters in sea urchin embryos. *Molecular Reproduction and Development*. 2014 Sep; 81 (9): 778-793. *Cover Article
6. Gökırmak T, Campanale JP, **Shipp LE**, Moy GW, Tao H, Hamdoun A. Localization and substrate selectivity of sea urchin multidrug (MDR) efflux transporters. *Journal of Biological Chemistry*. 2012 Dec; 287 (52): 43876-83.
5. **Shipp LE**, Hamdoun A. ATP-binding cassette (ABC) transporter expression and localization in sea urchin development. *Developmental Dynamics*. 2012 Jun; 241 (6); 1111-1124.
4. **Shipp LE**, Lee JV, Yu CY, Pufall M, Zhang P, Scott DK, Wang JC. Transcriptional regulation of human dual specificity protein phosphatase 1 (DUSP1) gene by glucocorticoids. *PLoS One*. 2010 Oct 29; 5 (10): e13754.
3. Roohk DJ, Varady KA, Turner SM, Emson CL, Gelling RW, Shankaran M, Lindwall G, **Shipp LE**, Scanlan TS, Wang JC, Hellerstein MK. Differential in vivo effects on target pathways of a novel arylpyrazole glucocorticoid receptor modulator compared with prednisolone. *Journal of Pharmacology and Experimental Therapeutics*. 2010 Apr; 333 (1): 281-289.
2. Koliwad SK, Kuo T, **Shipp LE**, Gray NE, Backhed F, So AY, Farese RV Jr, Wang JC. Angiopoietin-like 4 (ANGPTL4, fasting-induced adipose factor) is a direct glucocorticoid receptor target and participates in glucocorticoid-

regulated triglyceride metabolism. *Journal of Biological Chemistry*. 2009 Sep 18; 284 (38): 25593-601.

1. Patek SN, **Shipp LE**, Staaterman E. The acoustics and acoustic behavior of the California spiny lobster (*Panulirus interruptus*). *Journal of the Acoustical Society of America*. 2009 May; 125 (5): 3434-43.

FELLOWSHIPS, GRANTS, & SCHOLARSHIPS

- 2014-2015 Philanthropic Educational Organization (P.E.O.) Scholarship.
2013-2015 National Science Foundation Graduate Research Fellowship (NSF GRF); awarded 2010, reserved 2010-11, declined 2012.
2012-2015 Achievement Rewards for College Scientists (ARCS) Scholarship.
2010-2013 National Defense Science & Engineering Graduate (NDSEG) Fellowship.
2006 Undergraduate Research Apprenticeship Summer Fellowship.
2001-2006 Full Athletic Scholarship, NCAA Division I Women's Gymnastics, UC Berkeley.

OTHER AWARDS & HONORS

- 2010 Equal Opportunity/Affirmative Action & Diversity Award, UC San Diego.
2006 Rhodes Scholarship Nominee.
2005 NCAA Academic All-American.
2005 Dean's Honor List, UC Berkeley.

RESEARCH EXPERIENCE

- 2009-2015 Graduate Student Researcher. Scripps Institution of Oceanography (SIO), UC San Diego.
Advisor: Amro Hamdoun, Ph.D.
2006-2009 Staff Research Associate & Laboratory Manager. Department of Nutritional Science & Toxicology, UC Berkeley.
Principal Investigator: Jen-Chywan (Wally) Wang, Ph.D.
2005-2006 Undergraduate Student Researcher. Department of Integrative Biology, UC Berkeley.
Advisor: Sheila Patek, Ph.D.

PRESENTATIONS & PUBLISHED ABSTRACTS

- **Shipp LE**, Hill RZ, Moy GW, Gökırmak T, Tresguerres M, Hamdoun A. ABCC5 is required for cAMP-mediated hindgut invagination in sea urchin embryos. Society for Developmental Biology, National Meeting. Snowbird, UT, USA. 2015 Jul. Poster. ****Best Student Presentation Award , 3rd place.**
- **Shipp LE**, Hill RZ, Moy GW, Gökırmak T, Hamdoun A. The ATP-binding cassette transporter ABCC5a in pigment cells is required for sea urchin gastrulation. Society for Developmental Biology, West Coast Meeting. Yosemite, CA, USA. 2015 Mar. Oral presentation. ****Best Student Talk Award.**
- **Shipp LE**, Hill RZ, Moy GW, Gökırmak T, Hamdoun A. Expression of the ATP-binding cassette transporter Sp-ABCC5a in pigment cells is required for sea urchin gastrulation. AAAS Pacific Division. Riverside, CA, USA. 2014 Jun. Oral presentation. ****Best Student Talk Award.**
- Shipp LE**, Hill RZ, Moy GW, Gökırmak T, Hamdoun A. Expression of the ATP-Binding Cassette transporter *Sp*-ABCC5a in pigment cells is required for sea urchin gastrulation. Developmental Biology of the Sea Urchin XXII. Woods Hole, MA, USA. 2014 Apr. Poster.
- Shipp LE**, Moy GW, Hamdoun A. The potential role of ABC transporters in glycosaminoglycan efflux during sea urchin development. Glycobiology Symposium. San Diego, CA, USA. 2013 Jan. Oral presentation.
- Shipp LE**, Moy GW, Gökırmak T, Hamdoun A. Spatial and temporal profiling of ATP-binding cassette (ABC) transporters reveals a role for ABCC5a in archenteron fusion. Developmental Biology of the Sea Urchin XXI. Woods Hole, MA, USA. 2012 Oct. Oral presentation.
- Shipp LE**, Hamdoun A. Developmental expression of ATP-binding cassette (ABC) transporters in sea urchins. FEBS-ABC2012. Innsbruck, Austria. 2012 Mar. Poster.
- Shipp LE**, Gökırmak T, Hamdoun A. Expression of ATP-binding cassette transporters in sea urchins. Pollution Responses in Marine Organisms. Long Beach, CA, USA. 2011 May. Poster.
- Shipp LE**, Patek SN. Two sides to sound production: behavior and pulse patterns in spiny lobsters. Society of Integrative & Comparative Biology. Phoenix, AZ, USA. 2007 Jan. Poster.

TEACHING, MENTORING, & CURRICULUM DEVELOPMENT

- 2011-2015 Faculty Instructor, *Fertilization and Early Development*. Oncofertility Science Academy High School program. UC San Diego.
- 2015 Educator for Birch Aquarium Eggstravaganza event. SIO, UC San Diego.

- 2014 Adjunct Faculty Lecturer, *Introduction to Cellular Processes*, BIOL 225. Department of Biology. University of San Diego. (*sole instructor of record*)
- 2011-2014 Mentor for undergraduate students Rose Hill & Lisa Mesrop. UC San Diego.
- 2013 Teaching Assistant, *Pollution, Health, and Environment*, SIO 189/289. UC San Diego.
Course Professor: Amro Hamdoun, Ph.D.
- 2010-14 Laboratory Instructor, *Biology*. Steele Canyon High School, Spring Valley, CA; High Tech Middle School, Chula Vista, CA; Helix High School, La Mesa, CA; High Tech High School, Point Loma, San Diego, CA.
- 2010,12 Mentor for high school students through Focus on the Future Summer program. UC San Diego.
- 2012 Curriculum designer for Marine Biology undergraduate major at SIO. UC San Diego.
Faculty organizer: Bianca Brahamsha, Ph.D.
Title of lab created: Sea urchin fertilization & embryonic protection
- 2011 Mentor for undergraduate student Saloni Mehta through Summer Undergraduate Research Fellowship (SURF) program. UC San Diego.
- 2006-2009 Mentor for undergraduate students Jason Chan, Olivia Dong, Lily Lam, Joyce Lee, Michelle Lew, Cam Phu, Sandy Sun, Joanna Tran, David Wei, and Mei Wu. UC Berkeley.
- 2006-2007 English as a Second Language teaching volunteer. East Bay Sanctuary, Berkeley, CA.

UNDERGRADUATE NCAA DIVISION I ATHLETIC HONORS

- 2001-2005 Scholarship Athlete on UC Berkeley Women's Gymnastics Team
- 2005-present All-Time School Record Holder on the Vault
- 2005 NCAA Strength & Conditioning All-American
- 2002-5 Letter Winner
- 2004-5 Women's Gymnastics Endowment Recipient
- 2004-5 Team Captain
- 2003,5 Pac-10 All-Academic Honors
- 2004 Voted Most Improved Gymnast

ABSTRACT OF THE DISSERTATION

The role of the ATP-binding cassette (ABC) transporter ABCC5a in hindgut invagination of sea urchin embryos

by

Lauren Elizabeth Shipp

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2015

Professor Amro Hamdoun, Chair

ATP-binding cassette (ABC) transporters are large membrane proteins found in all organisms. They use ATP to power transport of diverse substrates across membranes, and the ABC-B, -C, and -G families make up the multidrug resistance (MDR) transporters. Here, I survey the expression of MDR transporters during development of the purple sea urchin, *Strongylocentrotus purpuratus*, and identify and characterize one transporter, *Sp-ABCC5a* (C5a) that has a role in developmental signaling required for hindgut morphogenesis.

From the unfertilized egg through the prism stage (~58 hours post-fertilization, hpf), I detected transcripts of 20 MDR transporters with predicted roles in cell signaling, lysosomal and mitochondrial homeostasis, potassium channel regulation, pigmentation, and xenobiotic efflux. The protective

transporter ABCB1a is ubiquitously expressed throughout embryogenesis in all cells, and the protein localizes to apical membranes at the interface between the embryo and external environment. In contrast, expression of C5a, a previously uncharacterized transporter, is first detected at hatching and peaks after gastrulation, and the protein localizes to basolateral membranes. I propose that profiling spatiotemporal expression and subcellular protein localization may identify transporters with roles in protection, homeostasis, and signaling.

As expected, efflux activity of C5a does not support a role in protection; C5a is not a broad-spectrum chemical effluxor, and it discriminates between structurally similar compounds. C5a expression is restricted to aboral non-skeletogenic mesoderm (NSM) cells (pigment cells) and is dependent on delta/notch signaling from the skeletogenic mesoderm (SM) as well as the aboral NSM regulatory gene, *glial cells missing*. During gastrulation, C5a protein traffics to the plasma membrane of pigment cells, while these cells migrate away from the archenteron to embed in the aboral ectoderm.

Knockdown of C5a leads to embryos with differentiated pigment cells but abnormal protrusion of the hindgut, which is seen in ~90% of embryos by the late prism stage (~eight hours after C5a protein expression peaks). The ABCC5 substrate cAMP rescues the prolapse in a dose-dependent manner, and in control embryos causes hyper-invagination. The cAMP-producing enzyme soluble adenylyl cyclase (sAC) is expressed in tandem with C5a, with peak expression in pigment cells just after gastrulation. Blocking sAC activity with the

specific inhibitor KH7 impairs gastrulation. Together these data suggest that C5a-mediated efflux of sAC-derived cAMP from pigment cells controls late invagination of the hindgut. This study elucidates a novel role for an ABCC/MRP transporter in mesoderm-endoderm signaling during embryogenesis.

CHAPTER 1.
INTRODUCTION AND BACKGROUND

1.1. INTRODUCTION

This dissertation began as a quest to identify the membrane proteins responsible for protecting embryos from harmful environmental chemicals. However, what emerged was the interesting story of a previously uncharacterized ATP-binding cassette (ABC) transporter, ABCC5a, and its unexpected role in developmental signaling. When this work began in 2009, the multidrug resistance (MDR) ABC transporters were thought to primarily function in protecting cells from harmful chemicals (e.g. chemotherapeutics and mercury). That same year brought the publication of the first major study showing a developmental role for an MDR transporter (MDR49 in *Drosophila*) (Ricardo and Lehmann, 2009). With the possibility of developmental cell signaling in mind, I focused on the role of ABCC5a in sea urchin embryogenesis.

ABC transporters are found in all organisms, and members of the ABC-B, -C, and -G subfamilies (the MDR transporters) are well-known to efflux exogenous chemicals and toxicants from cells (Dean et al., 2001). The sea urchin, *Strongylocentrotus purpuratus*, has an expansion of MDR transporters in its genome, with 82 gene models (Gökirmak et al., 2014) compared to 49 in humans (Barbet et al., 2012). These transporters are extensively expressed throughout embryogenesis (Goldstone et al., 2006; Shipp and Hamdoun, 2012). This, in addition to technical tractability, makes *S. purpuratus* an ideal organism in which to study MDR transporters.

A growing body of research is showing that ABC transporters play prominent roles in development. For example, *Drosophila melanogaster* MDR49 (an ABCB transporter) effluxes a chemoattractant from the somatic gonad that directs germ cell migration to the somatic gonad (Ricardo and Lehmann, 2009). Additionally, ABC transporters are overexpressed in many stem cells compared to their differentiated descendants (Dean et al., 2005). This overexpression can contribute to multipotency, as is the case with ABCG2 in hematopoietic stem cells (Bunting, 2002; Zhou et al., 2001). In other cases, overexpression of ABC transporters can promote differentiation (Raaijmakers, 2007). Endogenous substrates of transporters include cyclic nucleotides, leukotrienes, and prostaglandins (Fletcher et al., 2010). These compounds function in chemoattraction (Dormann and Weijer, 2006; Yokomizo et al., 1997), cell signaling (Funk, 2001; Lucas et al., 2000), developmental signaling (Moroki et al., 2000; Yamamoto et al., 2003), and stem cell biology (Möhle and Drost, 2012). However, direct links between specific ABC transporters, their substrates and regulation, and downstream physiological and/or developmental events remain to be comprehensively studied.

Like many multidrug resistance proteins (MRPs), human ABCC5 (MRP5, MOAT-C, pABC11, sMRP) has been the subject of many studies in drug resistance (Belinsky et al., 1998; Chen and Tiwari, 2011; Kool et al., 1997; McAleer et al., 1999). But unlike many MRPs, ABCC5 has yet to be demonstrated as toxicologically important (Chen and Tiwari, 2011; Leslie et al.,

2001). Expression, regulation, and activity of the ABCC5 protein are not well characterized, and its function remains poorly understood. ABCC5 has been reported to efflux cGMP and cAMP (Jedlitschky et al., 2000; Sager and Ravna, 2009; Wielinga et al., 2003), heme (Korolnek et al., 2014), hyaluronan (Schulz et al., 2007), and *N*-lactoyl-amino acids (Jansen et al., 2015). However, its exact affinity for cGMP, its most studied endogenous substrate, remains unclear (de Wolf et al., 2007; Pratt et al., 2005; Reid et al., 2003; Wielinga et al., 2003), and there are conflicting findings about its potential drug substrates. Finally, ABCC5 inhibitors are nonspecific and minimally effective compared to their effects on related ABC transporters (Borst et al., 2007). Overall, ABCC5 has proved to be a challenging transporter to characterize, and substantial room exists for broadening knowledge of its expression, structure, function, substrates, and regulation in any organism. To date, no one has attempted to systematically study ABCC5 in a developmental system. Here, I use the sea urchin embryo to broaden our knowledge of this elusive transporter.

Chapter 2 consists of a gene expression screen of MDR transporters during embryogenesis. I survey expression of 20 genes throughout the first 58 hours of development and bin the expression profiles into three categories: (1) expressed throughout development with a decrease at hatching (~21 hpf), (2) rapidly expressed only after a specific developmental stage, and (3) expressed increasingly throughout development (0-58 hpf). Group 1 transporters include the known protective gene *Sp-ABCB1a* (B1a), and Group 2 transporters, which

exhibit the tightest temporal regulation reminiscent of developmentally important genes, include the uncharacterized transporter gene *Sp-ABCC5a* (*C5a*). *In situ* hybridization and fluorescent-tagged over-expressions of these two transporters reveal that *B1a* is expressed in all cells of the embryo, and the protein localizes to apical membranes at the embryo/external environment interface. In contrast, *C5a* transcripts are detected only in a subset of cells likely to be non-skeletogenic mesoderm, and the protein localizes to basolateral membranes between cells. From these findings, I hypothesized that the tight temporal and spatial regulation of the *C5a* gene, as well as the localization of the protein at the interface between cells, suggest a role in developmental signaling rather than cellular protection.

In Chapter 3, I experimentally develop this hypothesis and investigate the role of *C5a* in embryogenesis, ultimately finding that it mediates cAMP signaling to direct invagination of the hindgut. I find that *C5a* is expressed in pigment cells, the aboral subset of non-skeletal mesenchyme (NSM) cells, and the gene is activated downstream of both delta/notch signaling from the SM, and *gcm*. *C5a* protein levels peak just after gastrulation, and the transporter localizes to the plasma membrane of migrating pigment cells. Knockdown of *C5a* causes the hindgut to abnormally protrude into a “prolapse”, and this phenotype is rescued by cAMP, a substrate of mammalian ABCC5. The cAMP-producing enzyme soluble adenylyl cyclase (sAC) is expressed in parallel with *C5a*, in pigment cells with transcript levels peaking after gastrulation. Blocking sAC activity with the specific inhibitor KH7 causes defects in gut formation. Together, these data

support a model in which C5a transports sAC-derived cAMP from pigment cells, which directs invagination of the hindgut.

The major finding of this dissertation research is a novel developmental role for an ABC transporter. As 2009 marked the first seminal study of a transporter with a developmental function (Ricardo and Lehmann, 2009), this dissertation adds to the growing body of evidence showing that ABC transporters are an underappreciated and understudied group of proteins involved in cellular signaling in embryos. Further work on ABC transporters in embryogenesis will broaden our understanding of how cells secrete directive signals during morphogenesis.

1.2. BACKGROUND

1.2.1. ABCC5/MRP5 TRANSPORTERS

ABCC5 has been the subject of many studies, but its substrates and physiological roles remain debated. Here I will summarize the existing literature on ABCC5/MRP5 transporters.

1.2.1.1. Expression

Ubiquitous tissue expression of the *ABCC5* gene, coupled with protein detection in all tissues tested to date, suggests the transporter is likely to be widely used in multicellular organisms. Human ABCC5 mRNA is widely expressed in adult tissues (Belinsky et al., 1998; Kool et al., 1997), with highest expression levels in skeletal muscle, brain (Belinsky et al., 1998; Kool et al., 1997), heart, kidney, and testis (Belinsky et al., 1998). *Hs-ABCC5a* is expressed in pre-term and term placentas, and its mRNA levels decrease with increasing gestational age (Meyer Zu Schwabedissen et al., 2005). In zebrafish, *Dr-ABCC5* is expressed throughout the embryo, with highest expression in the developing nervous system (Korolnek et al., 2014).

No studies have comprehensively addressed the tissue distribution of the ABCC5 protein, and several groups disagree about the quality of available expression data generated with different antibodies (Borst et al., 2007; Schinkel and Jonker, 2012). The polyclonal AMF antibody (raised in rabbits, targeting 14

C-terminal amino acids) showed ABCC5 protein expression in muscle cells of the corpus cavernosum, bladder, and ureter with specific localization in the urothelium and lamina propria mucosae of the ureter and urethra (Nies et al., 2002). Nies et al., 2002 also used a monoclonal rat antibody to ABCC5 (mAb M₅I-1, from Wijnholds et al., 2000) to colocalize ABCC5 with phosphodiesterase 5 in epithelial cells of urothelium and within lamina propria. Their comparisons between the two ABCC5 antibodies suggested AMF had a higher affinity for ABCC5 than M₅I-1 did. Also with AMF antibody, *Hs*-ABCC5 was detected in cultured cytotrophoblasts (placental cells), and expression increased as the cells differentiated into syncytiotrophoblasts (Meyer Zu Schwabedissen et al., 2005). ABCC5 is expressed in human heart in endothelial cells, cardiomyocytes, and smooth muscle cells (AMF antibody) (Dazert et al., 2003). Finally, *Hs*-ABCC5 is expressed in the brain, primarily in the pyramidal neurons, capillary endothelium, and astrocytes (detected with both AMF and M₅I-1 antibodies) (Nies et al., 2004).

ABCC5 knockout mice have been generated, though no detailed characterization of their physiology, reproductive capacity, or development has been published. Nonetheless, they are reportedly healthy and fertile (de Wolf et al., 2007; Wijnholds et al., 2000), which has called into question the physiological necessity of ABCC5. It has been suggested that ABCC4, the most closely related transporter to ABCC5 (36% amino acid identity), could be upregulated in these mice, which could rescue the effects of eliminating ABCC5 (Adachi et al., 2002). There is precedent for this type of compensatory ABC transporter expression, as

the ABCB1 knockout mouse upregulates expression of *ABCB1b* (Schinkel et al., 1994).

1.2.1.2. Localization

ABCC5 is primarily found on basolateral plasma membranes, although apical and intracellular localizations have also been reported. *Hs*-ABCC5 is primarily detected on basolateral plasma membranes in polarized cells (Borst et al., 2007; Korolnek et al., 2014). A stable line of HEK293 (non-polarized human embryonic kidney) cells expressing an eGFP-ABCC5 fusion protein showed the fluorescent protein was located on the plasma membrane (McAleer et al., 1999). In MDCKII (polarized epithelial) cells transduced to overexpress ABCC5, the protein was detected with mAb M₅I-1 on basolateral membranes (Wijnholds et al., 2000). This study showed that using the same antibody and overexpression protocol in non-polarized HEK293 cells, ABCC5 was predominantly intracellular, with only slight localization on the plasma membrane (Wijnholds et al., 2000). This contradicts the findings of McAleer et al., 1999, though the two studies' methods of protein visualization differ. In human placenta, AMF antibody was used to detect ABCC5 on basal membranes of syncytiotrophoblasts (Meyer Zu Schwabedissen et al., 2005). AMF antibody also showed that *Hs*-ABCC5a resides on the luminal (apical) side of brain capillary endothelial cells (Nies et al., 2004). Interestingly, ABCC5 was immunolocalized to both apical and basolateral surfaces of human amniotic epithelium (M₅I-1) (Aye et al., 2007).

1.2.1.3. Gene splicing and paralogs

Organization of ABCC5-type genes in the human and *S. purpuratus* genomes suggest that alternate splicing and/or paralog abundance may be factors in its regulation. The gene structure of *Hs-ABCC5* created some confusion in initial characterizations, and later it was determined that sMRP (short MRP) (Suzuki et al., 1997) was actually an N-terminally truncated variant of ABCC5 (Suzuki et al., 2000). mRNA of sMRP was detected in similar patterns to that of full-length ABCC5, suggesting the short splice variant has a physiological function (Suzuki et al., 2000). Three more isoforms of *ABCC5* were characterized in human retina (Stojic et al., 2007), suggesting that regulated unproductive splicing and translation (RUST) (Lewis et al., 2003) is likely a mechanism of controlling tissue-specific ABCC5 expression (Stojic et al., 2007). Splice variation could be a control mechanism for sea urchin ABCC5 expression, and additionally in *S. purpuratus*, expression of ABCC5 transporters is influenced by gene duplication. This produced 12 *ABCC5* gene paralogs with >80% nucleotide sequence similarity, representing the most abundant group of ABC transporters in the genome (Sea Urchin Genome Sequencing Consortium et al., 2006).

1.2.1.4. Structure

Based on homology modeling, the protein structure of C5a is predicted to be similar to that of *Hs-ABCC5*, which represents the most common topology of human ABC transporters (Dean et al., 2001). On western blots, *Hs-ABCC5* is detected at a range of molecular masses; 160-180 kDa (Wijnholds et al., 2000), 185 kDa (Jedlitschky et al., 2000), 220 kDa (eGFP-tagged) (McAleer et al., 1999), 160-200 kDa (Nies et al., 2002), and 200 kDa (Aye et al., 2007). This range of apparent molecular masses is likely due to tissue-specific differences in glycosylation (Nies et al., 2002).

Although no crystal structure has been published for *Hs-ABCC5*, robust molecular models exist (Ravna et al., 2008; Sager et al., 2012). *ABCC5* has two membrane spanning domains (MSDs), each composed of six transmembrane helices (TMHs) connected by both extracellular loops (ELs) and cytoplasmic loops (CLs). Two intracellular nucleotide binding domains (NBDs) contain canonical Walker A, Walker B, and Walker C domains, and these NBDs are the sites of ATP hydrolysis, which provides energy for the conformational change that translocates substrates across membranes. *ABCC5* lacks the N-terminal MSD0 characteristic of other *ABCC* transporters (such as *ABCC1/MRP1*) and is instead similar in structure to *ABCB1* (P-glycoprotein, P-gp), a well-characterized multidrug effluxor that has few if any overlapping substrates with *ABCC5* (Fletcher et al., 2010). Little of the protein is in the extracellular domain, as both the N- and C-terminal ends are intracellular.

The two ligand binding pockets (LBPs, a.k.a. drug binding pockets) of *Hs-ABCC5* are composed of (LBP1) TMHs 1, 2, 11, and 12, and (LBP2) TMHs 5, 6, 7, and 8. These LBPs each have a positively charged amino acid, Arg232 (LBP1) and Lys448 (LBP2), that are possible recognition points for anionic substrates (Ravna et al., 2008).

1.2.1.5. Substrates and inhibitors

1.2.1.5.1. Exogenous substrates

Many chemical substrates have been suggested for *ABCC5*, but their affinities and biological relevance remain in question. *ABCC5* may (McAleer et al., 1999) or may not (Wijnholds et al., 2000) confer resistance to cadmium chloride, as it may (Davidson et al., 2002) or may not (Reid et al., 2003) efflux the anticancer drugs cytarabine and gemcitabine. 5'-fluorouracil (5-FU) is another reported substrate (Nambaru et al., 2011; Pratt et al., 2005) that was also shown not to be effluxed by *ABCC5* (Reid et al., 2003; Wijnholds et al., 2000). *ABCC5*, along with *ABCC4*, is unique among other MRPs in that it may transport nucleoside monophosphate analogs such as the antiviral compound PMEA (9-(2-phosphonylmethoxyethyl)adenine) (Ritter et al., 2005; Wijnholds et al., 2000). Overall, the drug/chemical efflux capacities of *ABCC5* remain in question, and this transporter has not been shown to be toxicologically relevant (Chen and Tiwari, 2011; Leslie et al., 2001).

1.2.1.5.2. Endogenous substrates

1.2.1.5.2.1. Cyclic nucleotides

ABCC5 has been reported to efflux cGMP (Boadu and Sager, 2004; Jedlitschky et al., 2000; Meyer Zu Schwabedissen et al., 2005; Sager and Ravna, 2009; Sager et al., 2012; Wielinga et al., 2003; Wijnholds et al., 2000), cAMP (Jedlitschky et al., 2000; Sager and Ravna, 2009; Wielinga et al., 2003), heme (Korolnek et al., 2014), hyaluronan (Schulz et al., 2007), and *N*-lactoyl amino acids (Jansen et al., 2015).

Through transport of cAMP, ABCC5 may elevate extracellular cAMP that likely functions as a signal received via GPCRs to elicit cellular responses in receiving cells (Godinho et al., 2015). In *Dictyostelium* development, ABCB3 effluxes cAMP, and ABCC5 is also a candidate cAMP transporter (Miranda et al., 2015). This cAMP excreted by ABC transporters specifies cell fate and stimulates chemotaxis (Miranda et al., 2015). Extracellular cAMP causes pseudopod extension at the leading edge (Dormann and Weijer, 2006), and stimulates elevation of intracellular cGMP to drive myosin-mediated contraction of the lagging edge (Bosgraaf et al., 2002; Veltman and Van Haastert, 2008). In this way, cAMP and cGMP synergize to produce directional movement. In axonal growth as well, cAMP and cGMP pathways likely interact to fine-tune cyclic nucleotide mediated migration (Song and Poo, 1999), and the ratio of cAMP/cGMP influences migration (Nishiyama et al., 2003; Song et al., 1998) by directing neuronal growth cones.

cGMP transport by ABCC5 affects several cellular processes, though its exact affinity remains disputed (Pratt et al., 2005; Reid et al., 2003; Wielinga et al., 2003). ABCC5 was reported to have a cGMP K_m of 2.1 μM (Jedlitschky et al., 2000), a value that was supported by several subsequent studies (Boadu and Sager, 2004; Meyer Zu Schwabedissen et al., 2005; Sager et al., 2012). However, other labs observed only mM affinity (Pratt et al., 2005; Reid et al., 2003; Wielinga et al., 2003). Notably, the methods used by labs measuring μM affinities differ from those of labs measuring mM affinities.

Showing biologically relevant cGMP affinity, Jedlitschky et al., 2000 used inside-out vesicles from V79 (Chinese hamster lung fibroblasts) transfected to overexpress ABCC5, and they measured intracellular ATP-dependent [^3H]cGMP accumulation. They found ATP-dependent transport was 9.3 pmol/mg protein, which was comparable to measurements from a similar experimental setup with inside-out trophoblastic vesicles from basal membranes expressing ABCC5 (Meyer Zu Schwabedissen et al., 2005). cGMP transport across erythrocyte membranes was reduced by 45% when ABCC5 was immunoprecipitated out of the solubilized extract (Boadu and Sager, 2004). Additionally, in GH₃ pituitary cells, cGMP efflux was associated with *ABCC5* expression, and knockdown of *ABCC5* decreased cGMP efflux by >30% (Andric et al., 2006).

Showing that cGMP is not a high-affinity substrate of ABCC5, Wielinga et al., 2003 used intact HEK293 cells transfected to overexpress ABCC5, and they showed that cGMP was only effluxed when cells were induced to produce very

high intracellular cGMP concentrations (~0.6 mM), suggesting cGMP is only a low affinity substrate of ABCC5. Additionally, studies that used cGMP to inhibit transport of other compounds found that half-maximal inhibition required ~1mM concentrations of cGMP (Pratt et al., 2005; Reid et al., 2003). Such concentrations are likely too high to be physiologically relevant.

Physiological importance of a cGMP transporter is supported by Nies et al., 2002, who suggested that expression of ABCC5 protein in the human genitourinary system, where ABCC5 colocalizes with phosphodiesterase 5, may work in concert with phosphodiesterase 5 to reduce intracellular cGMP levels in smooth muscle. Additionally, ABCC5 expression is reduced in murine models of septic shock, suggesting that disruption of the normal cGMP-NO pathway could participate in heart failure from sepsis (Meissner et al., 2007). Finally, it has been suggested that ABCC5-mediated export of cGMP could generate extracellular pools of cyclic nucleotide that may function in paracrine signaling (Ritter et al., 2005).

1.2.1.5.2.2. Heme

A recent study suggested heme as a substrate (Korolnek et al., 2014), and knockdown of ABCC5 in the heme auxotroph *Caenorhabditis elegans* caused embryonic lethality. The same study showed that ABCC5 knockdown in *Danio rerio* caused defects in erythropoiesis, also suggesting that ABCC5 participates in heme homeostasis. Interestingly, another study in *D. rerio* reported that

ABCC5 is up-regulated when heme synthesis is perturbed by knockdown of *klf4*, a transcription factor necessary for hematopoiesis (Gardiner et al., 2007).

1.2.1.5.2.3. Hyaluronan

ABCC5 has also been shown to transport hyaluronan (Schulz et al., 2007), a glycosaminoglycan (GAG) component of the extracellular matrix (ECM) that has both structural and cell signaling functions (Toole, 2004). Efflux of hyaluronan was measured from fibroblasts, and siRNA-mediated knock-down of *ABCC5* protein produced a dose-dependent reduction in hyaluronan efflux. Schulz et al., 2007 also showed that cGMP inhibited hyaluronan transport and concluded that cGMP may be a regulator of *ABCC5*-mediated hyaluronan efflux. This mechanism diverges from the predominant view that hyaluronan synthase (HAS), a membrane bound enzyme, itself mediates translocation of the growing hyaluronan polymer into the extracellular domain as it is being synthesized (Medina et al., 2012). Medina et al., 2012 provided further support for the latter mechanism, demonstrating evidence of an intraprotein pore in HAS. However, no direct evidence has contradicted the possibility that *ABCC5* transports hyaluronan.

1.2.1.5.2.4. N-lactoyl amino acids

Just this year, a metabolomics screen identified *N*-lactoyl amino acids as substrates of ABCC5 (Jansen et al., 2015). These metabolites are unfortunately not characterized, so the physiological effects of this transport remain unknown.

1.2.1.5.3. Inhibitors

Specific inhibitors of ABCC5 have been difficult to identify (Borst et al., 2007; Ritter et al., 2005). Inhibition of ABCC5 may be achieved with high concentrations of the nonspecific organic anion transport inhibitors benzebromarone, probenecid, sulfapyrazone, and MK571 (Borst et al., 2007). The phosphodiesterase inhibitors sildenafil (aka Viagra) and trequinsin may also inhibit ABCC5 due to their cGMP-like shape and binding ability (Ritter et al., 2005). Recently, seven sildenafil-analog compounds were identified with higher inhibitory activity than sildenafil (Sager et al., 2012), but they have not yet been tested in follow-up studies.

1.2.2. SEA URCHIN DEVELOPMENT

The bilaterally symmetric sea urchin embryo develops indirectly into a radially symmetric adult. Yet the early development of the sea urchin, a basal deuterostome, closely resembles that of chordates. Indeed nearly all vertebrate gene families are represented in the ~23,300 gene genome of *S. purpuratus* (Sea Urchin Genome Sequencing Consortium et al., 2006), and many of these genes' functions are thought to be conserved.

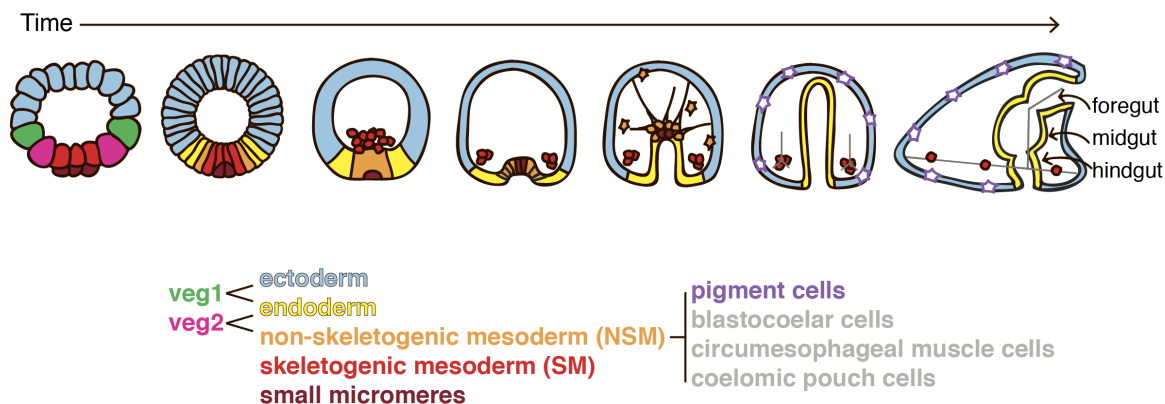


Figure 1.1. Development of the sea urchin from the 60-cell stage (~10 hpf) to the pluteus larva stage (~72 hpf). Cell and tissue specification is color-coded.

Early cleavage gives rise to a 60-cell embryo (~10 hpf in *S. purpuratus*) in which six cell types are specified, including the veg1 and veg2 cells (Fig. 1.1). Veg1 cells will become a portion of both the ectoderm and endoderm, while veg2 cells will make up the rest of the endoderm and the entire non-skeletogenic mesoderm (NSM) (Peter and Davidson, 2009). Part of the endoderm, which forms the gut during gastrulation, is specified from veg2 during Wnt-controlled endomesoderm differentiation (Wikramanayake et al., 2004). The remaining veg2-derived cells, the NSM, are the second group of cells (preceded by skeletogenic mesoderm (SM)) to ingress and transition from epithelium to mesenchyme. The NSM differentiates into four cell types: pigment cells, blastocoelar cells, coelomic pouch cells, and circumesophageal muscle (Cameron et al., 1991; Ruffins and Etensohn, 1996). These cells are important for gastrulation, neurogenesis, and immunity.

Gastrulation occurs in two phases: primary invagination and secondary invagination. Primary invagination involves actin rearrangement and formation of bottle cells (which may be pigment cells (Takata and Kominami, 2004)), causing the vegetal pole of the embryo to fold inward and create the early archenteron (gut) (Burke et al., 1991; Davidson et al., 1995; Kimberly and Hardin, 1998; Kominami and Takata, 2004; Lane et al., 1993; Nakajima and Burke, 1996). Pigment cells ingress from the vegetal plate during early gastrulation and continue to disperse once they are in the ectoderm (Gibson and Burke, 1987).

Secondary invagination results from pseudopodia extension, cell rearrangement, regionalized cell division, and cell recruitment (Hardin, 1989; Kominami and Takata, 2004). Directionality of archenteron movement depends on filopodia-mediated recognition of the animal pole, where the stomodeum (mouth) will form (Hardin and McClay, 1990). This is dependent on oral-aboral axis specification, which is patterned by Nodal signaling (Wei et al., 2012).

Following gastrulation, the ventral (oral) ectoderm of the prism stage embryo invaginates to produce the stomodeum. An oral hood is formed on the ventral ectoderm, and the archenteron and stomodeum fuse to make the mouth. The gut differentiates into a tripartite larval digestive system, composed of the muscular esophagus (foregut), large stomach (midgut), and tubular intestine (hindgut). The foregut and oral midgut are *veg2*-derived, while the aboral midgut and hindgut are *veg1* descendants (Logan and McClay, 1997; Ransick and Davidson, 1998). The regulation and development of larval gut compartments are

less understood than the earlier events of endoderm specification and gastrulation. However, larval gut morphogenesis is now being explored from a gene regulatory perspective (Annunziata et al., 2014), and its differentiation is governed by ParaHox genes (Cole et al., 2009).

1.3. REFERENCES

- Adachi, M., Reid, G. and Schuetz, J. D.** (2002). Therapeutic and biological importance of getting nucleotides out of cells: a case for the ABC transporters, MRP4 and 5. *Adv Drug Deliv Rev* **54**, 1333–1342.
- Andric, S. A., Kostic, T. S. and Stojilkovic, S. S.** (2006). Contribution of multidrug resistance protein MRP5 in control of cyclic guanosine 5'-monophosphate intracellular signaling in anterior pituitary cells. *Endocrinology* **147**, 3435–3445.
- Annunziata, R., Perillo, M., Andrikou, C., Cole, A. G., Martinez, P. and Arnone, M. I.** (2014). Pattern and process during sea urchin gut morphogenesis: The regulatory landscape. *Genesis* **52**, 251–268.
- Aye, I. L. M. H., Paxton, J. W., Evseenko, D. A. and Keelan, J. A.** (2007). Expression, localisation and activity of ATP binding cassette (ABC) family of drug transporters in human amnion membranes. *Placenta* **28**, 868–877.
- Barbet, R., Peiffer, I., Hutchins, J. R. A., Hatzfeld, A., Garrido, E. and Hatzfeld, J. A.** (2012). Expression of the 49 human ATP binding cassette (ABC) genes in pluripotent embryonic stem cells and in early- and late-stage multipotent mesenchymal stem cells: possible role of ABC plasma membrane transporters in maintaining human stem cell pluripotency. *Cell Cycle* **11**, 1611–1620.
- Belinsky, M. G., Bain, L. J., Balsara, B. B., Testa, J. R. and Kruh, G. D.** (1998). Characterization of MOAT-C and MOAT-D, new members of the MRP/cMOAT subfamily of transporter proteins. *J Natl Cancer Inst* **90**, 1735–1741.
- Boadu and Sager** (2004). Reconstitution of ATP-dependent cGMP transport into proteoliposomes by membrane proteins from human erythrocytes. *Scand J Clin Lab Invest* **64**, 41–48.
- Borst, P., de Wolf, C. and van de Wetering, K.** (2007). Multidrug resistance-associated proteins 3, 4, and 5. *Pflugers Arch* **453**, 661–673.
- Bosgraaf, L., Russcher, H., Smith, J. L., Wessels, D., Soll, D. R. and Van Haastert, P. J. M.** (2002). A novel cGMP signalling pathway mediating myosin phosphorylation and chemotaxis in *Dictyostelium*. *EMBO J* **21**, 4560–4570.
- Bunting, K.** (2002). ABC transporters as phenotypic markers and functional

regulators of stem cells. *Stem Cells* **20**, 11–20.

Burke, R. D., Myers, R. L., Sexton, T. L. and Jackson, C. (1991). Cell movements during the initial phase of gastrulation in the sea urchin embryo. *Dev Biol* **146**, 542–557.

Cameron, R. A., Fraser, S. E., Britten, R. J. and Davidson, E. H. (1991). Macromere cell fates during sea urchin development. *Development* **113**, 1085–1091.

Chen, Z.-S. and Tiwari, A. K. (2011). Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases. *FEBS J* **278**, 3226–3245.

Cole, A. G., Rizzo, F., Martinez, P., Fernandez-Serra, M. and Arnone, M. I. (2009). Two ParaHox genes, SpLox and SpCdx, interact to partition the posterior endoderm in the formation of a functional gut. *Development* **136**, 541–549.

Davidson, J. D., Ma, L., Iverson, P. W., Lesoon, A., Jin, S., Horwitz, L., Gallery, M., and Slapak, C. A. (2002) Human multi-drug resistance protein 5 (MRP5) confers resistance to gemcitabine. *Proc Am Assoc Cancer Res* **43**, 3868.

Davidson, L. A., Koehl, M. A., Keller, R. and Oster, G. F. (1995). How do sea urchins invaginate? Using biomechanics to distinguish between mechanisms of primary invagination. *Development* **121**, 2005–2018.

Dazert, P., Meissner, K., Vogelgesang, S., Heydrich, B., Eckel, L., Böhm, M., Warzok, R., Kerb, R., Brinkmann, U., Schaeffeler, E., et al. (2003). Expression and localization of the multidrug resistance protein 5 (MRP5/ABCC5), a cellular export pump for cyclic nucleotides, in human heart. *Am J Pathol* **163**, 1567–1577.

de Wolf, C. J. F., Yamaguchi, H., van der Heijden, I., Wielinga, P. R., Hundscheid, S. L., Ono, N., Scheffer, G. L., de Haas, M., Schuetz, J. D., Wijnholds, J., et al. (2007). cGMP transport by vesicles from human and mouse erythrocytes. *FEBS J* **274**, 439–450.

Dean, M., Fojo, T. and Bates, S. (2005). Tumour stem cells and drug resistance. *Nat Rev Cancer* **5**, 275–284.

Dean, M., Rzhetsky, A. and Allikmets, R. (2001). The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* **11**, 1156–1166.

- Dormann, D. and Weijer, C. J.** (2006). Chemotactic cell movement during Dictyostelium development and gastrulation. *Curr Opin Genet Dev* **16**, 367–373.
- Fletcher, J. I., Haber, M., Henderson, M. J. and Norris, M. D.** (2010). ABC transporters in cancer: more than just drug efflux pumps. *Nat Rev Cancer* **10**, 147–156.
- Funk, C. D.** (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294**, 1871–1875.
- Gardiner, M. R., Gongora, M. M., Grimmond, S. M. and Perkins, A. C.** (2007). A global role for zebrafish klf4 in embryonic erythropoiesis. *Mech Dev* **124**, 762–774.
- Gibson, A. W. and Burke, R. D.** (1987). Migratory and invasive behavior of pigment cells in normal and animalized sea urchin embryos. *Exp Cell Res* **173**, 546–557.
- Godinho, R. O., Duarte, T. and Pacini, E. S. A.** (2015). New perspectives in signaling mediated by receptors coupled to stimulatory G protein: the emerging significance of cAMP efflux and extracellular cAMP-adenosine pathway. *Front Pharmacol* **6**, 1–9.
- Goldstone, J. V., Hamdoun, A., Cole, B. J., Howard-Ashby, M., Nebert, D. W., Scally, M., Dean, M., Epel, D., Hahn, M. E. and Stegeman, J. J.** (2006). The chemical defensome: environmental sensing and response genes in the *Strongylocentrotus purpuratus* genome. *Dev Biol* **300**, 366–384.
- Gökirmak, T., Shipp, L. E., Campanale, J. P., Nicklisch, S. C. T. and Hamdoun, A.** (2014). Transport in technicolor: mapping ATP-binding cassette transporters in sea urchin embryos. *Mol Reprod Dev* **81**, 778–793.
- Hardin, J.** (1989). Local shifts in position and polarized motility drive cell rearrangement during sea urchin gastrulation. *Dev Biol* **136**, 430–445.
- Hardin, J. and McClay, D. R.** (1990). Target recognition by the archenteron during sea urchin gastrulation. *Dev Biol* **142**, 86–102.
- Jansen, R. S., Addie, R., Merkx, R., Fish, A., Mahakena, S., Bleijerveld, O. B., Altelaar, M., IJlst, L., Wanders, R. J., Borst, P., et al.** (2015). N-lactoyl-amino acids are ubiquitous metabolites that originate from CNBP2-mediated reverse proteolysis of lactate and amino acids. *Proc Natl Acad Sci USA* **201424638–6**.

- Jedlitschky, G., Burchell, B. and Keppler, D.** (2000). The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *J Biol Chem* **275**, 30069–30074.
- Kimberly, E. L. and Hardin, J.** (1998). Bottle cells are required for the initiation of primary invagination in the sea urchin embryo. *Dev Biol* **204**, 235–250.
- Kominami, T. and Takata, H.** (2004). Gastrulation in the sea urchin embryo: A model system for analyzing the morphogenesis of a monolayered epithelium. *Dev Growth Differ* **46**, 309–326.
- Kool, M., de Haas, M., Scheffer, G. L., Scheper, R. J., van Eijk, M. J., Juijn, J. A., Baas, F. and Borst, P.** (1997). Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* **57**, 3537–3547.
- Korolnek, T., Zhang, J., Beardsley, S., Scheffer, G. L. and Hamza, I.** (2014). Control of metazoan heme homeostasis by a conserved multidrug resistance protein. *Cell Metab* **19**, 1008–1019.
- Lane, M. C., Koehl, M. A., Wilt, F. and Keller, R.** (1993). A role for regulated secretion of apical extracellular matrix during epithelial invagination in the sea urchin. *Development* **117**, 1049–1060.
- Leslie, E. M., Deeley, R. G. and Cole, S. P.** (2001). Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. *Toxicology* **167**, 3–23.
- Lewis, B. P., Green, R. E. and Brenner, S. E.** (2003). Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. *Proc Natl Acad Sci USA* **100**, 189–192.
- Logan, C. Y. and McClay, D. R.** (1997). The allocation of early blastomeres to the ectoderm and endoderm is variable in the sea urchin embryo. *Development* **124**, 2213–2223.
- Lucas, K. A., Pitari, G. M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K. P. and Waldman, S. A.** (2000). Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* **52**, 375–414.
- McAlear, M. A., Breen, M. A., White, N. L. and Matthews, N.** (1999). pABC11 (also known as MOAT-C and MRP5), a member of the ABC family of proteins, has anion transporter activity but does not confer multidrug resistance when overexpressed in human embryonic kidney 293 cells. *J Biol*

Chem **274**, 23541–23548.

Medina, A. P., Lin, J. and Weigel, P. H. (2012). Hyaluronan synthase mediates dye translocation across liposomal membranes. *BMC Biochem.* **13**, 2.

Meissner, K., Kessler, W., Meyer Zu Schwabedissen, H. E. U., Schuster, K., Saalfeld, K., Grube, M., Buck, A., Jedlitschky, G., Maier, S., Traeger, T., et al. (2007). Sepsis affects cardiac expression of multidrug resistance protein 5 (MRP5, ABCC5), an ABC-type CGMP export pump. *Shock* **28**, 564–569.

Meyer Zu Schwabedissen, H. E. U., Grube, M., Heydrich, B., Linnemann, K., Fusch, C., Kroemer, H. K. and Jedlitschky, G. (2005). Expression, localization, and function of MRP5 (ABCC5), a transporter for cyclic nucleotides, in human placenta and cultured human trophoblasts: effects of gestational age and cellular differentiation. *Am J Pathol* **166**, 39–48.

Miranda, E. R., Nam, E. A., Kuspa, A. and Shaulsky, G. (2015). The ABC transporter, AbcB3, mediates cAMP export in *D. discoideum* development. *Dev Biol* **397**, 203–211.

Moroki, Y., Takeuchi, S., Igarashi, K., Hara, H. and Shiokawa, K. (2000). Effects of bromo-cyclic GMP and bromo-cyclic AMP on embryonic development of *Xenopus laevis*. *Zool Sci* **17**, 191–200.

Möhle, R. and Drost, A. C. (2012). G protein-coupled receptor crosstalk and signaling in hematopoietic stem and progenitor cells. *Ann N Y Acad Sci* **1266**, 63–67.

Nakajima, Y. and Burke, R. D. (1996). The initial phase of gastrulation in sea urchins is accompanied by the formation of bottle cells. *Dev Biol* **179**, 436–446.

Nambaru, P. K., Huebner, T., Koeck, K., Mews, S., Grube, M., Payen, L., Guittou, J., Sendler, M., Jedlitschky, G., Rimmbach, C., et al. (2011). Drug efflux transporter Multidrug Resistance-Associated Protein 5 affects sensitivity of pancreatic cancer cell lines to the nucleoside anticancer drug 5-fluorouracil. *Drug Metab Dispos* **39**, 132–139.

Nies, A. T., Jedlitschky, G., König, J., Herold-Mende, C., Steiner, H. H., Schmitt, H. P. and Keppler, D. (2004). Expression and immunolocalization of the multidrug resistance proteins, MRP1–MRP6 (ABCC1–ABCC6), in human brain. *Neuroscience* **129**, 349–360.

Nies, A. T., Spring, H., Thon, W. F., Keppler, D. and Jedlitschky, G. (2002).

Immunolocalization of multidrug resistance protein 5 in the human genitourinary system. *J Urol* **167**, 2271–2275.

Nishiyama, M., Hoshino, A., Tsai, L., Henley, J. R., Goshima, Y., Tessier-Lavigne, M., Poo, M.-M. and Hong, K. (2003). Cyclic AMP/GMP-dependent modulation of Ca²⁺ channels sets the polarity of nerve growth-cone turning. *Nature* **423**, 990–995.

Peter, I. S. and Davidson, E. H. (2009). Genomic control of patterning. *Int J Dev Biol* **53**, 707–716.

Pratt, S., Shepard, R. L., Kandasamy, R. A., Johnston, P. A., Perry, W. and Dantzig, A. H. (2005). The multidrug resistance protein 5 (ABCC5) confers resistance to 5-fluorouracil and transports its monophosphorylated metabolites. *Mol Cancer Ther* **4**, 855–863.

Raaijmakers, M. H. G. P. (2007). ATP-binding-cassette transporters in hematopoietic stem cells and their utility as therapeutical targets in acute and chronic myeloid leukemia. *Leukemia* **21**, 2094–2102.

Ransick, A. and Davidson, E. (1998). Late specification of veg(1) lineages to endodermal fate in the sea urchin embryo. *Dev Biol* **195**, 38–48.

Ravna, A. W., Sylte, I. and Sager, G. (2008). A molecular model of a putative substrate releasing conformation of multidrug resistance protein 5 (MRP5). *Eur J Med Chem* **43**, 2557–2567.

Reid, G., Wielinga, P., Zelcer, N., de Haas, M., Van Deemter, L., Wijnholds, J., Balzarini, J. and Borst, P. (2003). Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. *Mol Pharmacol* **63**, 1094–1103.

Ricardo, S. and Lehmann, R. (2009). An ABC transporter controls export of a *Drosophila* germ cell attractant. *Science* **323**, 943–946.

Ritter, C. A., Jedlitschky, G., Meyer Zu Schwabedissen, H., Grube, M., Köck, K. and Kroemer, H. K. (2005). Cellular export of drugs and signaling molecules by the ATP-binding cassette transporters MRP4 (ABCC4) and MRP5 (ABCC5). *Drug Metab Rev* **37**, 253–278.

Ruffins, S. W. and Ettensohn, C. A. (1996). A fate map of the vegetal plate of the sea urchin (*Lytechinus variegatus*) mesenchyme blastula. *Development* **122**, 253–263.

Sager, G. and Ravna, A. W. (2009). Cellular efflux of cAMP and cGMP - a

question about selectivity. *Mini Rev Med Chem* **9**, 1009–1013.

- Sager, G., Ørvoll, E. Ø., Lysaa, R. A., Kufareva, I., Abagyan, R. and Ravna, A. W.** (2012). Novel cGMP efflux inhibitors identified by virtual ligand screening (VLS) and confirmed by experimental studies. *J Med Chem* **55**, 3049–3057.
- Schinkel, A. H. and Jonker, J. W.** (2012). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* **55**, 3-29.
- Schinkel, A. H., Smit, J. J., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Mol, C. A., van der Valk, M. A., Robanus-Maandag, E. C. and Riele, te, H. P.** (1994). Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77**, 491–502.
- Schulz, T., Schumacher, U. and Prehm, P.** (2007). Hyaluronan export by the ABC transporter MRP5 and its modulation by intracellular cGMP. *J Biol Chem* **282**, 20999–21004.
- Sea Urchin Genome Sequencing Consortium, Sodergren, E., Weinstock, G. M., Davidson, E. H., Cameron, R. A., Gibbs, R. A., Angerer, R. C., Angerer, L. M., Arnone, M. I., Burgess, D. R., et al.** (2006). The genome of the sea urchin *Strongylocentrotus purpuratus*. *Science* **314**, 941–952.
- Shipp, L. E. and Hamdoun, A.** (2012). ATP-binding cassette (ABC) transporter expression and localization in sea urchin development. *Dev Dyn* **241**, 1111–1124.
- Song, H. and Poo, M.** (1999). Signal transduction underlying growth cone guidance by diffusible factors. *Curr Opin Neurobiol* **9**, 355–363.
- Song, H., Ming, G., He, Z., Lehmann, M., McKerracher, L., Tessier-Lavigne, M. and Poo, M.** (1998). Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* **281**, 1515–1518.
- Stojic, J., Stöhr, H. and Weber, B. H. F.** (2007). Three novel ABCC5 splice variants in human retina and their role as regulators of ABCC5 gene expression. *BMC Mol Biol* **8**, 42.
- Suzuki, T., Nishio, K., Sasaki, H., Kurokawa, H., Saito-Ohara, F., Ikeuchi, T., Tanabe, S., Terada, M. and Saijo, N.** (1997). cDNA cloning of a short type of multidrug resistance protein homologue, SMRP, from a human lung cancer cell line. *Biochem Biophys Res Commun* **238**, 790–794.

- Suzuki, T., Sasaki, H., Kuh, H. J., Agui, M., Tatsumi, Y., Tanabe, S., Terada, M., Saijo, N. and Nishio, K.** (2000). Detailed structural analysis on both human MRP5 and mouse *mrp5* transcripts. *Gene* **242**, 167–173.
- Takata, H. and Kominami, T.** (2004). Pigment cells trigger the onset of gastrulation in tropical sea urchin *Echinometra mathaei*. *Dev Growth Differ* **46**, 23–35.
- Toole, B. P.** (2004). Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer* **4**, 528–539.
- Veltman, D. M. and Van Haastert, P. J. M.** (2008). The role of cGMP and the rear of the cell in *Dictyostelium* chemotaxis and cell streaming. *Journal of Cell Science* **121**, 120–127.
- Wei, Z., Range, R., Angerer, R. and Angerer, L.** (2012). Axial patterning interactions in the sea urchin embryo: suppression of nodal by Wnt1 signaling. *Development* **139**, 1662–1669.
- Wielinga, P. R., van der Heijden, I., Reid, G., Beijnen, J. H., Wijnholds, J. and Borst, P.** (2003). Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells. *J Biol Chem* **278**, 17664–17671.
- Wijnholds, J., Mol, C. A., van Deemter, L., de Haas, M., Scheffer, G. L., Baas, F., Beijnen, J. H., Scheper, R. J., Hatse, S., De Clercq, E., et al.** (2000). Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. *Proc Natl Acad Sci USA* **97**, 7476–7481.
- Wikramanayake, A., Peterson, R., Chen, J., Huang, L., Bince, J., McClay, D. and Klein, W.** (2004). Nuclear beta-catenin-dependent Wnt8 signaling in vegetal cells of the early sea urchin embryo regulates gastrulation and differentiation of endoderm and mesodermal cell lineages. *Genesis* **39**, 194–205.
- Yamamoto, T., Yao, Y., Harumi, T. and Suzuki, N.** (2003). Localization of the nitric oxide/cGMP signaling pathway-related genes and influences of morpholino knock-down of soluble guanylyl cyclase on medaka fish embryogenesis. *Zool Sci* **20**, 181–191.
- Yokomizo, T., Izumi, T., Chang, K., Takawa, Y. and Shimizu, T.** (1997). A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. *Nature* **387**, 620–624.

Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., Lagutina, I., Grosveld, G. C., Osawa, M., Nakauchi, H., et al. (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* **7**, 1028–1034.

CHAPTER 2.
ATP-BINDING CASSETTE (ABC) TRANSPORTER EXPRESSION AND
LOCALIZATION IN SEA URCHIN DEVELOPMENT

2.1. ABSTRACT

ATP-binding cassette (ABC) transporters are membrane proteins that regulate intracellular concentrations of myriad compounds and ions. There are >100 ABC transporter predictions in the *Strongylocentrotus purpuratus* genome, including 40 annotated ABCB, ABCC, and ABCG “multidrug efflux” transporters. Despite the importance of multidrug transporters for protection and signaling, their expression patterns have not been characterized in deuterostome embryos. Sea urchin embryos expressed 20 ABCB, ABCC, and ABCG transporter genes in the first 58 hours of development, from unfertilized egg to early prism. We quantified transcripts of *ABCB1a*, *ABCB4a*, *ABCC1*, *ABCC5a*, *ABCC9a*, and *ABCG2b*, and found that *ABCB1a* mRNA was 10-100 times more abundant than other transporter mRNAs. *In situ* hybridization showed *ABCB1a* was expressed ubiquitously in embryos, while *ABCC5a* was restricted to secondary mesenchyme cells and their precursors. Fluorescent protein fusions showed localization of ABCB1a on apical cell surfaces, and ABCC5a on basolateral surfaces. Embryos utilize many ABC transporters with predicted functions in cell signaling, lysosomal and mitochondrial homeostasis, potassium channel regulation, pigmentation, and xenobiotic efflux. Detailed characterization of ABCB1a and ABCC5a revealed that they have different temporal and spatial gene expression profiles and protein localization patterns that correlate to their predicted functions in protection and development, respectively.

2.2. INTRODUCTION

ATP-Binding Cassette (ABC) transporters are a conserved family of membrane proteins that use ATP to move compounds across membranes in both adult and embryonic cells (Borst and Elferink, 2002; Dean et al., 2001; Elbling et al., 1993; Higgins, 1992). They transport peptides, metals, xenobiotics, and ions necessary for homeostasis, protection, and signaling. The ABC transporter family comprises eight subfamilies in sea urchins (ABCA to ABCH) and seven in mammals (ABCA to ABCG). Much of the functional information about these transporters pertains to the ABCB, ABCC, and ABCG “multidrug efflux” subfamilies and their roles in diseases including cancer, cholestasis, and cystic fibrosis. For example, increased expression of ABCB1 (P-glycoprotein, Pgp), ABCC1 (Multidrug resistance protein 1, MRP1), and ABCG2 (Breast cancer resistance protein, BCRP) in cancer cells leads to acquired drug resistance (Borst and Elferink, 2002). In contrast, reduced surface expression of the ABC transporters ABCB11 (Bile salt export pump, BSEP) and ABCC7 (Cystic fibrosis transmembrane conductance regulator, CFTR) leads to cholestasis and cystic fibrosis, respectively (Borst and Elferink, 2002; Dean et al., 2001).

While ABC transporters are well studied in disease, relatively little is known about their functions in embryos. In the *Strongylocentrotus purpuratus* genome, there are >100 ABC transporter gene predictions (<http://spbase.org>). Whole-genome tiling arrays revealed that these genes are extensively expressed

in the first five days of embryonic development. For example, >80% of *ABCC* genes were detected, a level that is ~30% higher than the overall level of gene utilization across the genome (Goldstone et al., 2006; Samanta et al., 2006). This high expression of transporters may be necessary to protect the embryo from xenobiotics. Consistent with this idea, sea urchin embryos possess both *ABCB* and *ABCC* transport activities that protect them from vinblastine (Hamdoun et al., 2004) and inorganic mercury (Bosnjak et al., 2009). Similarly, *ABCB1* (*Pgp*) protects mouse embryos from xenobiotics such as doxorubicin and mitomycin C (Elbling et al., 1993).

This high utilization of ABC transporters could indicate that they also function in cell specification through efflux of morphogens. For example, *Drosophila melanogaster* *mdr49* (an *ABCB* transporter) protects flies from colchicine toxicity (Wu et al., 1991), and it also transports signaling molecules. *Dm-mdr49* is expressed in the embryonic mesoderm where it effluxes a chemoattractant that directs germ cell migration to the somatic gonad (Ricardo and Lehmann, 2009). Similarly, human *ABCC1* (*MRP1*) is a xenobiotic transporter, but when expressed heterologously in *Caenorhabditis elegans*, it rescues defects in dauer larva formation induced by removal of the endogenous transporter (Yabe et al., 2005).

The goal of this study was to characterize the gene expression and protein localization of ABC transporters during embryonic development of sea urchins. Of the 40 manually annotated *ABCB*, *ABCC*, and *ABCG* genes, we found 20 to

be expressed during the first three days of development. We quantified the number of transcripts per egg/embryo for six transporter genes and found that mRNA of *ABCB1a* was 10-100 times more abundant than that of other measured transporters. *In situ* hybridization of *ABCB1a* and *ABCC5a* revealed that *ABCB1a* was ubiquitously expressed in all cells, while *ABCC5a* was expressed only in a subset of mesodermal precursors. ABCB1a protein primarily localized to the apical membrane of polarized epithelial cells, while ABCC5a was found on basolateral membranes. The spatial gene expression and protein localization patterns of ABCB1a and ABCC5a are consistent with predicted differences in protection versus signaling, respectively. Our results highlight the importance of ABCB, ABCC, and ABCG transporters in a wide range of developmental functions, ranging from protection against xenobiotics to control of cell signaling and differentiation.

2.3. RESULTS

2.3.1. ABC transporter genes expressed in early development.

We measured the dynamics of *ABCB*, *ABCC*, and *ABCG* gene expression during the first three days of sea urchin development, from unfertilized egg through the early prism stage. Embryos expressed 20 transporters including those potentially responsible for xenobiotic efflux, ion movement, and transport of signaling molecules (Table 2.1). To assess similarity of the detected genes to other well-characterized ABC transporters, we used Blastp to compare their predicted peptide sequences (Snpbase.org, genome version 3.1) to other proteins in NCBI. Embryos expressed eight *ABCB* transcripts including three genes related to multidrug pumps, one of which could be related to bile salt export pump (BSEP), four mitochondrial transporters, and one transporter associated with antigen processing (Tap)-like gene. Among the eight *ABCC* genes detected, one was a homolog of a well-known multidrug pump, four genes encoded transporters with both xenobiotic and signaling molecule substrates, and three genes were similar to the potassium channel regulating protein, sulfonylurea receptor-2 (SUR2). Finally, four *ABCG* genes were expressed, including one encoding a xenobiotic pump, one similar to uncharacterized transporters from insects, and two transporters homologous to *Drosophila* White.

Table 2.1. ABC Transporter genes detected in sea urchin development and functions of their homologs.

Gene	Gene ID	Annotated peptide length (aa)	Homologs	Blastp score	Also known as	Cellular membrane localization	Function (substrates)	References
<i>Sp-ABCB1a</i>	<u>SPU_001752</u>	1329	<i>H. sapiens</i> : ABCB1	0.0	CLCS; MDR1; P-GP; PGY1	Apical	Xenobiotic efflux (anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, mitoxantrone)	(Dean et al., 2001; Leslie et al., 2005; Fletcher et al., 2010)
<i>Sp-ABCB1b</i>	<u>SPU_002431</u>	1079	<i>H. sapiens</i> : ABCB11	0.0	BSEP; PGY4; SPCP	Apical	Bile salt transport (Taurocholate, Glycocholate, Taurochenodeoxycholate)	(Stieger et al., 2007)
<i>Sp-ABCB4a</i>	<u>SPU_007014</u>	1235	<i>H. sapiens</i> : ABCB1	0.0	CLCS; MDR1; P-GP; PGY1	Apical	Xenobiotic efflux (anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, mitoxantrone)	(Dean et al., 2001; Leslie et al., 2005; Fletcher et al., 2010)
<i>Sp-ABCB6</i>	<u>SPU_018342</u>	776	<i>H. sapiens</i> : ABCB6	0.0	ABG; FRP; MTABC3	Outer mitochondrial	Iron transport	(Dean et al., 2001; Zutz et al., 2009)
<i>Sp-ABCB7</i>	<u>SPU_003241</u>	651	<i>H. sapiens</i> : ABCB7	0.0	ABC7; ASAT; Atm1p	Inner mitochondrial	Cytosolic Fe/S transport; Iron homeostasis	(Dean et al., 2001; Zutz et al., 2009)
<i>Sp-ABCB8</i>	<u>SPU_024666</u>	469	<i>H. sapiens</i> : ABCB8	4e-97	MABC1; M-ABCI	Inner mitochondrial	Oxidative stress protection	(Zutz et al., 2009)
<i>Sp-ABCB9a</i>	<u>SPU_026825</u>	398	<i>H. sapiens</i> : ABCB9	8e-160	TAPL	Lysosomal	Peptide transport	(Zhao et al., 2006; Bangert et al., 2011)
<i>Sp-ABCB10a</i>	<u>SPU_016850</u>	623	<i>H. sapiens</i> : ABCB10	0.0	M-ABC2; MTABC2	Inner mitochondrial	Iron transport; possible peptide transport & antigen processing	(Herget and Tämpé, 2007; Chen et al., 2009; Zutz et al., 2009)
<i>Sp-ABCC1</i>	<u>SPU_026395</u>	1025	<i>H. sapiens</i> : ABCC1	0.0	MRP; ABCC; GS-X; MRP1	Basolateral	Xenobiotic efflux (GSH-conjugates, anthracyclines, mitoxantrone, vinca alkaloids, imatinib, epipodophyllotoxins, camptothecins, colchicines, metals, methotrexate, Etoposide-glucuronide, Doxorubicin-GS, glutathione disulfide (GSSG)); Signaling & homeostasis (GSH-conjugates, leukotrienes, prostaglandins, sphingosine-1-phosphate, bilirubin, estradiol 17 β -D-glucuronide)	(Leslie et al., 2005; Fletcher et al., 2010; Chen and Tiwari, 2011; He et al., 2011)

Table 2.1. ABC Transporter genes detected in sea urchin development and functions of their homologs, Continued.

Gene	Gene ID	Annotated peptide length (aa)	Homologs	Blastp score	Also known as	Cellular membrane localization	Function (substrates)	References
<i>Sp-ABCC4a</i>	<u>SPU_020669</u>	1411	<i>H. sapiens</i> ABCC4	0.0	MRP4; MOATB	Apical or Basolateral (tissue dependent)	Xenobiotic efflux (nucleosides, thiopurines, PMEA, methotrexate, anti-HIV nucleoside analogues, camptothecins); Signaling & homeostasis (leukotrienes, prostaglandins, thromboxane, cyclic nucleotides)	(Fletcher et al., 2010; Chen and Tiwari, 2011)
<i>Sp-ABCC4b</i>	<u>SPU_024191</u>	1214	<i>H. sapiens</i> ABCC4	1e-103				
<i>Sp-ABCC4c</i>	<u>SPU_002411</u>	1174	<i>H. sapiens</i> ABCC4	0.0				
<i>Sp-ABCC5a</i>	<u>SPU_023723</u>	1424	<i>H. sapiens</i> ABCC5	0.0	MRP5; SMRP; MOATC	Basolateral	Organic anion transport (acidic organic dyes, dinitrophenylglutathione); Xenobiotic efflux (methotrexate, cisplatin, doxorubicin, gemcitabine); Signaling & homeostasis (cyclic nucleotides)	(Borst and Elferink, 2002; Fletcher et al., 2010; Chen and Tiwari, 2011)
<i>Sp-ABCC9a</i>	<u>SPU_025903</u>	1585	<i>H. sapiens</i> ABCC9	0.0	SUR2			
<i>Sp-ABCC9b</i>	<u>SPU_028797</u>	1497	<i>H. sapiens</i> ABCC9	0.0				
<i>Sp-ABCC9d</i>	<u>SPU_007764</u>	1481	<i>H. sapiens</i> ABCC9	0.0				
<i>Sp-ABCG2b</i>	<u>SPU_014013</u>	448	<i>H. sapiens</i> ABCG2	7e-138	MRX; MXR; ABCP; BCRP; BMDP	Apical	Potassium channel regulation	(Bryan et al., 2007)
<i>Sp-ABCG9</i>	<u>SPU_012874</u>	485	<i>D. melanogaster</i> E23	1e-81	Early gene at 23		Xenobiotic efflux (mitoxantrone, camptothecins, anthracyclins, bisantrene, imatinib, methotrexate, flavopiridol, epipodophyllotoxins); Stem cell protection & maintenance	(Leslie et al., 2005; Krishnamurthy and Schuetz, 2006; Fletcher et al., 2010)
<i>Sp-ABCG11</i>	<u>SPU_020849</u>	590	<i>D. melanogaster</i> White	5e-109	white; DMWHITE; EG:BACN33B1.1	Pigment granular	Ecdysone signaling, circadian rhythm	(Itoh et al., 2011)
<i>Sp-ABCG12</i>	<u>SPU_015080</u>	677	<i>D. melanogaster</i> White	0.0			Eye color determinant (pigment metabolites)	(Ewart et al., 1994; Mackenzie et al., 2000)

^aBlastp score indicates E-value.

2.3.2. Temporal patterns of ABC transporter gene expression.

All of the ABCB, ABCC, and ABCG mRNAs showed little change in abundance from the egg to early blastula stage (Fig. 2.1, Fig. 2.2, Supplemental Table S2.1). The first significant changes in expression (fold change <0.5 or >2) occurred at hatching, consistent with the large synthesis and turnover of mRNA that occurs at this time (Davidson, 1986). For example, transcripts of 13 ABC transporters decreased at hatching, presumably due to turnover of maternally derived mRNA (i.e. synthesized during oogenesis and present in the egg prior to fertilization).

All 20 ABC transporter genes were generally expressed in one of three temporal patterns: (1) transporter expression is present from egg on, decreases at hatching, then is restored thereafter (Fig. 2.2, blue lines), (2) transporter transcripts are absent in early development and rapidly appear at a distinct developmental time point (Fig. 2.2, orange lines), and (3) transporter expression is robust from egg on and increases steadily throughout development (Fig. 2.2, green lines). Thirteen genes showed Group 1 patterns (blue, Fig. 2.2A-H, J, N-P, R) including *ABCB1a*, *ABCB1b*, *ABCB4a*, *ABCB6*, *ABCB7*, *ABCB8*, *ABCB9a*, *ABCB10a*, *ABCC4a*, *ABCC9a*, *ABCC9b*, *ABCC9d*, and *ABCG9*. After hatching, *ABCB1b*, *ABCB4a*, *ABCB7*, *ABCB10a*, *ABCC9b*, *ABCC9d*, and *ABCG9* all increased >2 fold. The second most common expression pattern was the Group 2 pattern (orange, Fig. 2.2M, Q, S-T) found for *ABCC5a*, *ABCG2b*, *ABCG11*, and *ABCG12*. *ABCC5a*, *ABCG11*, and *ABCG12* were first detected at the hatching

blastula stage while *ABCG2b* was not detected until the early prism stage. Group 3 (green, Fig. 2.2I, K-L) included three transporters, *ABCC1*, *ABCC4b*, and *ABCC4c*, that increased >2 fold by hatching (*ABCC1*, *ABCC4c*) or early gastrula (*ABCC4b*).

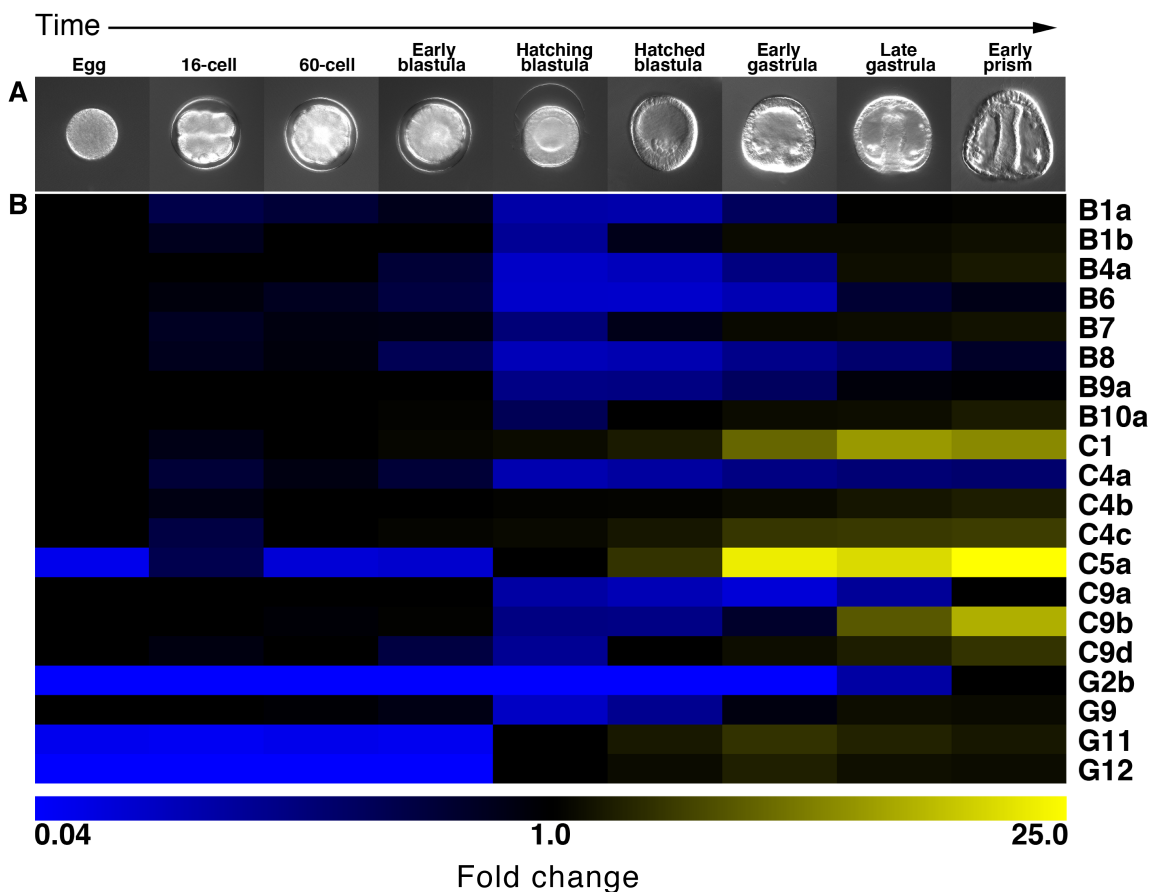


Figure 2.1. Developmental stages surveyed and relative ABC transporter gene expression. (A) DIC micrographs depict the developmental stages included in the gene expression survey. (B) Heat map of quantitative real-time PCR data expressed as fold change from the reference stage (i.e. earliest detectable stage). Reference stage is egg in all transporters except *ABCC5a*, *ABCG2b*, *ABCG11*, and *ABCG12*. Reference stage is hatching blastula for *ABCC5a*, *ABCG11*, and *ABCG12*. Reference stage is early prism for *ABCG2b*. All data represent the average of progeny from four females (N=4).

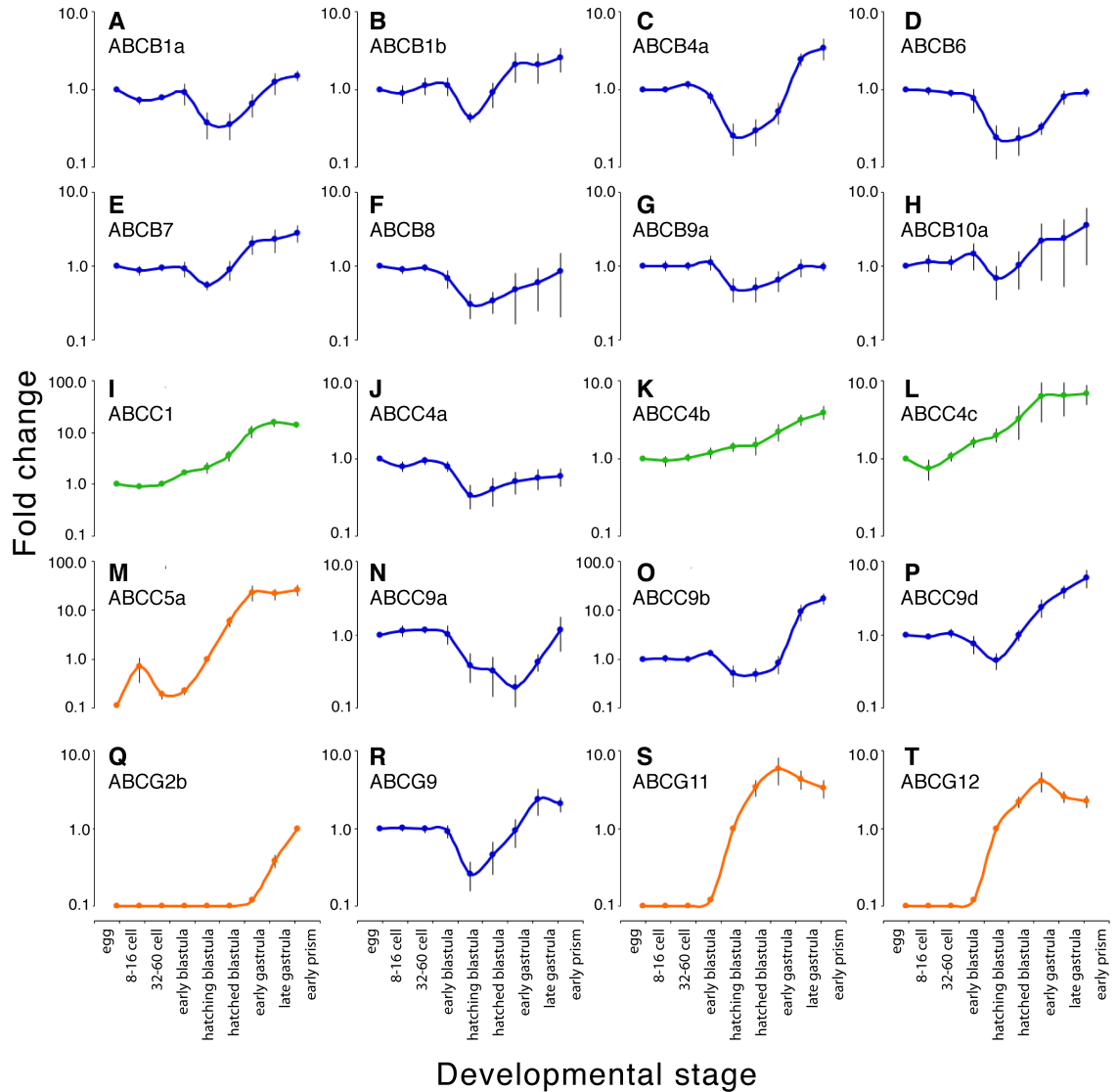


Figure 2.2. Relative ABC transporter gene expression during sea urchin development. Data depicted in Fig. 2.1 is presented as individual graphs on a logarithmic scale. Expression profiles are shown in three color-coded groups. Group 1 is shown in blue, Group 2 in orange, and Group 3 in green. N = 4, error bars represent standard error.

2.3.2.1. Temporal expression patterns of paralogs

Many *S. purpuratus* ABC transporters have multiple paralogs. For example, *ABCB1* has 10 paralogs, *ABCC5* has 16, *ABCC9* has 14, and *ABCG2* has five. To gain insight into the potential functions of these paralogs, we

examined whether the expression patterns of detected paralogs were temporally synchronous. For *ABCB1a* and *ABCB1b*, expression was fairly uniform with both decreasing at hatching (Fig. 2.2A-B). In contrast, the *ABCC4* paralogs were asynchronous with *ABCC4a* decreasing at hatching then slowly restoring expression levels, while *ABCC4b* increased >2 fold at early gastrula, and *ABCC4c* increased >2 fold at hatching (Fig. 2.2J-L). Finally, while the *ABCC9* paralogs all decreased expression at hatching and restored expression levels later in development, only *ABCC9b* and *ABCC9d* increased >2 fold (Fig. 2.2N-P).

2.3.3. Quantification of ABC transporter mRNAs.

We quantified six ABC transporter transcripts using cDNA standards (Fig. 2.3, Supplemental Table S2.1) to determine their abundance. These six genes represented each of the three common expression profiles. The genes included *ABCB1a*, *ABCB4a*, and *ABCC9a* from Group 1, *ABCC5a* and *ABCG2b* from Group 2, and *ABCC1* from Group 3. *ABCB1a* was the most abundantly expressed transporter, averaging 12,878 transcripts per egg/embryo and ranging from 6,223 copies in hatched blastula to 20,135 copies per late gastrula embryo (Fig. 2.3A, Supplemental Table S2.1). *ABCB4a* ranged from 465 copies in hatching blastulae to 4,157 copies per early prism embryo with an average of 1,643 copies (Fig. 2.3B, Supplemental Table S2.1). *ABCC9a* had an average of 1,532 transcripts per egg/embryo, ranging from 468 copies in early gastrulae to 2,481 copies in 16-cell embryos (Fig. 2.3E, Supplemental Table S2.1). Both

ABCC5a and *ABCG2b* could not be detected until they exceeded ~100 transcripts per embryo. *ABCC5a* reached this threshold with 343 copies at hatching, and it peaked at the early prism stage with 7,871 copies per embryo (Fig. 2.3D, Supplemental Table S2.1). *ABCG2b* transcripts could only be accurately quantified at the latest stage surveyed, when they reached 186 copies per early prism embryo (Fig. 2.3F, Supplemental Table S2.1). *ABCC1* transcripts were present at 590 copies per egg and 517 copies per 16-cell embryo, then increased through development peaking at 7,471 copies per late gastrula stage embryo (Fig. 2.3C, Supplemental Table S2.1).

2.3.4. Spatial patterns of ABC transporter gene expression.

Next, we used whole mount *in situ* hybridization (WMISH) to characterize spatial expression of five ABC transporter genes. Two genes, *ABCB1a* and *ABCC5a*, showed clear localization patterns. *ABCB4a*, *ABCC1*, and *ABCC9a* were not detected, presumably because their messages were insufficiently abundant and/or widely dispersed. Temporal expression analyses described above showed that *ABCB1a*, which has predicted protective functions, was the most abundant mRNA (Fig. 2.3A). In contrast, *ABCC5a* mRNA was absent in early development but increased dramatically at a specific developmental stage (Fig. 2.3D), a pattern that is commonly observed for developmental genes such as *Nodal*, *HesC*, and *Delta* (Nam et al., 2007; Revilla-i-Domingo et al., 2007). Consistent with a predicted function in protection against toxicants, WMISH

showed *ABCB1a* was ubiquitously expressed in all cells throughout development (Fig. 2.4E-H). At the gastrula stage, *ABCB1a* was detected with such intense staining on the ectoderm and endoderm that it was difficult to determine if there was uniform mesodermal expression (Fig. 2.4H).

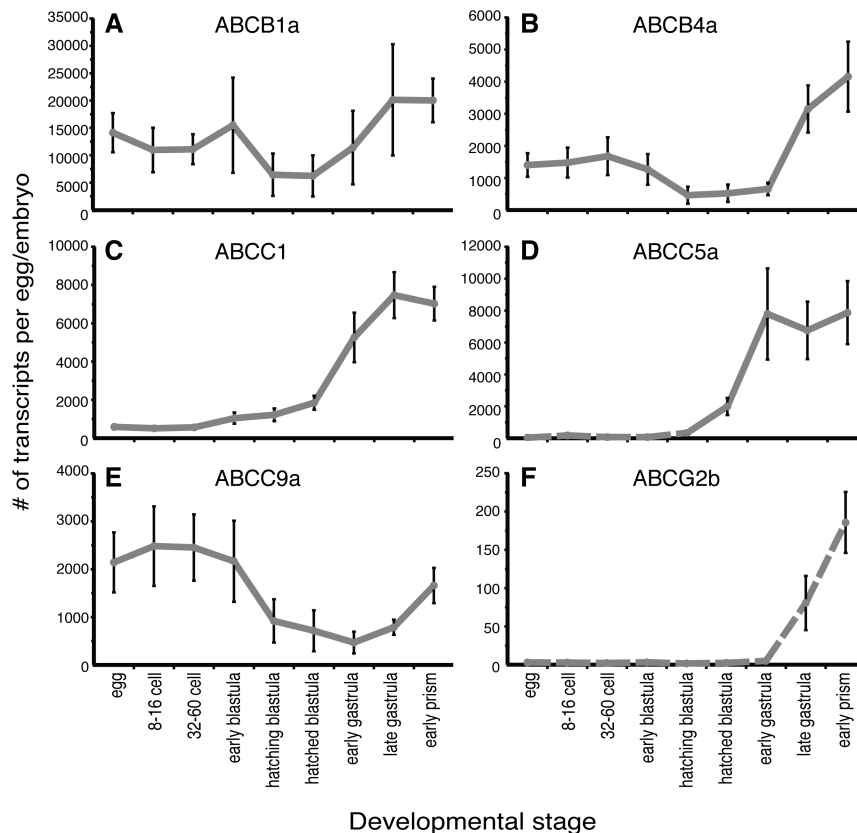


Figure 2.3. Number of ABC transporter transcripts per egg/embryo. Number of mRNA copies per egg/embryo is shown. Transcript copies were determined using a standard curve to quantify the reference point (egg for *ABCB1a*, *ABCB4a*, *ABCC1*, and *ABCC9a*; hatching blastula for *ABCC5a*; early prism for *ABCG2b*), and by applying fold change values (Figs 2.1, 2.2, Supplemental Table S2.1) to quantify all other stages. Dashed lines (**D**, **F**) indicate where transcripts were below the threshold of detection using qPCR (i.e. <100 copies per egg/embryo). N=4, bars represent standard error.

In contrast, *ABCC5a* transcripts were undetectable early in development (Fig. 2.4I), then were expressed only in a subset of embryonic cells after hatching

(Fig. 2.4J-L). *ABCC5a* was expressed in mesodermal cells (Fig. 2.4K-L) in a pattern consistent with the *veg2* lineage and their descendants, the (non-skeletogenic) secondary mesenchyme cells (SMC) (Peter and Davidson, 2009a). In hatched mesenchyme blastulae, *ABCC5a* mRNA was detected in the vegetal pole of the embryo and was absent from primary mesenchyme cells (Fig. 2.4J). During gastrulation, *ABCC5a*-expressing cells dispersed and were ultimately incorporated into the ectoderm in a pattern similar to that of pigment cells (Fig. 2.4K-L) (Peter and Davidson, 2009a; Ransick et al., 2002). In addition, the temporal expression pattern of *ABCC5a* matches those of *ABCG11* and *ABCG12*, homologs of the transporter necessary for eye pigmentation in *Drosophila*, *Dm-White* (Ewart et al., 1994; Mackenzie et al., 2000). Thus, *ABCC5a* could be essential for the formation or function of pigment cells in sea urchin embryos.

2.3.5. Cellular localization of ABCB1a and ABCC5a proteins.

We expressed fluorescent-protein fusions of ABCB1a and ABCC5a in the sea urchin embryo to determine their cellular localization (Fig. 2.5). ABCB1a-mCitrine protein localized to the apical membrane of the ectoderm in hatched mesenchyme blastulae (Fig. 2.5Aii). This transporter coated the outside surface of the embryo and was seen on the surfaces of apical microvilli (Fig. 2.5Bii). In contrast, ABCC5a-mCherry protein was absent from the apical cell surface and was instead localized on the basolateral cell membranes (Fig. 2.5Aiii, 2.5Biii). No

co-localization of ABCB1a-mCitrine and ABCC5a-mCherry proteins was observed in polarized epithelial cells (Fig. 2.5Biv), except for some slight overlap at the vegetal pole of the embryo (Fig. 2.5Aiv).

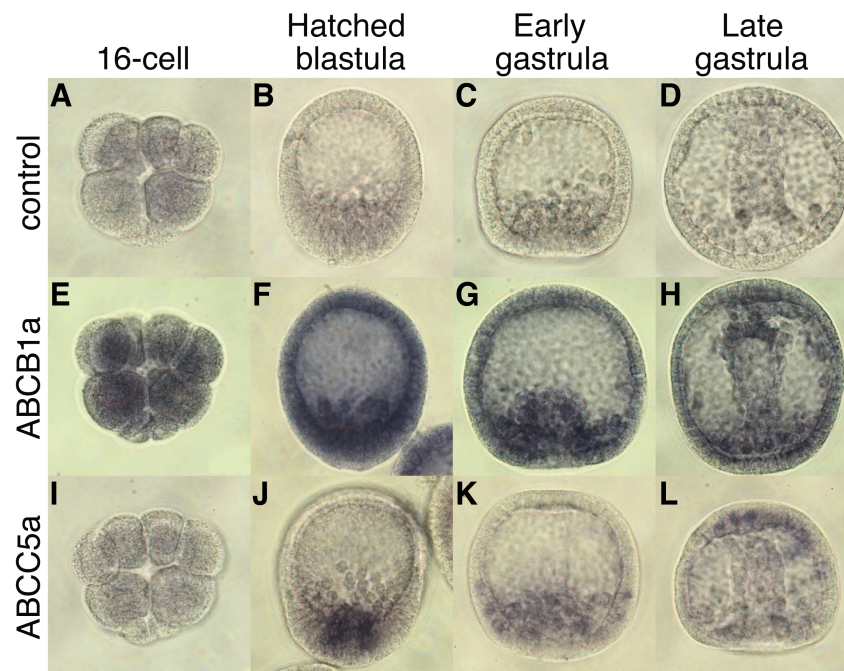


Figure 2.4. Spatial expression of ABC transporter genes. Whole mount *in situ* hybridization depicts endogenous expression of *ABCB1a* and *ABCC5a*. (A-D) For controls, DIG-labeled sense probe was used. (E-H) *ABCB1a* is expressed in all cells of embryos. (I) *ABCC5a* mRNA is not detected prior to hatching, after which it is detected (J) in the vegetal pole of hatched mesenchyme blastulae, and (K, L) in mesodermal cells in later embryos. (A, E, I) 16-cell embryos were fixed at 7 hpf, (B, F, J) hatched mesenchyme blastulae at 36 hpf, (C, G, K) early gastrulae at 42 hpf, and (D, H, L) late gastrulae at 48 hpf.

Our findings from both WMISH and FP-overexpressions indicate that at the hatched blastula stage, endogenous *ABCB1a* transcripts are present in all embryonic cells (Fig. 2.4F, Fig. 2.6A), while ABCB1a-mCitrine protein localizes on the apical membrane of all ectodermal cells (Fig. 2.5Aii). This indicates that in

blastulae, endogenous ABCB1a protein is also present on the apical side of all ectodermal cells (Fig. 2.6B, green). This places ABCB1a proteins in direct contact with the environment, where they can directly efflux unwanted chemicals from the embryo.

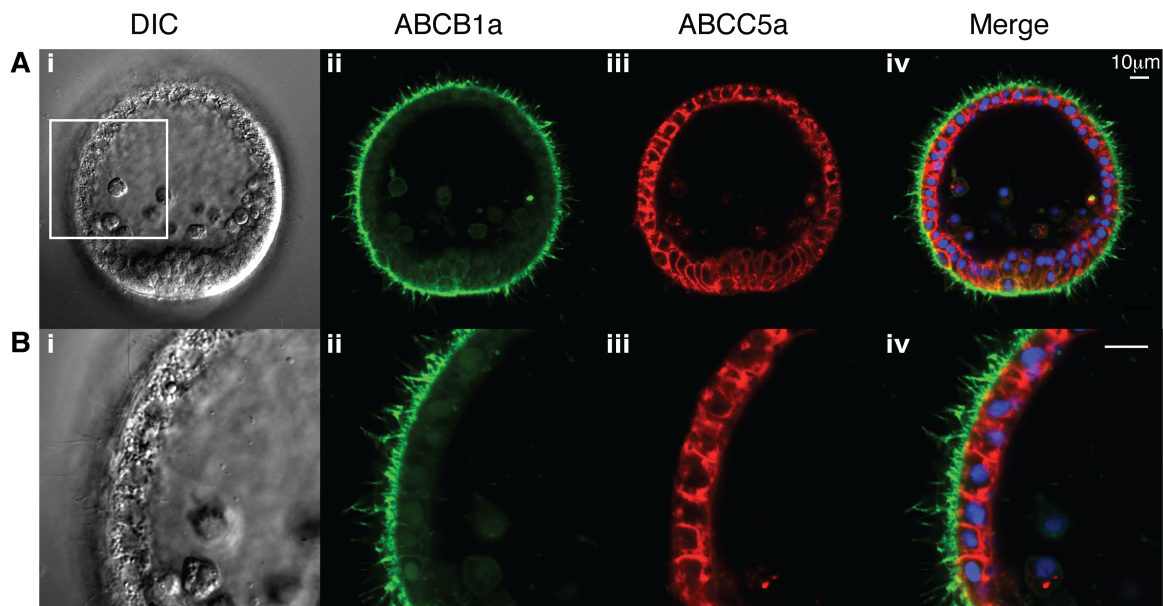


Figure 2.5. Localization of ABCB1a and ABCC5a proteins. Micrographs show exogenous ABCB1a and ABCC5a localization from expression of fluorescent-protein fusions. Representative embryos are shown. **B** is an inset from the embryo shown in **A**. mRNA from ABCB1a-mCitrine (**ii**, green) and ABCC5a-mCherry (**iii**, red) were injected into fertilized eggs, then embryos were grown to mesenchyme blastulae for imaging. (**iv**) Merged channel includes Histone H2B-CFP (blue), injected as a nuclear marker. ABCB1a-mCitrine protein localizes to the apical surface of the embryo, while ABCC5a-mCherry localizes on the basolateral cell surfaces.

In contrast, at the same developmental stage, *ABCC5a* transcripts are detected exclusively in a subset of vegetal cells (Fig. 2.4J, Fig. 2.6A) likely to be part of the Veg2 lineage (Peter and Davidson, 2009a). ABCC5a-mCherry protein is not detected at the apical surface of the embryo but is instead localized on basolateral membranes of polarized cells, which are not in direct contact with the

environment (Fig. 2.5Aiii). Thus, we propose that endogenous ABCC5a protein is expressed on basolateral membranes of vegetal cells in hatched blastulae (Fig. 2.6B, red). This position in the embryo suggests that ABCC5a is not involved in protective efflux of environmental chemicals.

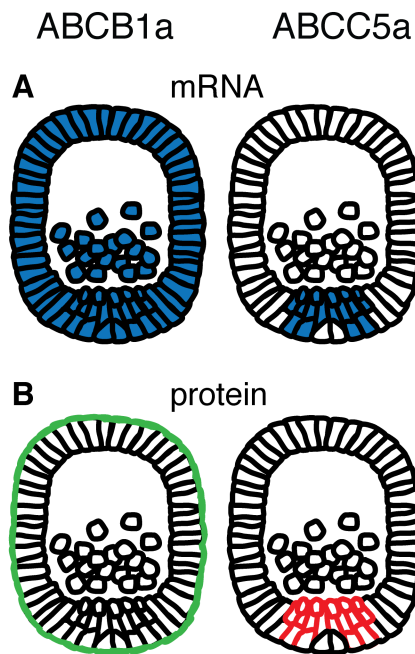


Figure 2.6. Model of endogenous ABCB1a and ABCC5a protein localization.

(A) A cartoon represents WMISH transcript localization shown in Figs 2.4F, 2.4J. By combining this with FP-fusion ABCB1a and ABCC5a protein localization data shown in Fig. 2.5A, we propose (B) a model for endogenous ABCB1a (green) and ABCC5a (red) expression. ABCB1a is primarily expressed on the apical membrane of polarized epithelial cells, while ABCC5a is expressed on the basolateral membranes of secondary (non-skeletogenic) mesenchyme cells.

2.4. DISCUSSION

Our results demonstrate that sea urchin embryos utilize many ABC transporters in early development. In the first three days of development, 20 ABC transporters were expressed including those with predicted functions in cell signaling, mitochondrial and lysosomal homeostasis, potassium channel regulation, pigmentation, and xenobiotic efflux (Table 1). *In situ* hybridization and fluorescent protein fusion expression of ABCB1a and ABCC5a revealed significant differences in location and timing of expression of these two transporters that correlate with predicted differences in their functions.

2.4.1. Xenobiotic transport

Sea urchin embryos expressed homologs of all three major types of multidrug transporters including ABCB1, ABCC1, and ABCG2. Among the detected ABCB transporters, *Sp-ABCB1a*, *Sp-ABCB1b*, and *Sp-ABCB4a* are similar to the human multidrug resistance transporter, P-glycoprotein (Pgp). This transporter protects cells by effluxing a wide range of mildly hydrophobic molecules (Dean et al., 2001; Fletcher et al., 2010), and its substrate poly-specificity is mediated by a large binding pocket with multiple drug binding sites (Aller et al., 2009). ABCB1/Pgp-mediated xenobiotic efflux activity is essential for protecting hematopoietic stem cells (Smeets et al., 1997) and various mammalian (Elbling et al., 1993) and invertebrate embryos including sea urchins (Hamdoun et al., 2004; Toomey and Epel, 1993).

Previous studies indicated that sea urchins have high levels of ABCC-like multidrug efflux activity (Hamdoun et al., 2004). We found that embryos expressed *Sp-ABCC1*, a homolog of Multidrug resistance protein 1 (MRP1), which could mediate this activity. In addition to direct efflux of toxic molecules, ABCC1/MRP1 effluxes glutathione-conjugates and therefore can also transport hydrophilic toxicants such as metals (Chen and Tiwari, 2011; Fletcher et al., 2010; He et al., 2011; Leslie et al., 2005). ABCC1 can also maintain cellular redox homeostasis via GSH and GSSG transport (Leslie et al., 2001), and it regulates inflammation and dendritic cell migration via transport of leukotriene LTC₄ (Leslie et al., 2005). Since ABCC1 performs both protective and signaling functions in mammals, it could also be a dual-functioning transporter in sea urchin development.

Finally, sea urchin embryos expressed *Sp-ABCG2b*, a homolog of human Breast cancer resistance protein (BCRP). ABCG2/BCRP is a xenobiotic transporter known to mediate drug resistance by effluxing anticancer drugs such as mitoxantrone (Krishnamurthy and Schuetz, 2006; Leslie et al., 2005). In addition to protection, ABCG2 may also maintain multipotency of hematopoietic stem cells (Bunting, 2002; Zhou et al., 2001). Consistent with this hypothesis, ABCG2 is involved in porphyrin homeostasis and contributes to self-renewal of mouse embryonic stem cells (Susanto et al., 2008).

2.4.2. Mitochondrial transport

An interesting finding of our study was the expression of four homologs of mitochondrial transporters, *Sp-ABCB6*, *Sp-ABCB7*, *Sp-ABCB8*, and *Sp-ABCB10a*. In sea urchin embryos, mitochondria are important for both energetics and oral-aboral specification (Coffman et al., 2009). The genes expressed are homologous to mammalian mitochondrial half-transporters likely to function in Fe/S cluster transport, iron homeostasis, heme biosynthesis, peptide transport, and oxidative stress protection (Herget and Tampé, 2007; Zutz et al., 2009) (Chen et al., 2009). In mammals, three of these transporters (ABCB7, ABCB8, and ABCB10) are expressed on the inner mitochondrial membrane, while ABCB6 is thought to localize to the outer mitochondrial membrane (Zutz et al., 2009).

2.4.3. Lysosomal transport

The final ABCB gene expressed was *Sp-ABCB9a*, which is homologous to the lysosomal Tap-like (TAPL) protein, a transporter of peptides from cytosol into the lysosome. ABCB9/TAPL may perform a homeostatic role such as disposing of accumulated cytosolic peptides (Bangert et al., 2011; Zhao et al., 2006), and it is possibly also involved in antigen processing (Bangert et al., 2011).

2.4.4. Multifunctional transport

Several of the expressed transporters could have dual functions in the embryo, including the aforementioned example of *Sp-ABCC1*. We detected other

predicted multifunctional transporters including *Sp-ABCC4a*, *Sp-ABCC4b*, *Sp-ABCC4c*, and *Sp-ABCC5a*, which are homologs of mammalian transporters with broad specificity for both signaling molecules (e.g. cGMP) and xenobiotics. Like ABCC1, ABCC4/MRP4 transports leukotrienes and is necessary for human dendritic cell migration (Fletcher et al., 2010).

One of the central transporters in this study was *Sp-ABCC5a*, whose homologs remain relatively poorly characterized in any organism. In humans, ABCC5/MRP5 localizes to basolateral membranes of polarized cells, and its mRNA is ubiquitously expressed in adult tissues, though it is highest in skeletal muscle, heart and brain (Chen and Tiwari, 2011). It has been suggested to be a cGMP transporter (Jedlitschky et al., 2000), although its affinity for this substrate is relatively low (de Wolf et al., 2007). Since *Hs-ABCC5* may export cyclic nucleotides, one possibility is that *Sp-ABCC5a* plays some role in signaling necessary for morphogenesis or migration of the mesenchyme cells. Alternatively, it may function in xenobiotic efflux, though in other systems it has yet to be demonstrated as toxicologically important (Chen and Tiwari, 2011; Leslie et al., 2001). One possibility is that *Sp-ABCC5a* may be involved in protection against some endogenously produced toxic metabolite.

2.4.5. Potassium channel conductance regulators

Three of the expressed transporter genes, *Sp-ABCC9a*, *Sp-ABCC9b*, and *Sp-ABCC9d*, are homologous to sulfonyl urea receptor 2 (SUR2), which

associates with inwardly rectifying potassium channels to regulate insulin secretion in humans (Bryan et al., 2007). Little is presently known about the functions of these channels in development. In *Drosophila*, ABCC9/SUR2 is expressed in trachea and dorsal vessels and is potentially involved in cell migration (Nasonkin et al., 1999).

2.4.6. White transporter homologs

Finally among the genes expressed, *Sp-ABCG11* and *Sp-ABCG12* are homologs of the White half-transporter, which transports precursors or metabolic intermediates of pigment to control eye color in *D. melanogaster* (Ewart et al., 1994; Mackenzie et al., 2000). Like the ABCB half-transporters, White protein is not localized on the cell membrane, but instead is found on intracellular vesicles (Evans et al., 2008; Mackenzie et al., 2000) where it may also transport cGMP (Evans et al., 2008). In *Drosophila* embryos, *white* transcripts are detected in the Malpighian tubules coincident with the onset of cell differentiation (Fjose et al., 1984).

2.4.7. Transporters with unresolved classification

Interestingly, we found two genes that may have different homologs than those indicated by their original names. For example, although the name *Sp-ABCB1b* indicates similarity to human ABCB1/Pgp, the top NCBI Blastp hits were chicken ABCB1 (CMDR1) (e=0.0) and human ABCB11 (Bile salt export pump,

BSEP) ($e=0.0$). BSEP transports bile salts across the canalicular plasma membrane (Stieger et al., 2007). Chicken *Mdr1* is expressed in the thymus and bursa of embryos and may participate in lymphoid differentiation of T and B cells (Petrini et al., 1995). Given that sea urchins lack a direct ABCB11 homolog, it is possible that *Sp*-ABCB1b transports sterols similar to bile salts.

Similarly, although *Sp*-ABCC9b is homologous to SUR2 ($e=0.0$), Blastp indicates it is equally similar to Multidrug resistance protein 2 (MRP2) ($e=0.0$). MRP2 is related to MRP1, and as such it effluxes both signaling molecules and xenobiotics (Fletcher et al., 2010; Leslie et al., 2001). Thus, one possibility is that *Sp*-ABCC9b has different functions than those predicted by its designation as an SUR.

2.4.8. Differential regulation of transporters

Given this great diversity of ABC transporters present in embryos, it seems plausible that multiple modes of regulation are employed to maintain and modulate their membrane activity through development. For example, our results with ABCB1a and ABCC5a indicate that they are likely to be under different modes of regulation.

ABCB1a transcripts are abundant throughout development (Fig. 2.3A, Supplemental Table S2.1) and are strongly detected in all cells of embryos including the primary mesenchyme (Fig. 2.4F). Yet, while ABCB1a-mCitrine accumulates to high levels on surfaces of ectodermal cells, it is expressed

weakly on surfaces of primary mesenchymal cells (Fig. 2.5Aii, Bii). This could indicate that ABCB1a is post-transcriptionally regulated and that levels of its mRNA do not necessarily correlate to surface levels of the protein. Additional evidence for post-transcriptional regulation of ABCB1a comes from the observation that efflux activity increases after fertilization of sea urchin eggs, even with exposure to inhibitors of transcription and translation (Hamdoun et al., 2004). This indicates that in very early development, efflux activity is post-translationally controlled, and it is possible that ABCB1a is similarly regulated throughout development. Alternatively, primary mesenchyme cells may have less ABCB1a-mCitrine due to membrane turnover associated with the epithelial to mesenchymal transition (EMT) (Wu et al., 2007).

In contrast, *ABCC5a* expression is tightly temporally and spatially controlled (Fig. 2.3D, Fig. 2.4J), expressed only after hatching and exclusively in a subset of veg2 cells and their descendants. This suggests that *ABCC5a* is transcriptionally regulated. Consistent with this hypothesis, in MCF7 cells, *Hs-ABCC5* expression is regulated by the EMT-inducing transcription factors Snail, Twist and FOXC2 (Saxena et al., 2011). These are important developmental transcription factors that could interact with *Sp-ABCC5a*. Our future studies will address this possibility and probe the role of *Sp-ABCC5a* in protection, specification, and/or functions of mesodermal cells.

2.4.9. Conclusions

Collectively, our results highlight the diversity of ABC transporters necessary for sea urchin development and provide a foundation for exploring their biology. The characterization of ABCB1a and ABCC5a emphasizes differences in spatial and temporal expression of ABC transporters, and the relation of these differences to predicted functions. Clearly, ABC transporters are more than protective transporters in embryogenesis, and exquisite regulation of membrane function by expression of transporters is likely central to homeostasis, protection, and signaling during development. Our future work will focus on major developmental transitions to address the regulation and function of ABCC5a in protection and/or specification of embryonic cells.

2.5. MATERIALS AND METHODS

2.5.1. Animals

Purple sea urchins, *Strongylocentrotus purpuratus*, were collected off the coast of San Diego, CA, USA, kept in 12°C running seawater, and fed *Macrocystis pyrifera*. Gametes were collected according to standard procedures (Foltz et al., 2004). Eggs were collected in raw seawater, passed through a 120 μ m nitex filter, and washed in filtered seawater (FSW). For RNA isolation and *in situ* analyses, a 500 ml solution of 1% packed eggs in FSW was fertilized with 5 μ l sperm (in FSW). Fertilization was visually confirmed, and only batches with >90% fertilization were used for experiments. Embryos were washed twice in FSW to remove excess sperm, and the culture was grown at a concentration of 500 embryos/ml FSW at 12°C.

2.5.2. RNA isolation

For all gene expression experiments, total RNA was isolated at nine developmental stages (approximate hours post-fertilization, hpf): 1. Unfertilized egg, 2. 16-cell (~6 hpf), 3. 60-cell (~8.5 hpf), 4. early blastula (12-13 hpf), 5. hatching blastula (21-23 hpf), 6. hatched blastula (27-29 hpf), 7. early gastrula (33-35 hpf), 8. late gastrula (50-52 hpf), 9. early prism (55-58 hpf). Experiments were replicated four times with progeny of four females.

Aliquots of cultures were hand-centrifuged to pellet eggs/embryos, and RNA was isolated using a Nucleospin RNA II isolation kit (Macherey-Nagel,

Bethlehem, PA, USA) according to the manufacturer's protocol. Yields varied (depending on the density of the pellet) from 3-56 μg total RNA. RNA concentration and purity was determined by spectrophotometry and agarose gel electrophoresis. Only samples with absorbance ratios of ~ 2.0 (260/280) and ~ 2.0 -2.2 (260/230) with clear major ribosomal subunit bands on gel visualization were used for experiments.

2.5.3. cDNA synthesis

Reverse transcription was performed using 500 ng total RNA, 1.5 μM random primer (New England Biolabs, Ipswich, MA, USA), 0.5 mM dNTPs (Fermentas, Glen Burnie, Maryland, USA), M-MuLV Reverse Transcriptase (New England Biolabs), and RNasin (Promega, Madison, WI, USA) at a final volume of 20 μl .

2.5.4. Quantitative real-time PCR (qPCR)

qPCR was performed on a Stratagene MX3000p thermal cycler (Agilent, Santa Clara, CA, USA) with EVA QPCR SuperMix Kit (Biochain, Hayward, CA, USA) according to the manufacturer's protocol. All reactions were run in duplicate. To each 20 μl cDNA synthesis reaction, 200 μl nuclease-free water was added for analysis with qPCR. A volume of 3.5 μl was used as template for qPCR with 625 nM of each (forward and reverse) primer in a total reaction volume of 20 μl per well.

2.5.5. Primer design and testing

We designed a total of 76 primer pairs: 24 in the ABCB family, 38 in the ABCC family, and 14 in the ABCG family. For each ABC transporter, primers were designed to avoid conserved regions (i.e. nucleotide binding domains). Two primer pairs (forward and reverse) were designed for each transporter gene, and the best pair was selected for each gene. Selected primer pairs are listed in Supplemental Table 2. Primers for control genes *Nodal*, *Nanos2*, and *Spz12* were generically designed from existing cDNA annotations. The *Ubiquitin* control primer pair was taken from <http://sugp.caltech.edu/SUGP/resources/methods/q-pcr.php>.

Primers were tested by amplification with serial dilutions of stock cDNA using the following criteria: confirmation of a “steep” amplification curve, single peak dissociation curve, and correct length (~100 bp) of a single amplicon on an agarose gel (Schmittgen and Livak, 2008). Serial dilutions of cDNA were made in 1x, 4x, 64x, 256x, and 1024x dilutions with water. Each primer was tested with these dilutions from eggs, 24 hpf and/or 55 hpf embryos to confirm that threshold cycle (Ct) increased 2 units for each dilution. Primer pairs that did not meet the above criteria were rejected from the study.

2.5.6. Confirmation of amplicon specificity to targeted genes

Amplicon specificity was confirmed for a subset of primers (*ABCB1a*, *ABCB4a*, *ABCC1*, *ABCC5a*, *ABCC9a*, and *ABCG2b*) by cloning and sequencing the qPCR products from embryos of two females. qPCR amplicons were purified and cloned into a pCR4-TOPO vector (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol, then sequenced (Retrogen, San Diego, CA, USA). The resulting sequences were searched using the Blastn algorithm on SpBase.org in the Sp genome v3.1 database. All inputs mapped exclusively to the targeted gene, confirming the qPCR primers were specific to individual targeted genes.

2.5.7. qPCR analyses

Gene expression changes are reported as fold differences with respect to the unfertilized egg. For *ABCC5a*, *ABCG2b*, *ABCG11*, and *ABCG12*, egg transcript levels were too low to quantify so expression is reported with respect to the earliest developmental stage at which quantification was possible (hatching blastula for *ABCC5a*, *ABCG11*, and *ABCG12*, and early prism for *ABCG2b*). The formula 2^x was used, where x is the threshold cycle (Ct) number difference between the reference stage (egg, hatching blastula or early prism) and the other stages of development ($\Delta\Delta\text{Ct}$ method). For example, the pre-normalized fold change for *ABCB1a* gene expression at the late gastrula stage is:

$$2^x_{ABCB1a} = 2^{(\text{Ct}_{\text{egg}} - \text{Ct}_{\text{late gastrula}})_{ABCB1a}}$$

Results were normalized to *Ubiquitin* according to (Juliano et al., 2006; Nemer et al., 1991; Peter and Davidson, 2009b) such that the reported value reflects the formula:

$$\text{Fold change} = 2^{\Delta\Delta\text{CT}_{\text{ABC1a}}} / 2^{\Delta\Delta\text{CT}_{\text{ubiquitin}}}$$

Reported data are an average of four females. Fold changes are significant if they are ± 2 -fold change from the reference point ($0.5 < \text{not significant} < 2$) (Peter and Davidson, 2009b).

2.5.8. Transcript copy number calculations

Transcript copy numbers per egg/embryo were calculated by quantifying the reference time point against a standard curve generated with dilutions of the sequenced TOPO-cloned qPCR amplicons. We calculated that each qPCR well contained the equivalent of 1.27 eggs/embryos per well based on the following: each *S. purpuratus* egg/embryo contains between 3.3 ng (Brandhorst, 2004) and 3.0 ± 0.2 ng total RNA through 60 hpf (Nemer et al., 1984). Using an average of these values (3.15 ng per egg/embryo) and the assumption that 50% of material is lost in converting mRNA to cDNA (Ransick, 2004), we converted 0.5 μg total RNA to cDNA to achieve ~ 80 embryos worth of cDNA in a 220 μl volume. We used 3.5 μl per qPCR well, corresponding to 1.27 eggs/embryos per well.

Using a DNA molecular weight calculator (www.bioinformatics.org), we determined the molecular weight of each TOPO-cloned qPCR amplicon. We made 4x serial dilutions of these plasmids in water at concentrations equivalent

to 1-1,048,576 copies per egg/embryo. We repeated qPCR with the serial dilution series and the reference time point cDNA samples. From the dilution series, we generated a standard curve by applying a nonlinear regression trendline fit (Microsoft Excel) in the format: $Y=ae^{-bX}$ where Y is copies per egg/embryo and X is Ct. Transcript numbers were calculated from these equations.

2.5.9. ABC transporter protein expression

Sp-ABCB1a and *Sp-ABCC5a* cDNAs were cloned from egg and gastrula stage RNA, respectively, by Rapid Amplification of cDNA Ends (RACE; Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Phusion High-Fidelity DNA polymerase (New England BioLabs) was used for all PCR reactions. Fluorescent proteins mCitrine and mCherry were subcloned into a modified pCS2 vector, and fusions were generated by inserting transporter cDNAs using XhoI for an N-terminal FP-ABCB1a fusion (ABCB1a-mCitrine), and SpeI for a C-terminal FP-ABCC5a fusion (ABCC5a-mCherry). All constructs were sequenced after cloning (Retrogen). Capped mRNA was made using the mMessage mMachine SP6 kit (Ambion, Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. mRNA was injected into fertilized eggs at 2-5% egg volume in a final concentration of 1 mg/ml in water (Lepage and Gache, 2004). Fluorescent protein localization was visualized on a Zeiss LSM-700 laser scanning confocal microscope using a Zeiss Plan APOChromat 20x air objective (numerical aperture, 0.8) (Zeiss, Thornwood, NY, USA). All

images were captured using the Zen software suite (Zeiss, revision 5.5) and processed with ImageJ freeware (NIH, Bethesda, MD, USA).

2.5.10. Whole mount *in situ* hybridization (WMISH)

Templates for *in situ* probes were generated by cloning ~1.5kb of *ABCB1a* or *ABCC5a* into dual promoter pCRII-Topo TA vector (Invitrogen) according to the manufacturer's protocol. PCR was carried out with Phusion High-Fidelity DNA polymerase (New England BioLabs) using *ABCB1a*-mCitrine and *ABCC5a*-mCherry as templates. Primers and probe sequences are listed in Supplemental Table 2. Probe templates were sequenced after cloning (Retrogen).

WMISH was performed following a modified protocol (Ransick, 2004). Briefly, hatched blastula and later stage embryos were cultured as described. Cleavage-stage embryos were fertilized in 1 mM para-aminobenzoic acid (PABA) and passed through a 60 μ m filter to remove the fertilization envelope, then cultured as described. Swimming embryos were pelleted by cooling and gentle centrifugation. A dense aliquot of eggs or embryos was distributed among wells of plastic round-bottom plates to form a monolayer at the base of each well. Specimens were fixed on ice in two steps: 1) 20 minutes with 0.625% glutaraldehyde in Fixation Buffer (FB: 32.5 mM MOPS buffer, pH 7.0; 162.5 mM NaCl; 32.5% FSW), then transferred to 2) 1.25% glutaraldehyde in FB overnight at 4°C.

Specimens were washed once in FB without glutaraldehyde then three times in Tris-buffered Saline + Tween-20 (TBST: 0.1 M Tris buffer, pH 7.5; 0.15 M NaCl; 0.1% Tween-20). Proteinase K was applied for 10-15 minutes at 50 ng/ μ l in TBST. The digestion was stopped with 25 mM glycine in TBST, then specimens were washed two times in TBST and post-fixed for 30 minutes at room temperature with 4% paraformaldehyde in 50 mM MOPS buffer, pH 7.0 + 150 mM NaCl. Preceding hybridization, specimens were washed three times in TBST then transitioned into Hybridization Buffer (HB: 50% formamide; 5x Saline-Sodium Citrate (SSC); 20 mM Tris-base, pH 7.5; 5 mM EDTA; 0.1% Tween-20; 2x Denhardt's Solution; 50 μ g/ml Heparin; 500 μ g/ml yeast tRNA) in three steps: 30% HB in TBST, 60% HB in TBST, then 100% HB. Specimens were incubated in HB for 1 h at 60°C to pre-hybridize.

Digoxigenin (DIG)-labeled antisense probes were made by *in vitro* transcription using Sp6 or T7 RNA polymerase (New England Biolabs) with DIG RNA Labeling Mix (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Sense probes were used as negative controls. Probes were diluted to 1 ng/ μ l in HB and heated to 70°C for 5 min, then added to specimens for 12-16 h hybridization at 60°C. Post-hybridization washes included 15-20 min incubations at 60°C in the following solutions: HB; 50% HB + 50% 2X SSCT (2X SSC + 0.2% Tween-20); 2X SSCT; 0.5X SSCT; 0.2X SSCT. Specimens were then returned to room temperature, transferred to clean wells, and washed three times in TBST.

Specimens were blocked for 30 min at room temperature with 10% sheep serum + 1 mg/ml Bovine Serum Albumin (BSA) in TBST. Anti-digoxigenin-AP, Fab fragments from sheep (Roche) were added in a 1:1000 dilution in 5% sheep serum + 1 mg/ml BSA in TBST, then incubated for 1 h at room temperature. Specimens were washed three times in TBST, then two times in Alkaline Phosphate Buffer (APB: 100 mM Tris-base pH 9.5; 100 mM NaCl; 50 mM MgCl₂; 1 mM levamisole; 0.1% Tween-20). Stain was developed in 0.3375 mg/ml NBT + 0.175 mg/ml BCIP in APB for 1-24 h depending on the probe and transcript abundance. Reactions were quenched with 50 mM EDTA in TBST. Specimens were transitioned into 50% glycerol in TBST with 5 mM Na-azide, and then photographed with a Canon EOS 60D camera (Canon, Lake Success, NY, USA) through a 40x air (0.75NA) Neofluar objective on a Zeiss Axiovert S100 microscope.

2.6. ACKNOWLEDGEMENTS

The authors would like to thank Drs. Victor Vacquier and Linda Holland, and members of the Hamdoun Lab for helpful discussions of the manuscript. We thank Dr. Andrew Ransick for generously providing the updated protocol and helpful suggestions for WMISH, and Drs. Demian Koop, Linda Holland, and Nick Holland for helpful feedback while implementing the WMISH protocol. H2B-CFP was kindly provided by Drs. Scott Fraser and Sean Megason. This work was supported by NICHD 058070 to AH. LES was supported by a National Science Foundation Graduate Research Fellowship (NSF GRF) and a National Defense Science and Engineering Graduate (NDSEG) Fellowship.

2.7. SUPPLEMENTARY MATERIAL

Table S2.2. Primers used for qPCR, *in situ* hybridization, RACE, and cloning.

Gene	Gene ID	Primers for qPCR
Sp-ABCB1a	SPU_001752	F: TACCGTTTCAGTCCCTCAACC R: AAGCTCCCTTGATCCCTTGT
Sp-ABCB1b	SPU_002431	F: GCTGGCCCTGTTACATTAT R: CTTGTTTCTGGGTGCCTGTT
Sp-ABCB4a	SPU_007014	F: CGCAAAGCTAGAGCAGGAGT R: CAGTCCTGACGCAAGACAAA
Sp-ABCB6	SPU_018342	F: GCCAAGGAAGAGCTACATGC R: GCCCACTCAGAGGATTTTCG
Sp-ABCB7	SPU_003241	F: CTCTGCAGATAGCCCTCAG R: GCTGTTCAAGGAGGTATCCA
Sp-ABCB8	SPU_024666	F: CTTCTGGCATCCAATGAGGT R: GATCGCTGGACATTCTGTGA
Sp-ABCB9a	SPU_026825	F: CATTGTGGCTGAAGAAACCA R: GGTAGGTGATGCGGAGCTTA
Sp-ABCB10a	SPU_016850	F: CGTACGCTCACTGTCCAGAA R: CACAGTTCGGATGCTGCTTA
Sp-ABCC1	SPU_026395	F: AGTCTTGGGTTGCTGCTCAT R: TATACGGCTGGCAAGTCTCC
Sp-ABCC4a	SPU_020669	F: AACCAACAGTCGGGAAGTTGG R: CGATCCAGACGTAGTGACAGA
Sp-ABCC4b	SPU_024191	F: CATGCAGACTCCCTCTCCTC R: GTTTCTTCCAGGGGTTCTCC
Sp-ABCC4c	SPU_002411	F: CTGTGCGCAGAATACAGGAA R: AAGGTTAGCATCGGACGAGA
Sp-ABCC5a	SPU_023723	F: GAAGGCAACGATCCTCTCAG R: CCCAAACCCTTCATCATTGT
Sp-ABCC9a	SPU_025903	F: CTGGGGAAAATCGACAAAAA R: CAATGAGCACAAACCCTGCT
Sp-ABCC9b	SPU_028797	F: AGCGGGTGCAGCTATAGAAA R: CTTTGTGGGTGTTATTGGT
Sp-ABCC9d	SPU_007764	F: TCCAGGCCAAGATTGCTAGT R: CATCTCGTTGGACAGCTTCA
Sp-ABCG2b	SPU_014013	F: CGAGCGATGTTTATTCACGA R: ATAGGTATGGCACGACGAGAC
Sp-ABCG9	SPU_012874	F: CGTACGCAACGACAGATAAA R: AAAGTTCCTGTGGGTCAACG
Sp-ABCG11	SPU_020849	F: GCAGTCGGTTATTTGGGAGA R: GATGTAGAACCACCGAGGA
Sp-ABCG12	SPU_015080	F: ACAGCACATCGATCAAGCAG R: TACCTGAACAGCAGCGAATG
Sp-Polyq (Ubiquitin)	SPU_021496	F: CACAGGCAAGACCATCACAC R: GAGAGAGTGCGACCATCCTC
Sp-Nodal	SPU_011064	F: ACCACGGAGCTACCAATCAG R: CGGAGAGAATGATCGGAGAG
Sp-Nanos	SPU_003591	F: GCAAGAACAACGGAGAGAGC R: CCGCATAATGACAGGTGTA
Spz12	SPU_003470	F: GGTACGGATGAGGAGAACGA R: CGGCAATGTATGGTCTTGTG

Gene	Gene ID	Primer purpose	Primer sequence	Notes
Sp-ABCB1a	SPU_001752	RNA <i>in situ</i> probe	F: CCAATGGCAATGGAACAGTGCAGA	location in gene (bp): 83
		RNA <i>in situ</i> probe	R: TCGCTCTCCAACCAATGTGGAGTA	location in gene (bp): 1710
		5' RACE*	R: CGTCAAGAGATTGCCTGGTTTGATGTC	
		5' nested RACE	R: GCTTACTTTCAAGTGGTGTGTGGGAC	
Sp-ABCC5a	SPU_023723	cDNA cloning	F: TTA CTCTCGAGCCTCCACAACATGGGTAGAGGGT	Xho1 NH2 clone
		cDNA cloning	R: CCTTGCTCGAGTTAATGCTGCATAGACTGGCA	Xho1 NH2 clone
		RNA <i>in situ</i> probe	F: ATGATTATAGAAGGCAACGATCCTCTCAG	location in gene (bp): 1
		RNA <i>in situ</i> probe	R: ATCCCAGCTGTATCGCATTGAGT	location in gene (bp): 1557
		5' RACE	R: TCAGTTCTCCGGGTGCTTCCCGTCCGT	
		3' RACE	F: TCCAGCAGCAACTTGAAGTCCGGGTGAGGGA	
cDNA cloning		F: GCGACTAGTCATGATTATAGAAGGCAACG	Spe1 COOH clone	
		R: GGGACTAGTATTGTGCCACAATGGTTG	Spe1 COOH clone	

*ABCB1a 3' sequence was obtained from annotation and verified by cloning.

2.8. REFERENCES

- Aller, S. G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., Harrell, P. M., Trinh, Y. T., Zhang, Q., Urbatsch, I. L., et al. (2009). Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **323**, 1718–1722.
- Bangert, I., Tumulka, F. and Abele, R. (2011). The lysosomal polypeptide transporter TAPL: more than a housekeeping factor? *Biol Chem* **392**, 61–66.
- Borst, P. and Elferink, R. O. (2002). Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **71**, 537–592.
- Bosnjak, I., Uhlinger, K. R., Heim, W., Smital, T., Franekić-Colić, J., Coale, K., Epel, D. and Hamdoun, A. (2009). Multidrug efflux transporters limit accumulation of inorganic, but not organic, mercury in sea urchin embryos. *Environ Sci Technol* **43**, 8374–8380.
- Brandhorst, B. P. (2004). Isolating DNA, RNA, polysomes, and protein. *Methods Cell Biol* **74**, 579–599.
- Bryan, J., Munoz, A., Zhang, X., Duefer, M., Drews, G., Krippeit-Drews, P. and Aguilar-Bryan, L. (2007). ABCC8 and ABCC9: ABC transporters that regulate K⁺ channels. *Pflugers Arch* **453**, 703–718.
- Bunting, K. (2002). ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* **20**, 11–20.
- Chen, W., Paradkar, P. N., Li, L., Pierce, E. L., Langer, N. B., Takahashi-Makise, N., Hyde, B. B., Shirihai, O. S., Ward, D. M., Kaplan, J., et al. (2009). Abcb10 physically interacts with mitoferrin-1 (Slc25a37) to enhance its stability and function in the erythroid mitochondria. *Proc Natl Acad Sci USA* **106**, 16263–16268.
- Chen, Z.-S. and Tiwari, A. K. (2011). Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases. *FEBS J* **278**, 3226–3245.
- Coffman, J. A., Coluccio, A., Planchart, A. and Robertson, A. J. (2009). Oral-aboral axis specification in the sea urchin embryo III. Role of mitochondrial redox signaling via H₂O₂. *Dev Biol* **330**, 123–130.
- Davidson, E. H. (1986). Gene activity in early development. *Academic Press*.
- de Wolf, C. J. F., Yamaguchi, H., van der Heijden, I., Wielinga, P. R.,

- Hundscheid, S. L., Ono, N., Scheffer, G. L., de Haas, M., Schuetz, J. D., Wijnholds, J., et al.** (2007). cGMP transport by vesicles from human and mouse erythrocytes. *FEBS J* **274**, 439–450.
- Dean, M., Rzhetsky, A. and Allikmets, R.** (2001). The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* **11**, 1156–1166.
- Eibling, L., Berger, W., Rehberger, A., Waldhor, T. and Micksche, M.** (1993). P-glycoprotein regulates chemosensitivity in early developmental stages of the mouse. *FASEB J* **7**, 1499–1506.
- Evans, J. M., Day, J. P., Cabrero, P., Dow, J. A. T. and Davies, S.-A.** (2008). A new role for a classical gene: white transports cyclic GMP. *J Exp Biol* **211**, 890–899.
- Ewart, G. D., Cannell, D., Cox, G. B. and Howells, A. J.** (1994). Mutational analysis of the traffic ATPase (ABC) transporters involved in uptake of eye pigment precursors in *Drosophila melanogaster*. Implications for structure-function relationships. *J Biol Chem* **269**, 10370–10377.
- Fjose, A., Polito, L. C., Weber, U. and Gehring, W. J.** (1984). Developmental expression of the white locus of *Drosophila melanogaster*. *EMBO J* **3**, 2087–2094.
- Fletcher, J. I., Haber, M., Henderson, M. J. and Norris, M. D.** (2010). ABC transporters in cancer: more than just drug efflux pumps. *Nat Rev Cancer* **10**, 147–156.
- Foltz, K. R., Adams, N. L. and Runft, L. L.** (2004). Echinoderm eggs and embryos: procurement and culture. *Methods Cell Biol* **74**, 39–74.
- Goldstone, J. V., Hamdoun, A., Cole, B. J., Howard-Ashby, M., Nebert, D. W., Scally, M., Dean, M., Epel, D., Hahn, M. E. and Stegeman, J. J.** (2006). The chemical defensome: environmental sensing and response genes in the *Strongylocentrotus purpuratus* genome. *Dev Biol* **300**, 366–384.
- Hamdoun, A. M., Cherr, G. N., Roepke, T. A. and Epel, D.** (2004). Activation of multidrug efflux transporter activity at fertilization in sea urchin embryos (*Strongylocentrotus purpuratus*). *Dev Biol* **276**, 452–462.
- He, S. M., Li, R., Kanwar, J. R. and Zhou, S. F.** (2011). Structural and functional properties of human Multidrug Resistance Protein 1 (MRP1/ABCC1). *Curr Med Chem* **18**, 439–481.
- Herget, M. and Tampé, R.** (2007). Intracellular peptide transporters in human--

compartmentalization of the "peptidome". *Pflugers Arch* **453**, 591–600.

- Higgins, C. F.** (1992). ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* **8**, 67–113.
- Jedlitschky, G., Burchell, B. and Keppler, D.** (2000). The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *J Biol Chem* **275**, 30069–30074.
- Juliano, C. E., Voronina, E., Stack, C., Aldrich, M., Cameron, A. R. and Wessel, G. M.** (2006). Germ line determinants are not localized early in sea urchin development, but do accumulate in the small micromere lineage. *Dev Biol* **300**, 406–415.
- Krishnamurthy, P. and Schuetz, J. D.** (2006). Role of ABCG2/BCRP in biology and medicine. *Annu Rev Pharmacol Toxicol* **46**, 381–410.
- Lepage, T. and Gache, C.** (2004). Expression of exogenous mRNAs to study gene function in the sea urchin embryo. *Methods Cell Biol* **74**, 677–697.
- Leslie, E. M., Deeley, R. G. and Cole, S. P.** (2001). Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. *Toxicology* **167**, 3–23.
- Leslie, E. M., Deeley, R. G. and Cole, S. P. C.** (2005). Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol Appl Pharmacol* **204**, 216–237.
- Mackenzie, S., Howells, A., Cox, G. and Ewart, G.** (2000). Sub-cellular localisation of the White/Scarlet ABC transporter to pigment granule membranes within the compound eye of *Drosophila melanogaster*. *Genetica* **108**, 239–252.
- Nam, J., Su, Y.-H., Lee, P. Y., Robertson, A. J., Coffman, J. A. and Davidson, E. H.** (2007). Cis-regulatory control of the nodal gene, initiator of the sea urchin oral ectoderm gene network. *Dev Biol* **306**, 860–869.
- Nasonkin, I., Alikasifoglu, A., Ambrose, C., Cahill, P., Cheng, M., Sarniak, A., Egan, M. and Thomas, P. M.** (1999). A novel sulfonyleurea receptor family member expressed in the embryonic *Drosophila* dorsal vessel and tracheal system. *J Biol Chem* **274**, 29420–29425.
- Nemer, M., Rondinelli, E., Infante, D. and Infante, A. A.** (1991). Polyubiquitin RNA characteristics and conditional induction in sea urchin embryos. *Dev Biol* **145**, 255–265.

- Nemer, M., Travaglini, E. C., Rondinelli, E. and D'Alonzo, J.** (1984). Developmental regulation, induction, and embryonic tissue specificity of sea urchin metallothionein gene expression. *Dev Biol* **102**, 471–482.
- Peter, I. S. and Davidson, E. H.** (2009a). Genomic control of patterning. *Int J Dev Biol* **53**, 707–716.
- Peter, I. S. and Davidson, E. H.** (2009b). The endoderm gene regulatory network in sea urchin embryos up to mid-blastula stage. *Dev Biol* **340**, 188–199.
- Petrini, M., Galimberti, S., Sabbatini, A., Bianchi, F., Bernardini, N., Dolfi, A. and Lupetti, M.** (1995). Expression of multidrug resistance (mdr) gene(s) in primary lymphoid organs of chicken immune system during embryonic development. *Experientia* **51**, 137–140.
- Ransick, A.** (2004). Detection of mRNA by in situ hybridization and RT-PCR. *Methods Cell Biol* **74**, 601–620.
- Ransick, A., Rast, J. P., Minokawa, T., Calestani, C. and Davidson, E. H.** (2002). New early zygotic regulators expressed in endomesoderm of sea urchin embryos discovered by differential array hybridization. *Dev Biol* **246**, 132–147.
- Revilla-i-Domingo, R., Oliveri, P. and Davidson, E. H.** (2007). A missing link in the sea urchin embryo gene regulatory network: hesC and the double-negative specification of micromeres. *Proc Natl Acad Sci USA* **104**, 12383–12388.
- Ricardo, S. and Lehmann, R.** (2009). An ABC transporter controls export of a *Drosophila* germ cell attractant. *Science* **323**, 943–946.
- Samanta, M. P., Tongprasit, W., Istrail, S., Cameron, R. A., Tu, Q., Davidson, E. H. and Stolc, V.** (2006). The transcriptome of the sea urchin embryo. *Science* **314**, 960–962.
- Saxena, M., Stephens, M. A., Pathak, H. and Rangarajan, A.** (2011). Transcription factors that mediate epithelial-mesenchymal transition lead to multidrug resistance by upregulating ABC transporters. *Cell Death Dis* **2**, e179.
- Schmittgen, T. and Livak, K.** (2008). Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* **3**, 1101–1108.
- Smeets, M., Raymakers, R., Vierwinden, G., Pennings, A., vandeLocht, L.,**

- Wessels, H., Boezeman, J. and deWitte, T.** (1997). A low but functionally significant MDR1 expression protects primitive haemopoietic progenitor cells from anthracycline toxicity. *Brit J Haematol* **96**, 346–355.
- Stieger, B., Meier, Y. and Meier, P. J.** (2007). The bile salt export pump. *Pflugers Arch* **453**, 611–620.
- Susanto, J., Lin, Y.-H., Chen, Y.-N., Shen, C.-R., Yan, Y.-T., Tsai, S.-T., Chen, C.-H. and Shen, C.-N.** (2008). Porphyrin homeostasis maintained by ABCG2 regulates self-renewal of embryonic stem cells. *PLoS ONE* **3**, e4023.
- Toomey, B. and Epel, D.** (1993). Multixenobiotic resistance in *Urechis caupo* embryos: protection from environmental toxins. *Biol Bull* **185**, 355.
- Wu, C. T., Budding, M., Griffin, M. S. and Croop, J. M.** (1991). Isolation and characterization of *Drosophila* multidrug resistance gene homologs. *Mol Cell Biol* **11**, 3940–3948.
- Wu, S.-Y., Ferkowicz, M. and McClay, D. R.** (2007). Ingression of primary mesenchyme cells of the sea urchin embryo: a precisely timed epithelial mesenchymal transition. *Birth Defects Res C Embryo Today* **81**, 241–252.
- Yabe, T., Suzuki, N., Furukawa, T., Ishihara, T. and Katsura, I.** (2005). Multidrug resistance-associated protein MRP-1 regulates dauer diapause by its export activity in *Caenorhabditis elegans*. *Development* **132**, 3197–3207.
- Zhao, C., Tampé, R. and Abele, R.** (2006). TAP and TAP-like--brothers in arms? *Naunyn Schmiedebergs Arch Pharmacol* **372**, 444–450.
- Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., Lagutina, I., Grosveld, G. C., Osawa, M., Nakauchi, H., et al.** (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* **7**, 1028–1034.
- Zutz, A., Gompf, S., Schägger, H. and Tampé, R.** (2009). Mitochondrial ABC proteins in health and disease. *Biochim Biophys Acta* **1787**, 681–690.

2.8. CHAPTER ACKNOWLEDGMENTS

Chapter 2, in full, is a reprint of Shipp, L.E. and Hamdoun A. ATP-binding cassette (ABC) transporter expression and localization in sea urchin development. *Developmental Dynamics* 2012, 241 (6): 1111-1124. It has been reformatted for this dissertation. The dissertation author was the primary investigator and author of this paper.

CHAPTER 3.
ABCC5 IS REQUIRED FOR cAMP-MEDIATED HINDGUT INVAGINATION IN
SEA URCHIN EMBRYOS

3.1. ABSTRACT

ATP-binding cassette (ABC) transporters are evolutionarily conserved proteins that pump diverse substrates across membranes. Many are known to transport signaling molecules and are extensively expressed during development. However, their role in secretion of extracellular signals that regulate embryogenesis is largely unexplored. Here we show that a mesodermal ABCC (MRP) transporter is necessary for orientation of endoderm movement in sea urchin embryos. This transporter, *Sp*-ABCC5a (*C5a*), is expressed in pigment cells and their precursors, which are a subset of the non-skeletogenic mesoderm (NSM) cells. *C5a* expression depends on Delta-Notch signaling from skeletogenic mesoderm and is downstream of *gcm* in the aboral NSM gene regulatory network. Long term imaging of development reveals *C5a* knockdown embryos gastrulate, but ~90% develop a prolapse of the hindgut by the late prism stage (~eight hours after *C5a* protein expression normally peaks). Since *C5a* orthologs efflux cyclic nucleotides, and cAMP-dependent protein kinase (*Sp*-CAPK/PKA) is expressed in pigment cells, we examined whether *C5a* could be involved in cAMP signaling. Consistent with this hypothesis, membrane-permeable pCPT-cAMP rescues the prolapse phenotype in *C5a*-knockdown embryos, and causes archenteron hyper-invagination in control embryos. In addition, the cAMP-producing enzyme soluble adenylyl cyclase (sAC) is expressed in pigment cells, and its inhibition impairs gastrulation. Together our data indicate that in pigment cells, *C5a*-mediated transport of sAC-derived cAMP

controls late invagination of the hindgut. Little is known about the ancestral functions of ABCC5/MRP5 transporters, and this study reveals a novel role for these proteins in mesoderm-endoderm signaling during embryogenesis.

3.2. INTRODUCTION

Morphogenesis is choreographed by extracellular signals. These include short-range signals, between neighboring cells, and long-range signals that work across the embryo. A central question is how long-range signals are distributed to act specifically. Active transport is an essential mechanism for secretion and organization of signals that act over long distances (Müller and Schier, 2011). For instance in plant root development, precise spatial and temporal distribution of transporters establishes directional gradients that direct tip growth (Robert and Friml, 2009), and in fly development active transporters secrete molecules that attract germ cells (Ricardo and Lehmann, 2009). Despite this importance of active signaling in development, transporters are largely unexplored in animal embryos. Tackling their biology is important, because it could help define the origin and destination of secreted signals.

In this study, we define novel functions of a member of the ATP-binding cassette (ABC) transporter family. This family includes active transporters that translocate signaling molecules across membranes. ABC transporters are found in all organisms and have a wide range of endogenous and exogenous substrates. Among the ABC family are the multifunctional “multidrug resistance” (MDR) transporters of the ABCB, -C, and -G subfamilies, which have primarily been studied for their xenobiotic efflux, because their overexpression in cancer cells leads to drug resistance (Chen and Tiwari, 2011; Cole, 2014). However, in addition to effluxing drugs, these transporters can also contribute to disease by

transporting signaling molecules that govern morphogenetic behaviors of cells (Fletcher et al., 2010; Henderson et al., 2011; Jin et al., 2014; van de Ven et al., 2009; Weekes et al., 2013).

We previously demonstrated that MDR ABC transporters are extensively expressed during embryogenesis (Goldstone et al., 2006; Shipp and Hamdoun, 2012). Among them, the ABC transporter ABCC5 (MRP5, MOAT-C, pABC11, sMRP) is unique in that, although being related to proteins involved in protective efflux and drug resistance (Belinsky et al., 1998; Chen and Tiwari, 2011; Kool et al., 1997; McAleer et al., 1999), it has not been demonstrated as toxicologically important (Chen and Tiwari, 2011; Leslie et al., 2001). ABCC5 has been reported to efflux cGMP and cAMP (Jedlitschky et al., 2000; Sager and Ravna, 2009; Wielinga et al., 2003), heme (Korolnek et al., 2014), and/or hyaluronan (Schulz et al., 2007), but its function remains poorly understood.

Our previous study indicated that a sea urchin homolog of ABCC5, *Strongylocentrotus purpuratus* (*Sp*)-ABCC5a (C5a), may be expressed in non-skeletogenic mesenchyme (NSM) cells and that its subcellular localization and the timing of its expression suggest a possible developmental function (Shipp and Hamdoun, 2012). NSM cells are specified by Delta/Notch (D/N) signaling, and subsets of these cells differentiate into pigment cells, blastocoelar cells, circumesophageal muscle cells, and coelomic pouch cells (Materna and Davidson, 2012; Sherwood and McClay, 1999; Sweet et al., 2002). Blastocoelar cells are specifically derived from the oral NSM, whereas the aboral NSM gives

rise to pigment cells (Ruffins and Ettensohn, 1996). Both pigment and blastocoelar cells generate larval immunocytes (Solek et al., 2013), but their regulatory states are distinct (Materna et al., 2013; Solek et al., 2013).

The timing of *C5a* expression, with peak mRNA levels just after gastrulation, is coincident with a 10-fold increase in levels of the C5 substrate cAMP, from 2 fmol/embryo in mid-gastrulae to ~20 fmol/larva in prism/early plutei (Soliman, 1984). However, despite high cAMP levels in gastrulae and plutei, as well as increased gene expression of cAMP-dependent protein kinase (*Sp*-CAPK/PKA) (Wei et al., 2006), activity of CAPK/PKA is low at these stages (Fujino and Yasumasu, 1981). This temporal uncoupling of cAMP levels and CAPK/PKA activity suggests that after gastrulation, cAMP has additional targets beyond CAPK (Rast et al., 2002).

Here we use knockdown, transporter expression, and long term imaging to dissect the function of *C5a*. Consistent with a developmental role, *C5a* is less “promiscuous” than other MDR transporters and appears to have a narrower range of substrates. *C5a* is expressed in aboral NSM cells at the convergence of events surrounding endomesoderm specification, and it is regulated by D/N signaling emanating from the skeletogenic mesoderm (SM). Morpholino (MASO)-knockdown of *C5a* does not block pigment cell differentiation but alters late stages of invagination, causing prolapse of the hindgut. This prolapse is rescued with the *C5a* substrate cAMP, which originates from soluble adenylyl cyclase (sAC) in pigment cells and mediates hindgut invagination. Collectively, the results

advance understanding of ABCC5 and shed light on developmental signaling in gastrulation.

3.3. RESULTS

3.3.1. C5a is a 210 kDa protein similar to human ABCC5/MRP5

The predicted tertiary structure of C5a is similar to that of *Homo sapiens* (*Hs*)-ABCC5/MRP5 (Fig. 3.1A) (Leslie et al., 2001). C5a has two membrane spanning domains (MSDs), each consisting of six transmembrane helices (TMHs) connected by extracellular loops (ELs) and cytoplasmic loops (CLs). Two intracellular nucleotide (i.e. ATP) binding domains (NBDs) contain canonical Walker A, Walker B, and Walker C domains (Fig. 3.1A and (Gokirmak et al., 2012)). As with *Hs*-ABCC5, C5a lacks the N-terminal MSD0 characteristic of “long” ABCC transporters (such as ABCC1/MRP1) (Leslie et al., 2001) and is instead similar in topology to *Sp*-ABCB1a (P-glycoprotein, P-gp) (Gokirmak et al., 2012). C5a has two predicted N-linked glycosylation sites, both in MSD2, between TMH7 and 8, and between TMH11 and 12. Endogenous C5a has a molecular weight of approximately 210 kDa, while recombinant C5a-mCherry runs as a ~230 kDa doublet, consistent with addition of the 28 kDa mCherry tag (Fig. 3.1B, supplementary material Fig. S3.1). Our antibody also recognized an 80 kDa band throughout development, however expression of the 80 kDa band was inconsistent with the timing, molecular weight, and morpholino knockdown of C5a expression, indicating it was not C5a (Fig. S3.1). Non-specific immunoreactivity was subsequently removed by adsorption of the antisera to early embryos, prior to immunolocalization experiments (supplementary material).

3.3.2. C5a strongly effluxes FDA but not other chemicals in the fluorone or bodipy classes

In most organisms, ABCC (MRP-type) transporters efflux a range of structurally diverse compounds, including signaling molecules (reviewed in (Chen and Tiwari, 2011)), toxicants (Bosnjak et al., 2009), and fluorescent dyes (Gokirmak et al., 2012; Gökirmak et al., 2014; Litman et al., 2001; Strouse et al., 2013). In blastula stage embryos exposed to fluorescent compounds, we find that overexpression of C5a strongly reduces accumulation of FDA, but not CMFDA, calcein-AM (C-AM), BCECF-AM, bodipy-verapamil (b-Ver), or bodipy-vinblastine (b-Vin) (Fig. 3.1C), suggesting that C5a is less promiscuous than typical xenobiotic transporters (Gökirmak et al., 2014). C5a-overexpressing embryos accumulated just 17.1% (s.e.m. 0.63) of FDA levels measured in uninjected embryos, as quantified by FDA fluorescence (Fig. 3.1D). C5a-overexpressing

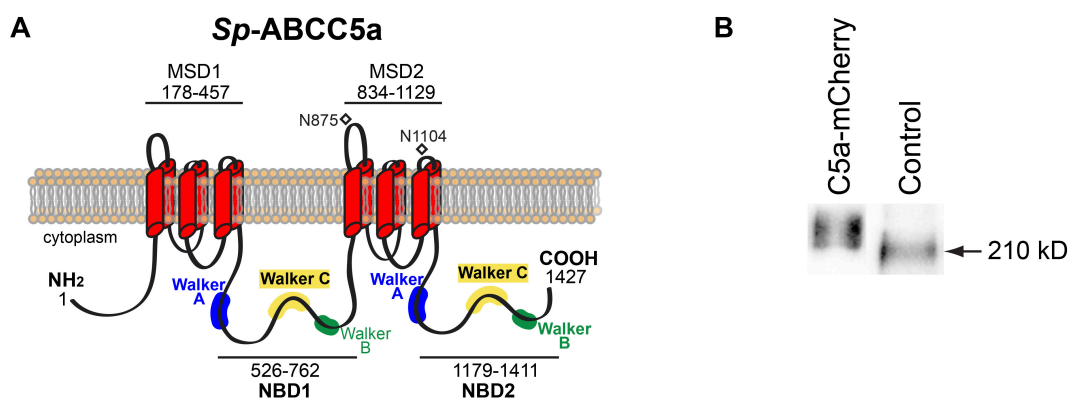


Figure 3.1. C5a is a 210 kDA membrane protein that is not a broad chemical transporter. (A) Topology model of C5a in a cell membrane. Predicted glycosylation sites are numbered and marked with diamonds. (B) Protein levels in 70 hpf embryos showing endogenous C5a (Control-injected; 200 embryos per lane) and overexpressed C5a (C5a-mCherry; 10 embryos per lane).

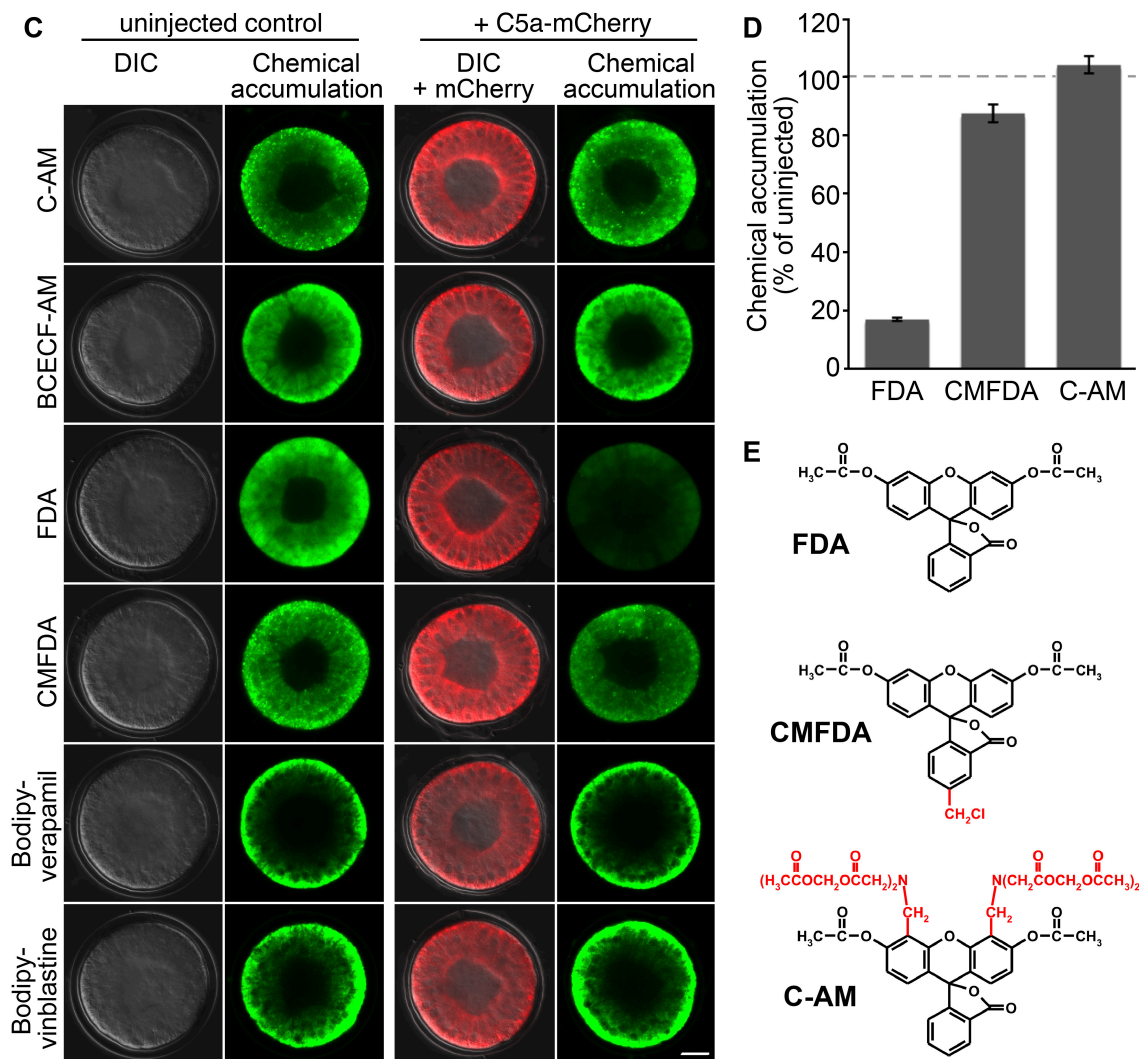


Figure 3.1. C5a is a 210 kDA membrane protein that is not a broad chemical transporter, Continued. (C) Representative micrographs showing accumulation of fluorone-based and bodipy-conjugated chemicals (green) in uninjected (differential interference contrast, DIC, only) and C5a-mCherry (red; DIC+mCherry) overexpressing embryos. (D) Mean accumulation (\pm s.e.m) of FDA, C-AM, and CMFDA in C5a-overexpressing embryos. $N \geq 3$. (E) Structures of dyes in (D).

embryos also had a weak but significant reduction of CMFDA (Fig. 3.1D), an FDA analog that is conjugated to glutathione prior to efflux (McAleer et al., 1999). C5a-overexpressing embryos accumulated 87.8% (s.e.m. 3.06) of control CMFDA

levels (Fig. 3.1D). Despite the structural similarities of FDA, CMFDA, and C-AM (Fig. 3.1E), accumulation of C-AM is not affected by C5a-overexpression (Fig. 3.1D). Overexpression of C5a also does not reduce accumulation of BCECF-AM, b-VER, or b-VIN (Fig. 3.1C).

3.3.3. C5a expression is highest during and immediately following gastrulation

Consistent with our previous report (Shipp and Hamdoun, 2012), C5a mRNA is first detected at hatching (21 hpf) and increases throughout gastrulation (Fig. 3.2A). *C5a* transcript levels peak at the late gastrula stage (42 hpf) with a 9.4-fold (± 2.6) increase compared to hatching, and transcript levels decrease from late gastrula to pluteus (74 hpf), with pluteus stage transcripts measuring 2.2-fold (± 0.7) of hatching levels (Fig. 3.2A). C5a protein levels follow that of the mRNA, with an eight-hour delay presumably reflecting the time needed for synthesis (Figs 3.2A,B). C5a protein is first reliably detected during early gastrulation (34 hpf; 48.9 ± 13.5 Arbitrary intensity units, AIU), and its levels increase throughout gastrulation, peaking at the prism stage (50 hpf; 79.3 ± 13.3 AIU). C5a protein levels decrease from prism to pluteus stage (74 hpf; 38.2 ± 13.0 AIU).

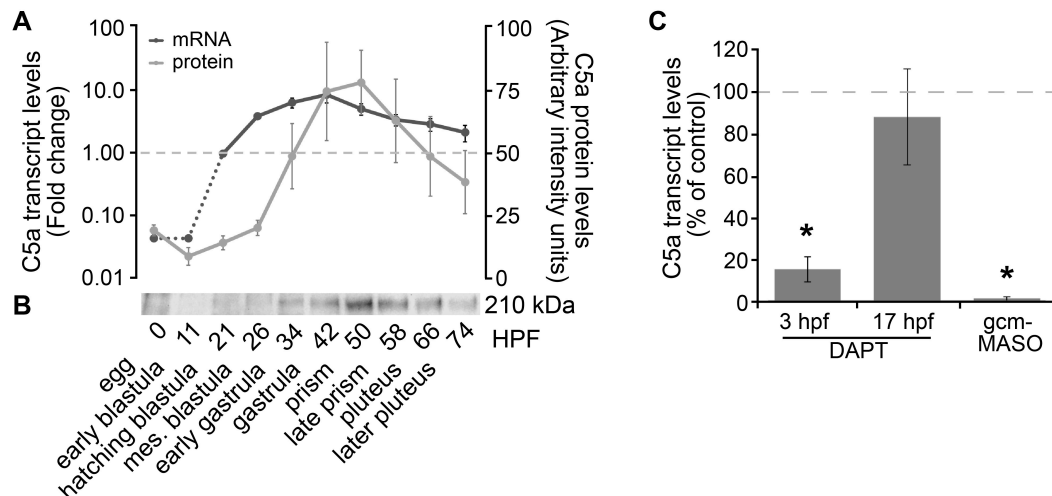


Figure 3.2. Expression of C5a peaks after gastrulation and is controlled by Delta/Notch signaling from the SM. (A) Mean levels (\pm s.e.m.) of C5a mRNA (dark gray, left axis) and protein (light gray, right axis). N = 4. (B) Representative Western blot. 200 embryos per lane. (C) Gastrula-stage *C5a* gene expression in response to D/N inhibition by DAPT or gcm-knockdown. Embryos were treated with DAPT at 3 hpf or 17 hpf, or injected with gcm-MASO. Transcript levels are shown as mean fold change (\pm s.e.m.) with respect to hatching-stage control (DMSO treated or uninjected). N = 3. Asterisks denote statistical significance ($p \leq 0.05$).

3.3.4. C5a is expressed in the pigment cell precursors

C5a is expressed in mesenchyme blastulae in a subset of vegetal cells likely to be NSM (Shipp and Hamdoun, 2012). Because two waves of D/N signaling specify the NSM (Materna and Davidson, 2012), we reasoned that *C5a* expression might be dependent on one of these waves. In the D/N signaling waves, the Notch ligand in NSM is activated by the Delta signal from either the SM (first wave) or a subset of the NSM (second wave) (Materna and Davidson, 2012). By exposing embryos to the γ -secretase inhibitor DAPT at 3 hpf and 17 hpf, Notch signaling is inhibited at the onset of either of the two waves. DAPT treatment at 3 hpf blocks *C5a* expression ($p \leq 0.008$; Fig. 3.2C), while 17 hpf DAPT treatment has no effect ($p \leq 0.32$). This indicates that Delta signaling

emanating from the SM between 3 and ~16 hpf is necessary for induction of *C5a*, which is consistent with *C5a* being expressed in NSM. In the aboral NSM, *Glial cells missing (gcm)* is the primary target of D/N signaling (Materna et al., 2013; Ransick and Davidson, 2006). Knockdown of *gcm* blocks expression of *C5a* ($p \leq 0.0001$; Fig. 3.2C), indicating that *C5a* is induced downstream of *gcm*.

In mesenchyme blastulae, *C5a* transcripts co-localize with those of *gcm* (Fig. 3.3A), which marks aboral NSM, the pigment cell precursors (Materna et al., 2013; Ransick and Davidson, 2006). In contrast, *Prox1*-expressing cells (blastocoelar cell precursors) (Materna et al., 2013) have little or no expression of *C5a* and *gcm* (Fig. 3.3A). A subset of *gcm*-positive pigment cells also express *C5a* protein, as shown in a mid-gastrula stage embryo (Fig. 3.3B) and prism stage embryos (supplementary material Fig. S3.1B,C). *C5a* protein is readily detected in the subset of *gcm*-positive cells that are distributed around the ectoderm at the mid-gastrula stage.

Collectively, these results indicate that *C5a* is expressed in pigment cells and their precursors. Pigment cells are mesodermal immunocytes (Solek et al., 2013) that emerge from the archenteron during gastrulation and distribute throughout the aboral ectoderm (Gibson and Burke, 1985; Gibson and Burke, 1987; Ransick and Davidson, 2006). *C5a*-expressing cells ingress during early gastrulation, and by mid/late gastrulation, they are no longer associated with the archenteron but have migrated across the blastocoel and are embedded in the

epithelium (Fig. 3.3C). By the prism stage, C5a protein is detected in cells distributed throughout the aboral ectoderm (Figs 3.3C,D), consistent in morphology and location with pigment cells (Gibson and Burke, 1985; Gustafson and Wolpert, 1967; Materna and Davidson, 2012; Ransick and Davidson, 2006).

In the transition from mesenchyme blastula to mid-gastrula stages, C5a moves from intracellular compartments to the plasma membrane (Fig. 3.3D). In mesenchyme blastula embryos, ingressing pigment cells have intracellular C5a protein and none on the cell periphery (Fig. 3.3D). Notably, at this stage C5a is undetectable by Western blot (Fig. 3.2B). By the mid-gastrula stage, a pigment

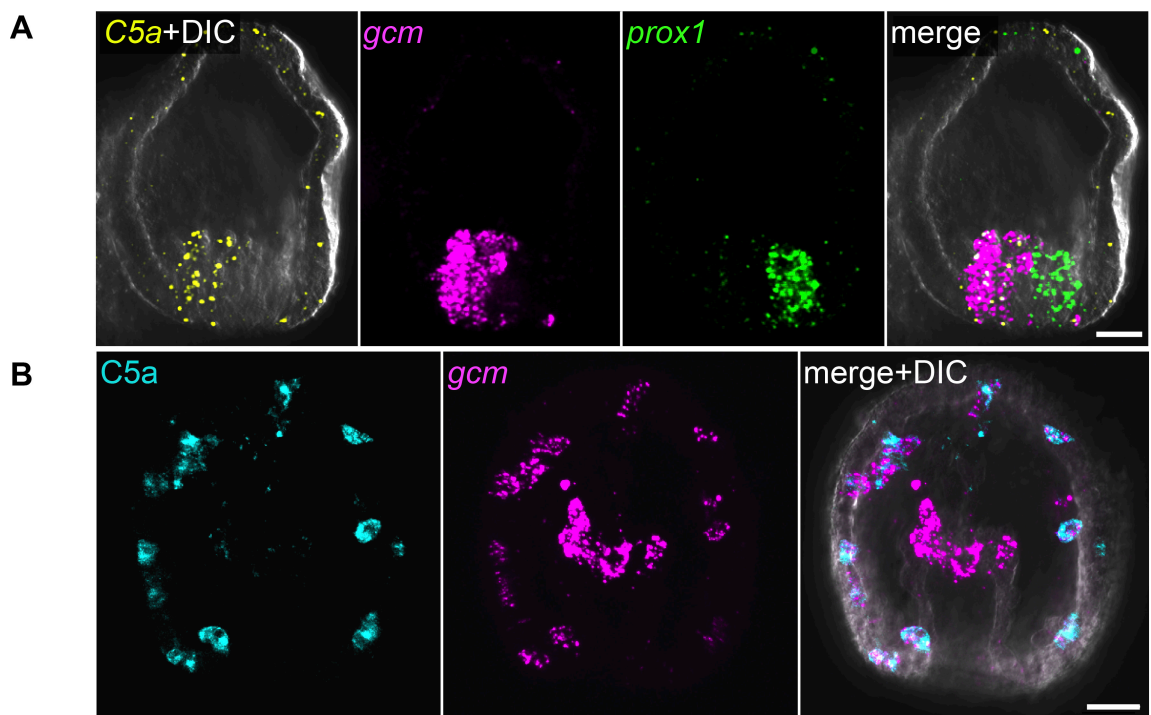


Figure 3.3. C5a is expressed in pigment cells that migrate away from the archenteron during early gastrulation. (A) Localization of *C5a* mRNA (yellow) with markers of aboral (*gcm*, magenta) and oral (*prox1*, green) NSM, shown by FISH in a mesenchyme blastula embryo. (B) Protein expression of C5a (cyan; immunolabeled) with a marker of aboral NSM (*gcm*, magenta; FISH) in a mid-gastrula stage embryo.

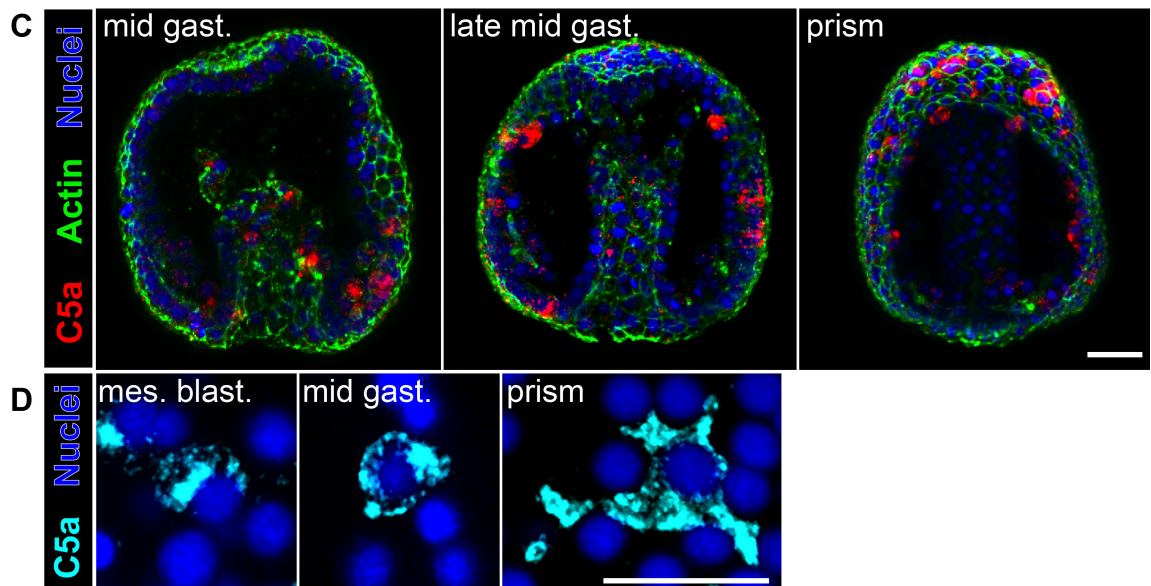


Figure 3.3. C5a is expressed in pigment cells that migrate away from the archenteron during early gastrulation, Continued. (C) Distribution of C5a-expressing pigment cells within the embryo, shown throughout gastrulation. C5a (red) is immunolabeled, phalloidin marks actin (green), and Hoechst marks nuclei (blue). (D) Subcellular localization of C5a (cyan; immunolabeled) from mesenchyme blastula to just after gastrulation (prism). C5a-expressing cells are located in their respective embryos as follows: ingressing from vegetal pole in the mesenchyme blastula; embedding in aboral ectoderm in the mid-gastrula; moving within aboral ectoderm in the prism.

cell embedded in the ectoderm shows that C5a has moved to the plasma membrane (Fig. 3.3D). This cell has both intracellular and plasma membrane localized C5a. By the prism stage, pigment cells extend motile pseudopodia (Gibson and Burke, 1987; Gustafson and Wolpert, 1967), and C5a is localized both intracellularly and along the plasma membrane of these projections (Fig. 3.3D).

3.3.5. C5a expression is required for gut formation, but not pigment cell formation

Two non-overlapping translation-blocking MASOs (supplementary material Fig. S3.2A) knock down C5a expression to levels that are undetectable on Western blots (supplementary material Fig. S3.1A). In embryos injected with either MASO1 or MASO2, a protrusion (or prolapse) of the gut is seen by the pluteus stage (supplementary material Fig. S3.2C), indicating that expression of C5a is necessary for gastrulation. The two MASO-induced phenotypes are indistinguishable, and results from MASO1 are shown throughout the remaining experiments.

Given that C5a is expressed in pigment cells, we hypothesized that knockdown of this protein might affect pigment cell formation. In contrast, pigment cells differentiated and produced echinochrome pigment (Griffiths, 1965) after C5a knockdown, suggesting that C5a is not essential for pigment cell formation. Pigment cells of C5a-knockdown embryos are distributed around the aboral ectoderm in similar patterns to those of control embryos (Figs 3.4Ai,Bi). However, in C5a-knockdown embryos, pigment cell pseudopodia and positioning relative to the ectoderm may be altered. In control embryos, pigment cells have long pseudopodia that extend from the cell, and the cells embed themselves within the ectoderm such that they are in contact with the external environment (Fig. 3.4Ai). In C5a-knockdown embryos, there is a reduction in pigment cell pseudopodia, and the cells often sit just beneath the surface of the ectoderm (Fig. 3.4Bi), much like the sub-ectodermal positions of pigment cells several hours earlier in normal development (Ransick and Davidson, 2006).

3.3.6. C5a expression is required for orientation of the hindgut

Since the most robust phenotype with either MASO1 or MASO2 was prolapse of the gut (Fig. 3.4Bii-iv), we measured the frequency of gut prolapse after knockdown. Prior to gastrulation, few if any abnormalities are evident in knockdown embryos (Figs 3.4C,D) apart from a slight developmental delay commonly seen with MASOs in sea urchins. Through 36 hpf, $\geq 93\%$ (± 7) of knockdown embryos are indistinguishable from controls (Figs 3.4C,D). By 48 hpf, control embryos are all full gastrulae, and 41% (± 26) of knockdown embryos are indistinguishable from controls, while 59% show an abnormal elongation of the

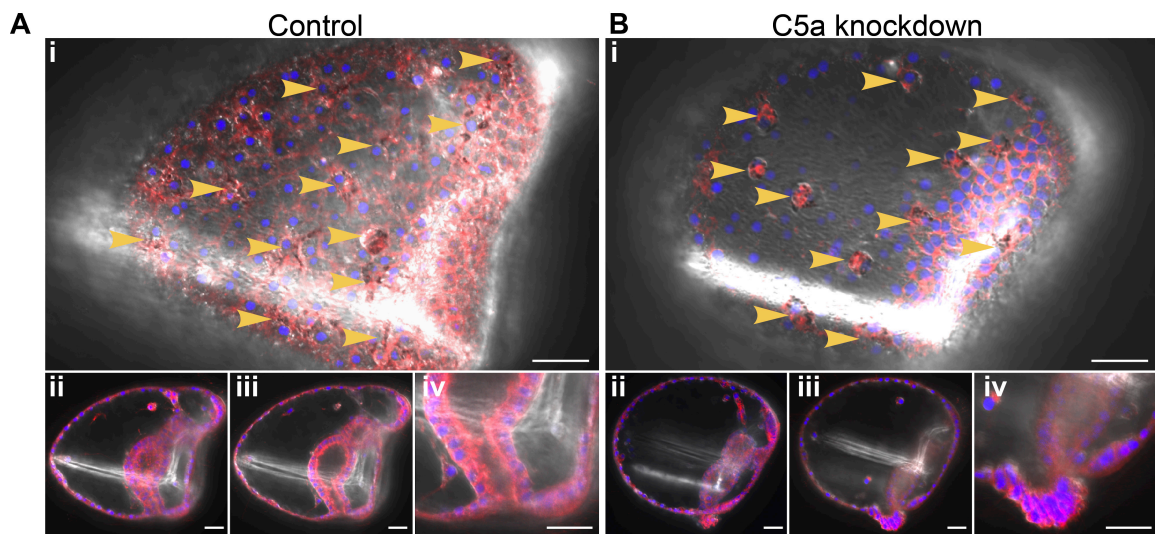


Figure 3.4. Gut prolapse is the major morphology in C5a-knockdown embryos. (A,B) Overview of the C5a-MASO phenotype shown with (A) a control embryo and (B) a C5a-knockdown embryo. Membranes (red) and nuclei (blue) are labeled with LCK-mCherry and H2B-CFP. Shown are: (i) distribution of pigment cells (yellow arrows) within the aboral ectoderm; (ii,iii) two deeper cross-sections showing gut and skeleton morphology; and (iv) higher magnification view of the hindgut region.

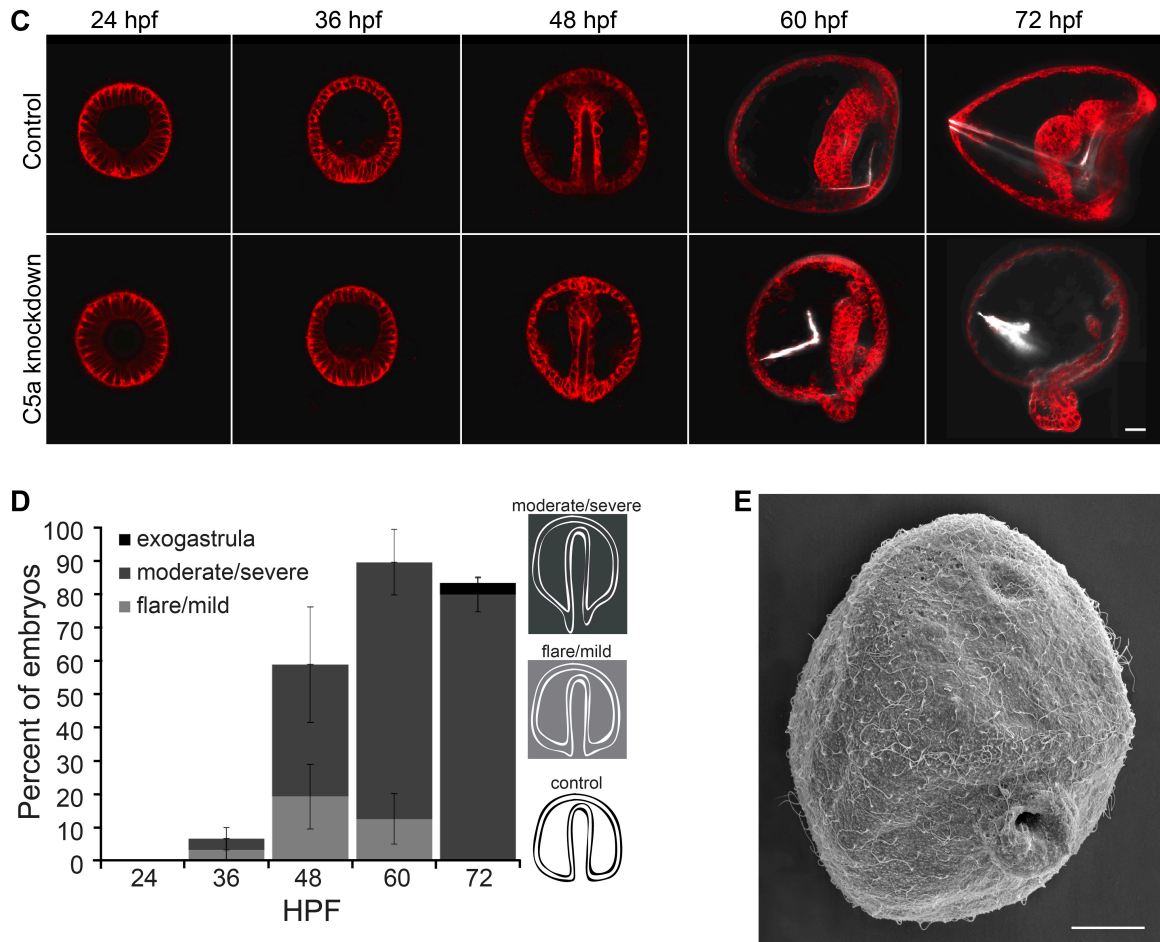


Figure 3.4. Gut prolapse is the major morphology in C5a-knockdown embryos, Continued. (C) A time-course of the C5a-knockdown phenotype is shown from 24-72 hpf with representative embryos. Membranes (red) are labeled with LCK-mCherry. (D) Mean (\pm s.e.m.) frequency of prolapse in C5a-knockdown embryos from 24-72 hpf. Cartoons depict the moderate/severe (dark gray) and flare/mild (light gray) categories. (E) Scanning electron micrograph of a representative C5a-knockdown embryo at 60 hpf. The invaginated stomodeum (mouth) is at the top, and the prolapsed anus is at the base of the image.

vegetal pole (Figs 3.4C,D). Nineteen percent (\pm 10) of embryos have only a mild prolapse, and 40% (\pm 17) have a moderate/severe prolapse (Fig. 4D).

At 60 hpf, control embryos are at the prism stage, with growing skeletal rods, and extended archenterons that may be beginning to differentiate into gut

compartments (Fig. 3.4C). At this time, 90% (± 6) of C5a-knockdown embryos have a clear prolapse of the gut. 77% (± 10) of knockdown embryos have a moderate/severe prolapse, and 13% (± 8) have a flare/mild prolapse (Fig. 3.4D). In some cases, we also observe absence or deformation of the skeletal rods. Fig. 4C shows an example of a 60 hpf moderate hindgut prolapse. By the 72 hpf pluteus stage, control embryos have elongated skeletal rods, a tripartite gut, and a fused mouth (Fig. 3.4C). In C5a-knockdown embryos, the severity of the prolapse and the degree of skeletal formation vary. 80% (± 5) of C5a knockdown embryos have a moderate/severe prolapse, and a small percent ($4\% \pm 2$) of embryos have guts that become everted into exogastrulae (Fig. 3.4D). Fig. 3.4C shows a severe prolapse, where the gut has collapsed against the oral/ventral wall of the embryo.

Finally, a scanning electron micrograph (EM) of a C5a-knockdown embryo shows a difference in ciliation of ectoderm tissue versus the prolapsed tissue (Fig. 3.4E). While the ectoderm is characteristically covered in long, uniform cilia, these long cilia are absent from the prolapsed tissue, suggesting the prolapse is not composed of ectoderm but is instead formed from the endoderm.

3.3.7. Live cell confocal time-lapse imaging of C5a-morphants

To further define the nature of the defect induced by removal of C5a, live cell confocal time-lapse movies were used to study changes in morphology through development of C5a-knockdowns. In these embryos, the archenteron

tips make contact with the stomodeums, and mesenchyme cells migrate across blastocoels (Fig. 3.5). In embryo 5 of Movie S1 (Fig. 3.5A), SM cells assume their appropriate places on either side of the vegetal poles of the embryos. Prolapses of the hindguts are evident after the archenterons have already elongated (Fig. 3.5A), indicating a distinction between this phenotype and typical exogastrulation effects.

In Movie S2 (Fig. 3.5B), a control and C5a knockdown embryo are shown side-by-side following the completion of gastrulation. In the control, the overall shape of the embryo remains circular/ovular, as the vegetal pole/hindgut remain flush with the ectoderm (yellow arrow). In the C5a knockdown embryo, the gut prolapses to create a protrusion from the vegetal pole (yellow arrow). This protrusion is shown in cross-section in Movie S3 (Fig. 3.5C), and isosurface

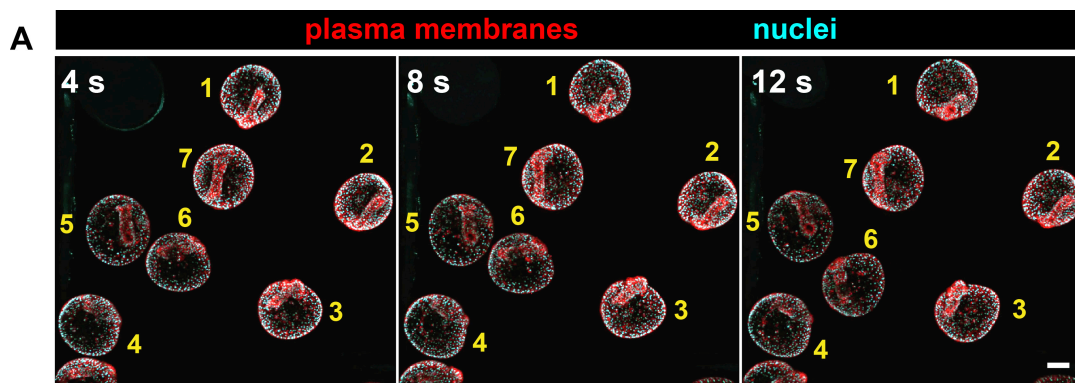


Figure 3.5. Long-term imaging of C5a-knockdown embryos reveals defects in hindgut formation. Long-term time-lapses are shown as still shots from the supplemental movies. Images are labeled with the corresponding time (seconds) on the movie. In all movies, membranes (red) are labeled with LCK-mCherry, and nuclei (blue) are labeled with H2B-CFP. **(A)** Movie S1. Maximum intensity projections (MIPs) of seven C5a-knockdown embryos. Scale bar is 50 μm .

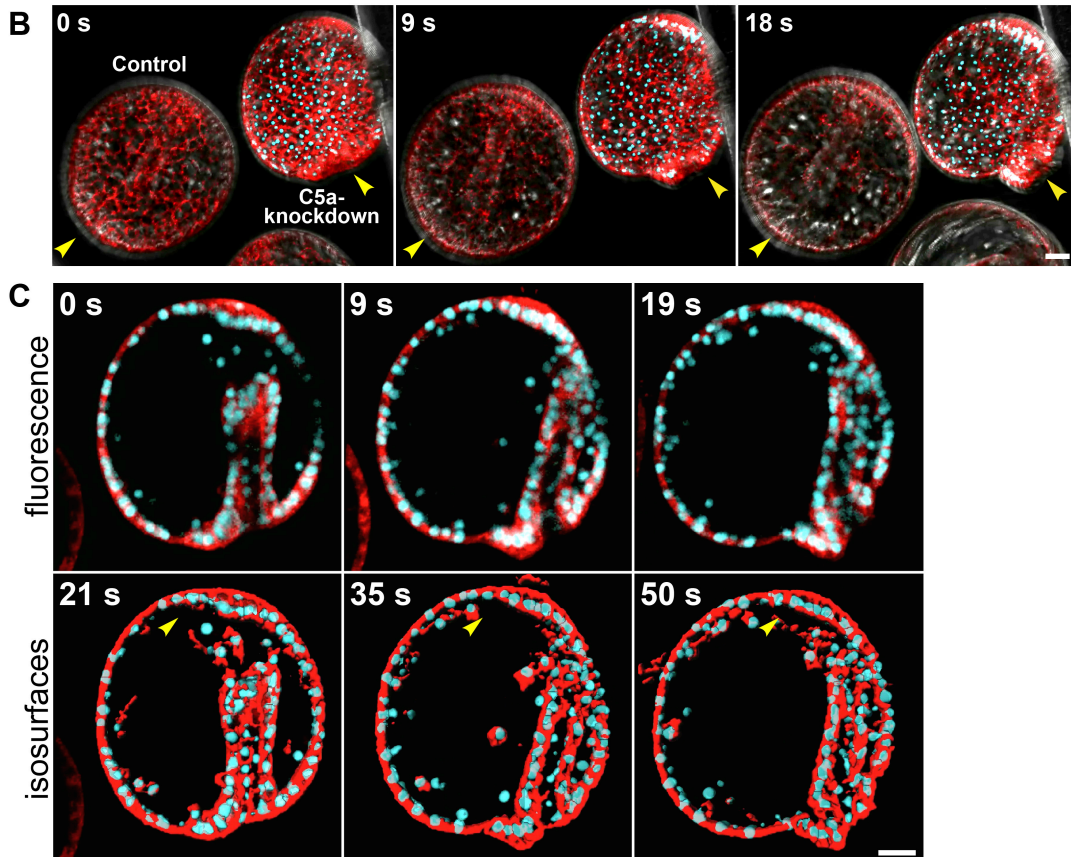


Figure 3.5. Long-term imaging of C5a-knockdown embryos reveals defects in hindgut formation, Continued. (B) Movie S2. MIPs of a control embryo (membrane labeled only) and a C5a-knockdown embryo (membrane and nuclei labeled) shown side-by-side. Vegetal poles are indicated with yellow arrows. (C) Movie S3. Cross-sections of the C5a-knockdown embryo from (C). The same three time points are shown both as raw fluorescence and as isosurfaces. Yellow arrows indicate the presumptive site of the anal sphincter.

rendering of this embryo highlights movements of NSM filopodia. At the beginning of the experiment, the archenteron is elongated and is oriented facing the presumptive oral hood. Only a subtle thickening is seen at the vegetal pole. Within 1 hour (1 s of movie), the archenteron tip has correctly changed orientation such that it contacts the presumptive stomodeum beneath the oral hood. NSM cells delaminate from the archenteron and migrate across the

blastocoel. The vegetal pole continues to thicken, and as the archenteron elongates, the hindgut protrudes. The site that should become the anal sphincter is visible at the interface between the thinner ectoderm cells and the thicker gut cells of the prolapse (Fig. 3.5C, yellow arrows).

3.3.8. C5a-mediated prolapse is rescued by cAMP, which is derived from sAC in pigment cells

Since mammalian ABCC5 effluxes cyclic nucleotides (Jedlitschky et al., 2000; Sager and Ravna, 2009; Wielinga et al., 2003), and there is evidence for cAMP signaling in C5a-expressing pigment cells (i.e. expression of *Sp*-cAMP-dependent protein kinase (CAPK/PKA) (Rast et al., 2002)), we tested if there is a link between cAMP signaling and C5a. When mesenchyme blastulae are exposed to membrane permeable cyclic nucleotide analogs, pCPT-cAMP or pCPT-cGMP, the hindguts hyper-invaginate (Fig. 3.6A). Exposing C5a-knockdown embryos to these analogs rescues hindgut prolapse (Fig. 3.6A) in a dose-dependent manner (Figs 3.6B,C). With the cGMP analog, the percent of embryos that prolapse ($84\% \pm 7$ in controls) is significantly reduced ($p \leq 0.05$) by both $25 \mu\text{M}$ ($45\% \pm 7$) and $100 \mu\text{M}$ ($18\% \pm 2$) pCPT-cGMP (Fig. 3.6B). However, the cAMP analog is more potent than the cGMP analog, and reduces the frequency of prolapse from 90% (± 4) in controls to 72% (± 14) at $1 \mu\text{M}$, 29% (± 14) at $10 \mu\text{M}$, and 13% (± 8) at $20 \mu\text{M}$ (Fig. 3.6C).

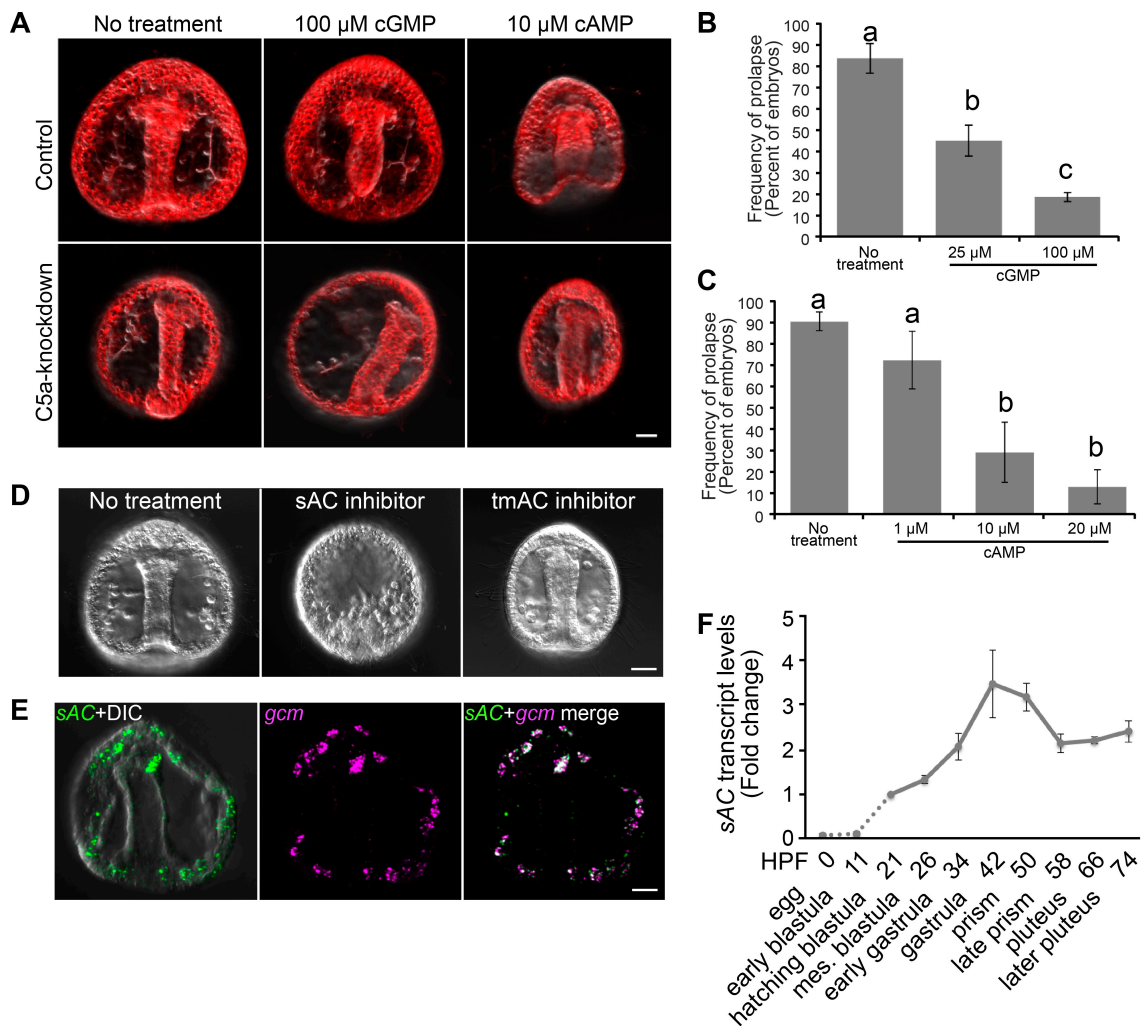


Figure 3.6. C5a-knockdown mediated prolapse is rescued by cAMP, which is derived from sAC in pigment cells. (A) Control (with membrane label) and C5a-knockdown (MASO and membrane label) embryos treated at the mesenchyme blastula stage with pCPT-cGMP or pCPT-cAMP, and imaged at the 68 hpf pluteus stage (oral view). (B,C) Mean (\pm s.e.m.) frequency of prolapse in C5a-knockdown embryos treated with pCPT-cGMP (B) or pCPT-cAMP (C). Letters denote statistical significance ($p \leq 0.05$). (D) cAMP production was perturbed by treating mesenchyme blastulae with the adenylyl cyclase inhibitors KH7 (10 μ M, sAC-specific) and 2',5'-dideoxyadenosine (100 μ M, tmAC-specific). 48 hpf prism larvae are shown. (E) Localization of sAC mRNA (green) with a marker of aboral NSM (*gcm*, magenta), shown by FISH in a gastrula embryo. (F) Mean levels (\pm s.e.m.) of sAC mRNA. N = 3.

Endogenous cAMP is produced by soluble adenylyl cyclase (sAC) and transmembrane adenylyl cyclase (tmAC), and these enzymes can be inhibited

with KH7 (sAC-specific) and 2',5'-dideoxyadenosine (tmAC-specific) (Beltrán et al., 2007; Tresguerres et al., 2011). In embryos exposed to these inhibitors, gut formation is impaired with 10 μ M KH7, but not 100 μ M 2',5'-dideoxyadenosine (Fig. 3.6D), indicating that sAC-produced cAMP is most important for gastrulation. In addition, *sAC* gene expression (Figs 3.6E,F) parallels that of *C5a* (Figs 3.2A,3.3A,3.3B) in that *sAC* mRNA localizes to *gcm*-positive pigment cells (Fig. 3.6E), and mRNA levels peak just after gastrulation (Fig. 3.6F).

3.4. DISCUSSION

ABC transporters are extensively utilized by the embryo (Goldstone et al., 2006; Gökirmak et al., 2014; Shipp and Hamdoun, 2012), yet most of their functions remain unknown. While some MDR transporters play important roles in xenobiotic efflux, others more likely have ancestral roles in sensing and signaling (Nigam, 2015). Here we report a novel developmental function for C5a in morphogenesis. The protein is expressed in migratory pigment cells, which are immunocytes derived from mesoderm, and knockdown of C5a leads to gut defects, including prolapse from the blastopore after gastrulation. The C5 substrate cAMP mediates invagination, causing hyper-invagination in control embryos, and rescuing the prolapse phenotype in C5a-knockdown embryos. Inhibition of sAC, which generates cAMP in pigment cells, blocks gastrulation. Together these data support a model in which C5a-mediated efflux of sAC-derived cAMP from pigment cells controls late invagination of the hindgut (Fig. 3.7).

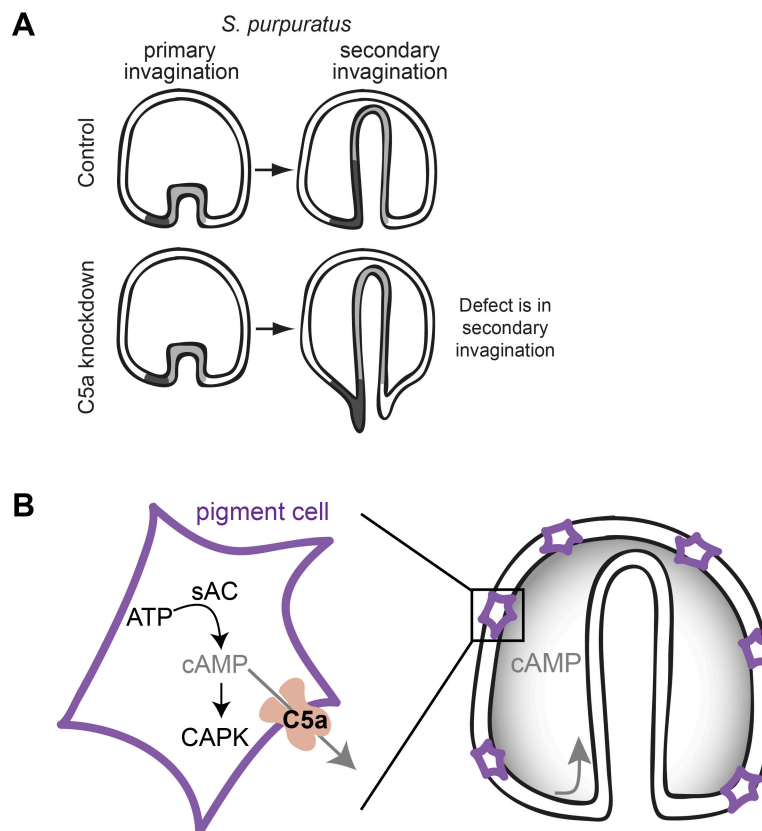


Figure 3.7. A model of C5a-mediated gut invagination. (A) In *S. purpuratus*, a subset of veg1 cells contribute to the elongating archenteron late in gastrulation, during secondary invagination (Barnet, 2011; Ransick and Davidson, 1998). In C5a-knockdown embryos, we observe defects in secondary, rather than primary invagination. (B) C5a-expressing pigment cells embed in the aboral ectoderm and efflux sAC-derived cAMP into the blastocoel. This cAMP promotes hindgut invagination and orientation.

3.4.1. Substrates of C5a

Consistent with its developmental role, C5a has narrower substrate selectivity than other sea urchin multidrug resistance protein (MRP/ABCC) transporters. For example, *Sp*-ABCC1, like C5a, is an MRP that localizes to basolateral membranes when overexpressed in sea urchin embryos. Unlike C5a, ABCC1 effluxes a wide variety of compounds, including C-AM, BCECF-AM, Fluo3-AM, CMFDA, FDA, b-VIN, and members of the cyanine and anthracene

dye families (Gökirmak et al., 2014). Of the six substrates we tested here (four fluorones and two bodipy-conjugates), only FDA is strongly effluxed from C5a-overexpressing embryos.

A number of studies have shown that mammalian ABCC5 transports cyclic nucleotides (Boadu and Sager, 2004; Jedlitschky et al., 2000; Meyer Zu Schwabedissen et al., 2005; Sager and Ravna, 2009; Sager et al., 2012; Wielinga et al., 2003; Wijnholds et al., 2000). cGMP is reported to be a higher-affinity substrate than cAMP, but the exact cGMP affinity remains unclear (de Wolf et al., 2007; Pratt et al., 2005; Reid et al., 2003; Wielinga et al., 2003). We find that while both pCPT-cGMP and pCPT-cAMP rescue the C5a-knockdown phenotype, cAMP is effective at lower concentrations than cGMP. Additionally, sAC activity, cAMP, and C5a, are all necessary for gut development, together suggesting that in sea urchins, cAMP is the relevant C5a substrate mediating hindgut morphogenesis. As cAMP and cGMP are structurally similar, the rescue by cGMP may be caused by off-target effects on cAMP-responsive pathways, or there may be an additional and/or synergistic role for cGMP.

3.4.2. C5a is expressed in motile pigment cells but is necessary for gastrulation

Although C5a is expressed in pigment cells and is downstream of *gcm*, it does not appear to be required for pigment cell differentiation. C5a-knockdown embryos have echinochrome-containing pigment cells, even in embryos with gut

prolapse. The positions of pigment cells in knockdown embryos – distributed around the aboral ectoderm – are similar to those of control embryos (Gibson and Burke, 1985; Gibson and Burke, 1987; Ransick and Davidson, 2006), though they are positioned slightly further from the apical surface than in controls.

Pigment cell specification is dependent on the Delta signal from SM cells, which activates Notch signaling in the NSM and establishes the aboral NSM gene regulatory network (GRN) (Materna and Davidson, 2012; Sherwood and McClay, 1999; Sweet et al., 2002). When we treated embryos at 3 hpf with DAPT to inhibit D/N signaling emanating from SM cells, *C5a* gene expression was blocked. Furthermore, knockdown of *Glial cells missing (gcm)*, the primary Notch target in aboral NSM cells (Materna et al., 2013; Ransick and Davidson, 2006), also blocked *C5a* expression, indicating that *C5a* gene expression is activated as part of the aboral NSM GRN.

Interestingly, when *gcm* is knocked down by MASOs, the resulting embryos have an ‘albino phenotype’ lacking pigment, but defects in gut morphogenesis are less prevalent and include exogastrulation at higher MASO concentrations (Ransick and Davidson, 2006). Perturbing D/N signaling from SM, the upstream inducer of *gcm* and *C5a*, resulted in a large fraction of embryos exogastrulating, consistent with previous reports (Materna and Davidson, 2012). However, while *C5a*-knockdown leads to embryos with differentiated pigment cells and prolapsed hindguts, *gcm*-knockdown leads to embryos with undifferentiated pigment cells and (usually) normal guts. One possibility is that

the gcm-knockdown is pleiotropic and that it induces an alternative pathway that bypasses the need for C5a.

3.4.3. Contributions of veg-lineage cells to the gut and timing of hindgut morphogenesis

Gastrulation in sea urchin embryos varies by species in regards to timing, the angle of the archenteron, and the contributions of different cell types to the archenteron (Barnet, 2011; Hardin, 1989; Hardin and McClay, 1990; Logan and McClay, 1997). In *S. purpuratus*, the mechanism of archenteron elongation resembles that of *L. variegatus* in that some veg1 cells ultimately contribute to the archenteron (Logan and McClay, 1997; Ransick and Davidson, 1998). Very late in gastrulation, a subset of veg1 cells (*brachyury*-expressing) involute and produce the anus/hindgut, while a distinct subset of veg1 cells remains a part of the vegetal ectoderm (Barnet, 2011; Gross and McClay, 2001). The timing of C5a-knockdown mediated gut prolapse is consistent with a defect in secondary, rather than primary, invagination (Fig. 3.7A). Given that a subset of veg1-derived cells form the *S. purpuratus* hindgut late in gastrulation, it is likely that C5a efflux activity in pigment cells is required for proper movement and orientation of veg1-derived hindgut cells. Interestingly, pigment cells have been reported to affect gastrulation in *Echinometra mathaei* (Takata and Kominami, 2004), a sea urchin closely related to *S. purpuratus* (Smith et al., 2006). However, unlike our findings,

pigment cells influence gastrulation movements during primary invagination in *E. mathaei*.

3.4.4. How does C5a in pigment cells affect the position of the hindgut?

Based on our observations, the relevant efflux activity is most likely from plasma membrane-localized C5a, which would efflux a compound into either intercellular space or the blastocoel (Fig. 3.7B). C5a may be necessary to establish a signaling gradient in the blastocoel, or it may directly signal to competent hindgut cells and direct/orient their movements or polarity. Either mechanism could alter cell adhesion, polarity, and/or chemokinesis.

If plasma membrane C5a effluxes cAMP from pigment cells, extracellular cAMP could function via GPCRs on the hindgut to induce invagination. In *Dictyostelium* development, cAMP secreted by ABC transporters stimulates chemotaxis (Miranda et al., 2015), and chemotaxis is an important mechanism of cell movement during deuterostome gastrulation (Dormann and Weijer, 2006). cAMP can activate GPCRs directly (Miranda et al., 2015) or can first be extracellularly metabolized to adenosine (Godinho et al., 2015). The absence of cAMP efflux in C5a-knockdowns may compromise the orientation of late-invaginating cells, leading them to fold outward as a consequence of gut elongation.

Based on the localization of C5a, we considered possibility that intracellular C5a could pump cAMP into membrane-bound compartments. cAMP

compartmentalization is necessary to selectively activate discrete cAMP-dependent signaling pathways within a cell, and ABC transporters can fine-tune cyclic nucleotide distribution (Cheepala et al., 2012). Compartmentalized cAMP could conceivably serve as a store similar to Ca^{++} in the endoplasmic reticulum, which could be released upon stimulation to trigger a rapid cellular response. We observe that pigment cells do not embed in the ectoderm as successfully in C5a-knockdowns as in control embryos, possibly suggesting that C5a could function cell-autonomously. However, since the hindgut defect is the most prominent C5a-knockdown phenotype, we hypothesize that the major role of C5a is in paracrine signaling through efflux of cAMP.

Finally, it is interesting to note that a phenotype similar to our observed hindgut prolapse was reported by (Burke et al., 1991), which was caused by antibodies disrupting apical lamina glycoproteins/fibropillins (Burke et al., 1998). A connection between C5a activity, pigment cell embedding, and the apical lamina is currently unclear, but it is conceivable that altered pigment cell embedding may alter the landscape of ectoderm adhesion molecules and amplify the effect caused by altered cAMP signaling.

3.4.5. Evolutionary implications of C5a-mediated gut morphogenesis

ABCC transporters often exhibit one-to-one orthology across broad phylogenetic spans (Goldstone et al., 2006; Whalen et al., 2012). For example, zebrafish, mouse, and human ABCC5 are more closely related to one another

than to other ABCC family members from the same organism (Korolnek et al., 2014), suggesting potential conservation of function. We find that the fluorescent substrates of sea urchin C5a are roughly similar to those reported for *Hs-ABCC5*. McAleer et al., 1999, showed that *Hs-ABCC5* strongly effluxes FDA and CMFDA, but does not efflux C-AM or rhodamine dyes. Similarly, we found that C5a strongly effluxes FDA, weakly effluxes CMFDA, and does not efflux C-AM. An exception is BCECF-AM, which is moderately effluxed by *Hs-ABCC5* (McAleer et al., 1999), but not by C5a, possibly indicating modest divergence in substrate selectivity.

The primary structure and subcellular localization of sea urchin C5a are also similar to those of its mammalian homologs. Based on our modeling, sea urchin C5a is predicted to have conserved topology in comparison with molecular models of *Hs-ABCC5* (Ravna et al., 2008; Sager et al., 2012). However, the size of C5a, ~210 kDa, is slightly larger than that of *Hs-ABCC5*, which has been detected at a range of molecular masses from 160-200 kDa (Aye et al., 2007; Jedlitschky et al., 2000; McAleer et al., 1999; Nies et al., 2002; Wijnholds et al., 2000), likely due to tissue-specific differences in glycosylation (Nies et al., 2002). In sea urchins, the over-expressed C5a localizes to basolateral membranes in polarized epithelial cells (Shipp and Hamdoun, 2012), and endogenous C5a is found both intracellularly and on the plasma membrane in polar (migratory) pigment cells. *Hs-ABCC5* primarily localizes to basal or basolateral plasma

membranes in polarized cells (Borst et al., 2007; Korolnek et al., 2014; Meyer Zu Schwabedissen et al., 2005; Wijnholds et al., 2000).

An unresolved question is whether the function of ABCC5 might also be evolutionarily conserved. In *C. elegans* and *D. rerio*, ABCC5 is thought to transport heme, and its knockdown causes embryonic lethality and reduced blood cell formation, respectively (Korolnek et al., 2014). In *Dictyostelium* development, ABCC5 is a candidate cAMP transporter, though it may be less critical than ABCB3 (Miranda et al., 2015). In mammals, studies of ABCC5 in development are sparse, but human ABCC5 is expressed in membranes of amniotic epithelia (Aye et al., 2007), and in cytotrophoblasts and syncytiotrophoblasts (Manceau et al., 2012; Meyer Zu Schwabedissen et al., 2005). Our study is the first detailed characterization of an ABCC5/MRP5 transporter in development. The highly regulated expression of this transporter and clear effects of its perturbation, underscore that better understanding of transporters is necessary for understanding signaling in development.

3.5. MATERIALS AND METHODS

3.5.1. Animals and reagents

Sea urchins, *Strongylocentrotus purpuratus*, were procured as described in Shipp and Hamdoun, 2012. Embryos were grown at 15°C. Stock solutions were prepared in dimethyl sulfoxide (DMSO) or Nanopure water and diluted to final concentrations in filtered seawater (FSW).

Efflux assays were performed with C-AM (calcein-AM) (Biotium, Hayward, CA); FDA (fluorescein diacetate) (Sigma, St. Louis, MO); and CMFDA (5-Chloromethylfluorescein diacetate), BCECF-AM (2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester), b-VER (bodipy-verapamil), and b-VIN (bodipy-vinblastine) (Life Technologies, Grand Island, NY). The γ -secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (EMD Millipore, Darmstadt, Germany) was used to assess the effects of D/N signaling on gene expression. Hoechst 33342 and Phalloidin-Alexa Fluor 488 (Life Technologies) were used to label nuclei and actin in immunolabeled embryos. Rescue experiments were performed with pCPT-cAMP and pCPT-cGMP (Sigma), and inhibition of adenylyl cyclase was performed with 2-(1H-benzimidazol-2-ylthio)-2-[(5-bromo-2-hydroxyphenyl)methylene]hydrazide, propanoic acid (KH7; sAC inhibitor) and 2',5'-dideoxyadenosine (tmAC inhibitor).

3.5.2. Gene expression analyses

RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction (qPCR) were performed according to Shipp and Hamdoun, 2012. Experiments were replicated with four separate batches of embryos for *C5a* and three batches for *sAC*. qPCR primers for *sAC* (SPU_012084) were: Fwd: 5'-AACTGGGACACAGAGGTTGG-3', Rev: 5'-CCTTCATTGCCTATGGTCGT-3'.

To assess the effect of D/N signaling on gene expression, embryos were treated with 8 μ M DAPT at 3 hpf or 17 hpf. DAPT inhibits cleavage of Notch, which blocks its ability to associate with Su(H) and activate transcription (Hughes et al., 2009; Materna and Davidson, 2012). RNA was isolated from unfertilized eggs (0 hpf; untreated), hatching blastulae (21 hpf), and gastrulae (43 hpf). *C5a* gene expression at the gastrula stage was quantified as fold change with respect to control samples at the earliest detectable stage: hatching (DMSO-treated, 21 hpf). The experiment was replicated with three batches. The effects of *gcm*-knockdown on *C5a* expression were determined similarly, only with *gcm*-MASO injected embryos from three separate batches.

Fluorescent RNA in situ hybridization (FISH) was performed with minor modifications from published protocols. Pre-hybridization washes, hybridization, and post-hybridization washes were performed according to Shipp and Hamdoun, 2012. All other steps were performed after Chen, Luo, and Su (Chen et al., 2011). *gcm* and *prox1* probes were used to mark aboral and oral NSM, respectively (Materna et al., 2013). Primers to generate the *sAC in situ* probe

were: Fwd: 5'-GATGTAGGTGAGAAGCCGTTAG-3', Rev: 5'-CGAGGAAGAGGCAACAAGAA-3'.

3.5.3. Microinjection of mRNAs and MASOs

Injections and mRNA syntheses were performed as described previously (Gokirmak et al., 2012; Shipp and Hamdoun, 2012). For C5a overexpression, we injected 0.8-1 mg/ml of C-terminal mCherry-tagged C5a mRNA (C5a-mCherry). For live-imaging, LCK-mCherry, LCK-mCitrine, and/or histone H2B-CFP mRNAs were injected at 0.05 mg/ml each to label membranes and nuclei.

To knock down C5a, morpholino antisense oligonucleotides (MASOs) were obtained from Gene Tools (Philomath, OR). C5a-MASO1: 5'-GAGGATCGTTGCCTTCTATAATCAT-3' (300 μ M). C5a-MASO2: 5'-TTATTTTCCCCGGCGTCATAAGTTT-3' (500-600 μ M). MASOs were co-injected with LCK-mCherry and/or histone H2B-CFP mRNA to label membranes and nuclei for live-imaging. *Gcm* was knocked down as described by Ransick and Davidson, 2006.

3.5.4. Western blot

Western blots were performed as described previously (Whalen et al., 2012) with affinity-purified anti-C5a antibody (described in supplementary material). For the developmental time-series, protein lysates were obtained from the same embryo batches and time points used for gene expression analyses.

Western blots were run with each of the four batches independently and probed with anti-C5a. Anti-C5a was used at 1:500, and secondary (goat anti-rabbit hrp) antibody was used at 1:2000. The 210 kDa C5a band was quantified by densitometry with ImageJ (NIH, Bethesda, MD).

3.5.5. Transporter efflux activity assays

Efflux assays were modified from Gokirmak et al., 2012. Briefly, uninjected (control) and C5a-CmCherry-injected (C5a overexpressing) embryos were grown to the ~14 hpf blastula stage, then incubated and washed prior to imaging as follows: 100 nM FDA or CMFDA (60 minutes, 10 washes, 30 minute FSW incubation); 125 nM b-VER or b-VIN (90 minutes, 10 washes); 250 nM C-AM or BCECF-AM (90 minutes). For each dye, experiments were replicated three or four times with different batches of embryos, and six embryos were measured per batch.

3.5.6. Immunohistochemistry (IHC)

Embryos were fixed in 4% PFA for 1 hour at 15°C and washed in 0.05% saponin in PBS, 50 mM glycine in PBS, and PBS. Samples were blocked for two hours at 15°C in 2% BSA with 5% goat serum in PBS, then washed with PBS and incubated in adsorbed anti-C5a primary antibody (described in supplementary material) at 14°C for ~14 hours. Samples that had already been

processed for FISH were blocked and treated with primary antibody, bypassing the initial fixation and wash steps. Embryos were washed in PBS then incubated at 14°C in 1:1000 goat anti-rabbit Alexa Fluor 594 (Life Technologies) secondary antibody in 2% BSA-PBS. Samples were washed in PBS and stained with 10 μ M Hoechst 33342 and 1.5 units/ml Phalloidin-Alexa Fluor 488 before imaging.

3.5.7. Quantification of C5a-knockdown phenotypes and rescue

To quantify the timing of the C5a-knockdown phenotype, embryos from three batches were injected with MASO1 and LCK-mCherry and/or histone H2B-CFP mRNAs for imaging. 18-19 embryos were used per batch for a total of 55 embryos, which were individually assessed at 24, 36, 48, 60, and 70 hpf by culturing one embryo per well (Costar 96-well round bottom plate, Fisher) in 100 μ l FSW. We classified gut prolapses as flare/mild, moderate/severe, or exogastrula (which was rare). Control injected (54 embryos, LCK and/or histone mRNA only) and uninjected (56 embryos) morphologies were also monitored and quantified, both of which resulted in 100% healthy embryos (not shown).

To quantify rescue of the C5a-knockdown phenotype, MASO1-injected embryos were treated with membrane permeable pCPT-cGMP or pCPT-cAMP at the mesenchyme blastula stage. Morphologies of 45-73 embryos from 4-5 batches per treatment were assessed at the 68 hpf pluteus stage, and any hindgut defect (mild to severe) was counted as prolapsed.

3.5.8. Microscopy

Unless otherwise indicated, all scale bars are 20 μm . Live-imaging for efflux assays was performed as described (Gokirmak et al., 2012), using a Zeiss LSM 700 laser scanning confocal microscope (Plan-Apochromat 20x air objective, 0.8 numerical aperture (NA); Zeiss, Thornwood, NY). Intracellular dye accumulation was quantified with the “measure” module of ImageJ. Efflux activity of C5a was determined by intracellular substrate fluorescence relative to uninjected control embryos.

To characterize embryo phenotypes, high-resolution images were captured on a Zeiss LSM 700 microscope with a Zeiss LDC-Apochromat 40X water objective (1.1 NA), and images were processed with ImageJ. To quantify C5a-knockdown phenotypes, embryos were monitored at 25-40X magnifications on a Leica M165FC stereoscope (Leica, Buffalo Grove, IL).

For FISH and IHC, embryos were mounted in TBST and imaged on a Zeiss LSM 700 microscope (20X or 40X objective). Time-lapse imaging of C5a-knockdown embryos is described in Supplementary Materials (Fig. S3.3), and all time-lapses were processed with Imaris 7.6.1 software (Bitplane, Zurich, Switzerland). For scanning electron microscopy (EM), embryos were fixed at 60 hpf in 2% glutaraldehyde in FSW, then processed and imaged according to Holland and Jespersen (Holland and Jespersen, 1973).

3.5.9. Statistics

Statistics were performed in JMP10 (SAS, Cary, NC) with one-way ANOVAs and Tukey-Kramer post-hoc comparisons ($p \leq 0.05$), blocked by female. For efflux assays, Arbitrary Fluorescence Units/area values were compared. For gene expression with DAPT treatment and gcm-MASO, fold-changes (with respect to Control hatching stage) were compared. For pCPT-cAMP and pCPT-cGMP rescue experiments, percentages of embryos prolapsed were compared.

3.6. ACKNOWLEDGEMENTS

We are grateful to Dr. Victor D. Vacquier, Joseph Campanale, Jose Espinoza, Lisa Mesrop, Dr. Sascha Nicklisch, Dr. Stefan Materna, Hannah Rosenblatt, and Alysha Vu for constructive discussions and assistance throughout this work. We thank Dr. Nicholas D. Holland for performing EM, Dr. Andrew Ransick for providing *gcm* and *prox1* FISH probes, Dr. Yi-Hsien Su for advice on FISH, and Dr. Martin Tresguerres (Scripps Institution of Oceanography, UCSD) for providing adenylyl cyclase inhibitors and advice on cAMP and sAC. Thank you to the Achievement Rewards for College Scientists (ARCS) Foundation and the Philanthropic Educational Organization (P.E.O.) for support of L.E.S.

3.7. AUTHOR CONTRIBUTIONS

L.E.S. and A.H. conceived and designed the experiments and wrote the manuscript. L.E.S., R.Z.H., G.W.M., and T.G. performed the experiments. L.E.S., R.Z.H., G.W.M., T.G., and A.H. analyzed the data.

3.8. FUNDING

This work was supported by NIH HD 058070 and ES 021985, and a UCSD Academic Senate Grant (A.H.). L.E.S. was supported by a NSF Graduate Research Fellowship and a National Defense Science and Engineering Graduate Fellowship.

3.9. SUPPLEMENTARY MATERIAL

3.9.1. Supplementary Methods

3.9.1.1. Sp-C5a antibody generation and validation

Rabbit polyclonal antibodies to C5a (anti-C5a) were produced against recombinant C5a expressed in bacteria. The following N-terminal fragment of C5a was cloned into the pProEx Htb expression vector (Invitrogen) and expressed in BL21 Codon Plus cells: MIEGNDPLSMTSPHRASEGDHNDGFGVQERSSSLEDQTVIEMDSQIDTALSY TDGKTPGELKDGRIGEQEDDPDETEQLLDKREEGDTEEQKSSNTGTKYWATG NFISSVTSQWLTPLFRAAKKRGLNDDDLHYHILPVDSAEKNAKIFAQLWEEEEIKHH GNAVKASLRRVILR. Recombinant protein was produced and purified at the PEP Core facility of The Scripps Research Institute. Purified protein was run on polyacrylamide gels, and the protein bands were excised and sent to Lampire Biological Laboratories (Pipersville, PA) for antigen preparation, rabbit immunization, and affinity purification. The recombinant protein was used to affinity-purify an aliquot of the C5a-specific IgG from antiserum. This affinity-purified antibody was used for Western blots, while adsorbed whole serum (described below) was used for immunohistochemistry (IHC) (and affinity-purified antibody was tested with IHC as a control).

To determine specificity of anti-C5a, we used Western blots to compare protein expression between embryos expressing endogenous C5a, overexpressing C5a-mCherry, and expressing no C5a (i.e. MASO knockdown)

(Fig. S3.1A). Western blotting was performed with 70 hpf embryos from the same batch, and samples included: (1) C5a-CmCherry overexpressing (0.8 mg/ml), (2) control-injected (0.05 mg/ml histone H2B-CFP), (3) control-uninjected, (4) C5a knockdown with MASO1 (300 μ M), and (5) C5a knockdown with MASO2 (600 μ M). Ten embryos were pooled for the C5a-CmCherry over-expressing lane, and 200 embryos were pooled per lane for the control-injected, control-uninjected, MASO1, and MASO2 samples.

3.9.1.1.1. Antibody adsorption for immunohistochemistry

Because an 80 kDa band inconsistent with mature C5a appeared strongly on blots of all developmental stages, while the 210 kDa C5a band appeared only after 26 hpf, anti-C5a whole serum was adsorbed to fixed and permeabilized 22 hpf embryos to remove immunoglobulins reacting with non-C5a antigens. Sera (diluted to 1:1500 in 2% BSA-PBS) were adsorbed for 24 hours at 15°C and then collected and stored at 4°C with 5 mM sodium azide prior to immunolocalization of C5a. Examples of C5a immunolocalizations are shown using pre- and post-adsorbed antibody (Fig. S3.1B,C). After adsorption, localization was restricted to *gcm*-expressing pigment cells, consistent with the localization of *C5a* transcripts (Fig. 3.3).

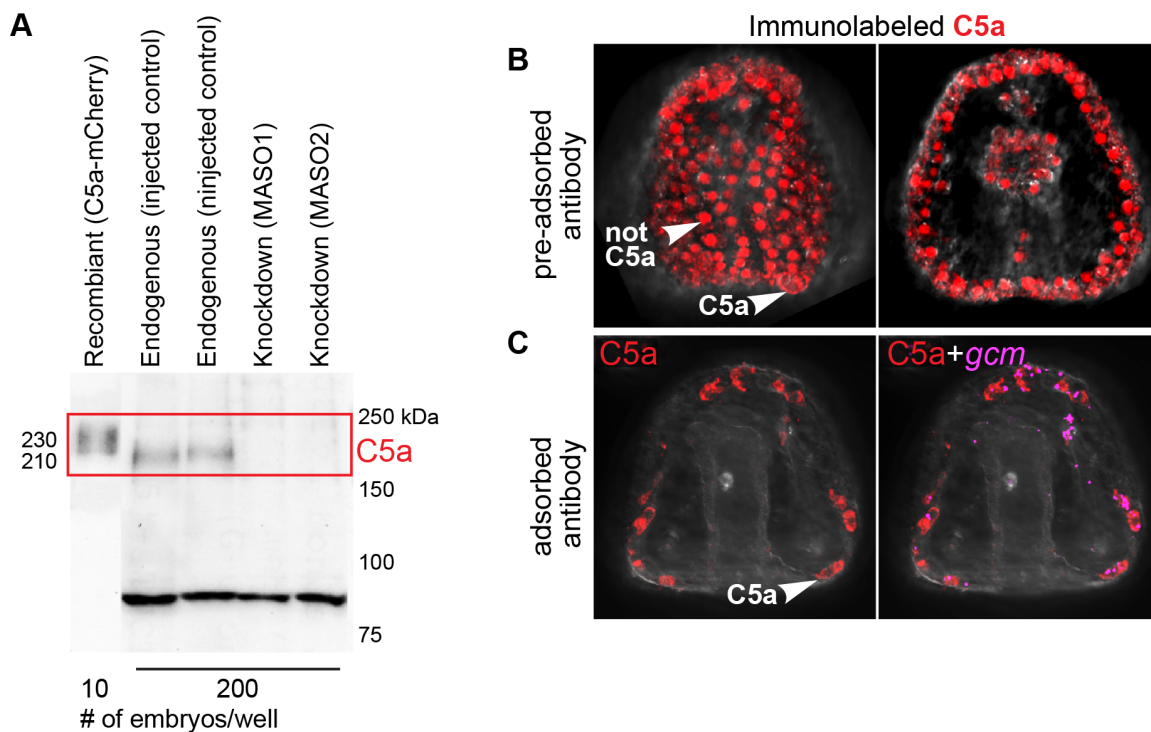


Figure S3.1. Detecting C5a with affinity-purified and adsorbed anti-C5a, and demonstrating knockdown with two MASOs. (A) Western blot using lysates from 70 hpf embryos, probed with affinity-purified anti-C5a. C5a-mCherry runs as a ~230 kDa doublet, while endogenous C5a runs at 210 kDa. Ten embryos were lysed and run per lane for the C5a-mCherry sample, while 200 embryos per lane were run for all other samples. Endogenous C5a (210 kDa band) is knocked down by both C5a MASO1 and MASO2, while an ~80 kDa band not corresponding to C5a is unaffected by both MASOs. Lysate from the ten C5a-mCherry overexpressing embryos had insufficient ~80 kDa antigen to be detected in the first lane. (B,C) Immunolocalization of C5a (red) in prism stage embryos using pre- and post-adsorbed anti-C5a (from whole serum). (B) Embryo is immunolabeled with pre-adsorbed anti-C5a serum. Two z-sections are shown. Pre-adsorbed anti-C5a recognizes a nuclear antigen (likely corresponding to the 80 kDa band seen in (A)) in most cells. In addition, in only cells with pigment cell-like distribution around the embryo, it recognizes a membrane-localized and intracellular signal corresponding to C5a. (C) Embryo is immunolabeled with adsorbed anti-C5a serum, and *gcm* transcripts (magenta) are labeled with FISH. Adsorbed anti-C5a does not detect a nuclear antigen, but recognizes membrane-localized and intracellular C5a in *gcm*-expressing pigment cells, which is consistent with the localization of *C5a* transcripts in *gcm*-expressing cells (Fig. 3.3).

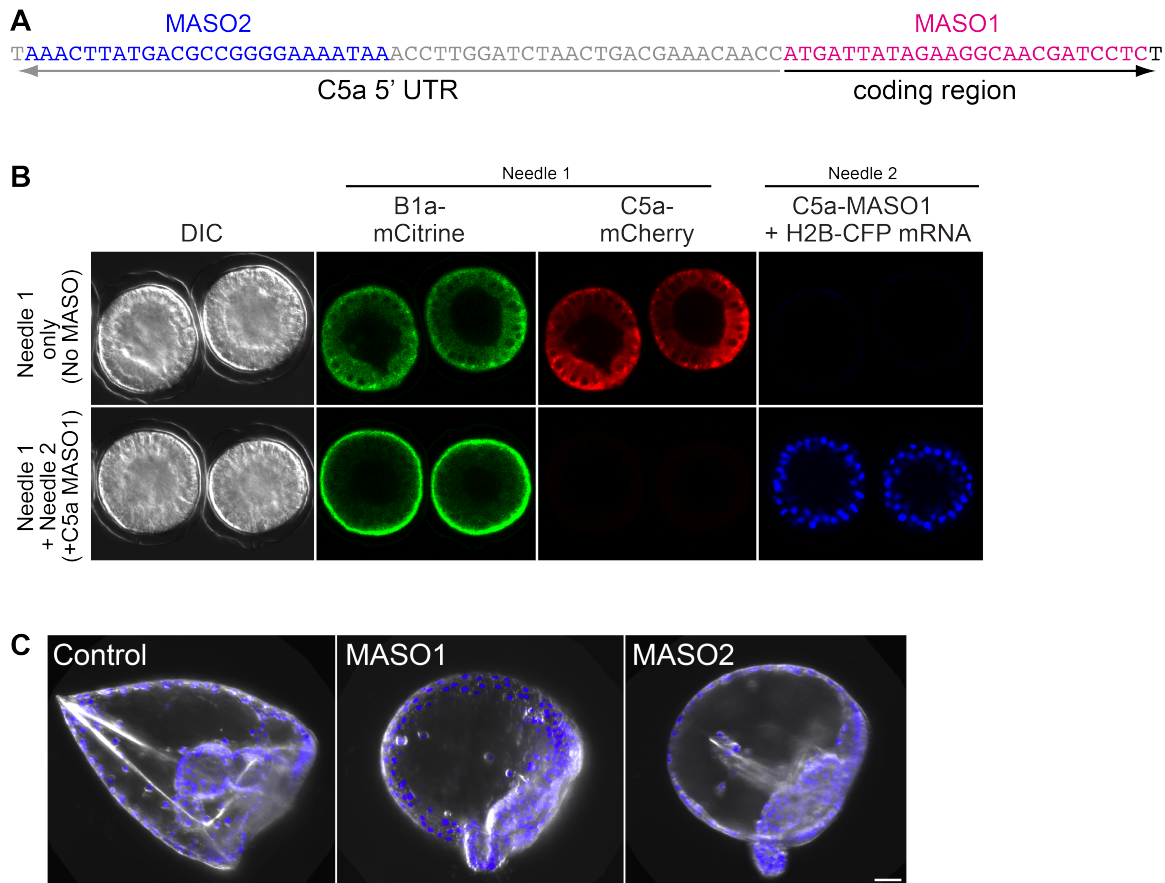


Figure S3.2. Two MASOs block translation of *C5a* and cause hindgut prolapse. (A) Two non-overlapping MASOs block translation of *C5a*. MASO1 targets the first 25 bases of the coding region, while MASO2 targets the 5' UTR. (B) *C5a*-MASO1 knocks down *C5a*-mCherry, but not B1a-mCitrine. Fertilized eggs were all injected with Needle 1, containing 1 mg/ml each of B1a-mCitrine and *C5a*-mCherry mRNA and imaged at the 20 hpf blastula stage. In the top panel (no MASO), both B1a-mCitrine and *C5a*-mCherry are expressed. In the bottom panel, samples were injected a second time with Needle 2, containing 0.3 mM *C5a*-MASO1 and H2B-CFP mRNA as an injection marker. In the presence of MASO1, no *C5a*-mCherry expression is detected, while B1a-mCitrine is robustly expressed. B1a-mCitrine localizes to apical membranes, presumably due to a relief on the protein sorting machinery, as *C5a*-mCherry is no longer present as a membrane-bound protein. Interestingly, in the absence of MASO, neither protein membrane-localizes, presumably because the trafficking machinery is overwhelmed. Consistent with this hypothesis, B1a trafficking is normal in the presence of *C5a*-MASO1. (C) Both MASO1 and MASO2 cause hindgut prolapse as shown in 70 hpf embryos. The phenotypes that result from injection of MASO1 and MASO2 are indistinguishable. Nuclei (blue) are labeled with H2B-CFP.

3.9.1.2. Time-lapse imaging of C5a-knockdown embryos

Embryos were mounted in FSW on protamine sulfate-coated Delta-T dishes (Bioptechs, Butler, PA) and retained in an enclosure of Scotch double-sided tape, with four $\sim 50 \mu\text{m}$ channels through which FSW could flow (Fig. S3.3). A coverslip was placed on top of the tape to restrict embryo movement. A chilled stage maintained embryos at 15°C , and images were captured on a Leica Sp8 confocal microscope with a Plan-Apochromat 20X objective (0.7 numerical aperture). In long time-lapses (> 15 hours), embryonic development was slowed to ~ 0.5 - 0.75 times the normal rate.

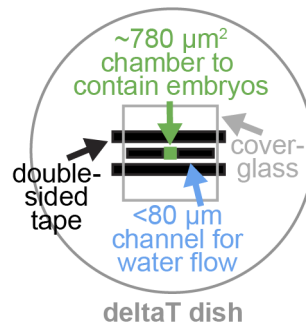


Figure S3.3. Chambers used to contain swimming embryos for long-term imaging.

3.10. REFERENCES

- Aye, I. L. M. H., Paxton, J. W., Evseenko, D. A. and Keelan, J. A.** (2007). Expression, localisation and activity of ATP binding cassette (ABC) family of drug transporters in human amnion membranes. *Placenta* **28**, 868–877.
- Barnet, M. E.** (2011). Dynamics of sea urchin gastrulation revealed by tracking cells of diverse lineage and regulatory state (Doctoral dissertation). *California Institute of Technology. Pasadena, CA.* **6146**.
- Belinsky, M. G., Bain, L. J., Balsara, B. B., Testa, J. R. and Kruh, G. D.** (1998). Characterization of MOAT-C and MOAT-D, new members of the MRP/cMOAT subfamily of transporter proteins. *J Natl Cancer Inst* **90**, 1735–1741.
- Beltrán, C., Vacquier, V. D., Moy, G., Chen, Y., Buck, J., Levin, L. R. and Darszon, A.** (2007). Particulate and soluble adenylyl cyclases participate in the sperm acrosome reaction. *Biochem Biophys Res Commun* **358**, 1128–1135.
- Boadu, E., and Sager, G.** (2004). Reconstitution of ATP-dependent cGMP transport into proteoliposomes by membrane proteins from human erythrocytes. *Scand J Clin Lab Invest* **64**, 41–48.
- Borst, P., de Wolf, C. and van de Wetering, K.** (2007). Multidrug resistance-associated proteins 3, 4, and 5. *Pflugers Arch* **453**, 661–673.
- Bosnjak, I., Uhlinger, K. R., Heim, W., Smital, T., Franekić-Colić, J., Coale, K., Epel, D. and Hamdoun, A.** (2009). Multidrug efflux transporters limit accumulation of inorganic, but not organic, mercury in sea urchin embryos. *Environ Sci Technol* **43**, 8374–8380.
- Burke, R. D., Lail, M. and Nakajima, Y.** (1998). The apical lamina and its role in cell adhesion in sea urchin embryos. *Cell Adhes. Commun* **5**, 97–108.
- Burke, R. D., Myers, R. L., Sexton, T. L. and Jackson, C.** (1991). Cell movements during the initial phase of gastrulation in the sea urchin embryo. *Dev Biol* **146**, 542–557.
- Cheepala, S., Hulot, J.-S., Morgan, J. A., Sassi, Y., Zhang, W., Naren, A. P. and Schuetz, J. D.** (2012). Cyclic nucleotide compartmentalization: contributions of phosphodiesterases and ATP-binding cassette transporters. *Annu Rev Pharmacol Toxicol* **53**, 231-253.

- Chen, J.-H., Luo, Y.-J. and Su, Y.-H.** (2011). The dynamic gene expression patterns of transcription factors constituting the sea urchin aboral ectoderm gene regulatory network. *Dev Dyn* **240**, 250–260.
- Chen, Z.-S. and Tiwari, A. K.** (2011). Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases. *FEBS J* **278**, 3226–3245.
- Cole, S.** (2014). Multidrug Resistance Protein 1 (MRP1, ABCC1), a “multitasking” ATP-binding cassette (ABC) transporter. *J Biol Chem* **289**, 30880–30888.
- de Wolf, C. J. F., Yamaguchi, H., van der Heijden, I., Wielinga, P. R., Hundscheid, S. L., Ono, N., Scheffer, G. L., de Haas, M., Schuetz, J. D., Wijnholds, J., et al.** (2007). cGMP transport by vesicles from human and mouse erythrocytes. *FEBS J* **274**, 439–450.
- Dormann, D. and Weijer, C. J.** (2006). Chemotactic cell movement during *Dictyostelium* development and gastrulation. *Curr Opin Genet Dev* **16**, 367–373.
- Fletcher, J. I., Haber, M., Henderson, M. J. and Norris, M. D.** (2010). ABC transporters in cancer: more than just drug efflux pumps. *Nat Rev Cancer* **10**, 147–156.
- Fujino, Y. and Yasumasu, I.** (1981). cAMP-dependent protein kinase in sea urchin embryos. *Gamete Res* **4**, 395–406.
- Gibson, A. W. and Burke, R. D.** (1985). The origin of pigment cells in embryos of the sea urchin *Strongylocentrotus purpuratus*. *Dev Biol* **107**, 414–419.
- Gibson, A. W. and Burke, R. D.** (1987). Migratory and invasive behavior of pigment cells in normal and animalized sea urchin embryos. *Exp Cell Res* **173**, 546–557.
- Godinho, R. O., Duarte, T. and Pacini, E. S. A.** (2015). New perspectives in signaling mediated by receptors coupled to stimulatory G protein: the emerging significance of cAMP efflux and extracellular cAMP-adenosine pathway. *Front Pharmacol* **06**, 1–9.
- Gokirmak, T., Campanale, J. P., Shipp, L. E., Moy, G. W., Tao, H. and Hamdoun, A.** (2012). Localization and substrate selectivity of sea urchin multidrug (MDR) efflux transporters. *J Biol Chem* **287**, 43876–43883.
- Goldstone, J. V., Hamdoun, A., Cole, B. J., Howard-Ashby, M., Nebert, D. W., Scally, M., Dean, M., Epel, D., Hahn, M. E. and Stegeman, J. J.** (2006).

The chemical defensome: environmental sensing and response genes in the *Strongylocentrotus purpuratus* genome. *Dev Biol* **300**, 366–384.

Gökirmak, T., Shipp, L. E., Campanale, J. P., Nicklisch, S. C. T. and Hamdoun, A. (2014). Transport in technicolor: mapping ATP-binding cassette transporters in sea urchin embryos. *Mol Reprod Dev* **81**, 778–793.

Griffiths, M. (1965). A study of the synthesis of naphthaquinone pigments by the larvae of two species of sea urchins and their reciprocal hybrids. *Dev Biol* **11**, 433–447.

Gross, J. M. and McClay, D. R. (2001). The role of Brachyury (T) during gastrulation movements in the sea urchin *Lytechinus variegatus*. *Dev Biol* **239**, 132–147.

Gustafson, T. and Wolpert, L. (1967). Cellular movement and contact in sea urchin morphogenesis. *Biol Rev Camb Philos Soc* **42**, 442–498.

Hardin, J. (1989). Local shifts in position and polarized motility drive cell rearrangement during sea urchin gastrulation. *Dev Biol* **136**, 430–445.

Hardin, J. and McClay, D. R. (1990). Target recognition by the archenteron during sea urchin gastrulation. *Dev Biol* **142**, 86–102.

Henderson, M. J., Haber, M., Porro, A., Munoz, M. A., Iraci, N., Xue, C., Murray, J., Flemming, C. L., Smith, J., Fletcher, J. I., et al. (2011). ABCB multidrug transporters in childhood neuroblastoma: clinical and biological effects independent of cytotoxic drug efflux. *J Natl Cancer Inst* **103**, 1236–1251.

Holland, N. D. and Jespersen, A. (1973). The fine structure of the fertilization membrane of the feather star *Comanthus japonica* (Echinodermata: Crinoidea). *Tissue Cell* **5**, 209–214.

Hughes, J. N., Dodge, N., Rathjen, P. D. and Rathjen, J. (2009). A novel role for gamma-secretase in the formation of primitive streak-like intermediates from ES cells in culture. *Stem Cells* **27**, 2941–2951.

Jedlitschky, G., Burchell, B. and Keppler, D. (2000). The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *J Biol Chem* **275**, 30069–30074.

Jin, D., Ni, T. T., Sun, J., Wan, H., Amack, J. D., Yu, G., Fleming, J., Chiang, C., Li, W., Papierniak, A., et al. (2014). Prostaglandin signalling regulates ciliogenesis by modulating intraflagellar transport. *Nat Cell Biol* **16**, 841–851.

- Kool, M., de Haas, M., Scheffer, G. L., Scheper, R. J., van Eijk, M. J., Juijn, J. A., Baas, F. and Borst, P.** (1997). Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* **57**, 3537–3547.
- Korolnek, T., Zhang, J., Beardsley, S., Scheffer, G. L. and Hamza, I.** (2014). Control of metazoan heme homeostasis by a conserved multidrug resistance protein. *Cell Metab* **19**, 1008–1019.
- Leslie, E. M., Deeley, R. G. and Cole, S. P.** (2001). Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. *Toxicology* **167**, 3–23.
- Litman, T., Druley, T. E., Stein, W. D. and Bates, S. E.** (2001). From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci* **58**, 931–959.
- Logan, C. Y. and McClay, D. R.** (1997). The allocation of early blastomeres to the ectoderm and endoderm is variable in the sea urchin embryo. *Development* **124**, 2213–2223.
- Manceau, S., Giraud, C., Declèves, X., Scherrmann, J. M., Artiguebelle, F., Goffinet, F., Chappuy, H., Vinot, C. and Tréluyer, J. M.** (2012). ABC drug transporter and nuclear receptor expression in human cytotrophoblasts: Influence of spontaneous syncytialization and induction by glucocorticoids. *Placenta* **33**, 927–932.
- Materna, S. C. and Davidson, E. H.** (2012). A comprehensive analysis of Delta signaling in pre-gastrular sea urchin embryos. *Dev Biol* **364**, 77–87.
- Materna, S. C., Ransick, A., Li, E. and Davidson, E. H.** (2013). Diversification of oral and aboral mesodermal regulatory states in pregastrular sea urchin embryos. *Dev Biol* **375**, 92–104.
- McAleer, M. A., Breen, M. A., White, N. L. and Matthews, N.** (1999). pABC11 (also known as MOAT-C and MRP5), a member of the ABC family of proteins, has anion transporter activity but does not confer multidrug resistance when overexpressed in human embryonic kidney 293 cells. *J Biol Chem* **274**, 23541–23548.
- Meyer Zu Schwabedissen, H. E. U., Grube, M., Heydrich, B., Linnemann, K., Fusch, C., Kroemer, H. K. and Jedlitschky, G.** (2005). Expression, localization, and function of MRP5 (ABCC5), a transporter for cyclic nucleotides, in human placenta and cultured human trophoblasts: effects of

gestational age and cellular differentiation. *Am J Pathol* **166**, 39–48.

Miranda, E. R., Nam, E. A., Kuspa, A. and Shaulsky, G. (2015). The ABC transporter, AbcB3, mediates cAMP export in *D. discoideum* development. *Dev Biol* **397**, 203–211.

Müller, P. and Schier, A. F. (2011). Extracellular movement of signaling molecules. *Dev Cell* **21**, 145–158.

Nies, A. T., Spring, H., Thon, W. F., Keppler, D. and Jedlitschky, G. (2002). Immunolocalization of multidrug resistance protein 5 in the human genitourinary system. *J Urol* **167**, 2271–2275.

Nigam, S. K. (2015). What do drug transporters really do? *Nat Rev Drug Discov* **14**, 29–44.

Pratt, S., Shepard, R. L., Kandasamy, R. A., Johnston, P. A., Perry, W. and Dantzig, A. H. (2005). The multidrug resistance protein 5 (ABCC5) confers resistance to 5-fluorouracil and transports its monophosphorylated metabolites. *Mol Cancer Ther* **4**, 855–863.

Ransick, A. and Davidson, E. (1998). Late specification of veg(1) lineages to endodermal fate in the sea urchin embryo. *Dev Biol* **195**, 38–48.

Ransick, A. and Davidson, E. H. (2006). cis-regulatory processing of Notch signaling input to the sea urchin glial cells missing gene during mesoderm specification. *Dev Biol* **297**, 587–602.

Rast, J., Cameron, R., Poustka, A. and Davidson, E. (2002). Brachyury target genes in the early sea urchin embryo isolated by differential macroarray screening. *Dev Biol* **246**, 191–208.

Ravna, A. W., Sylte, I. and Sager, G. (2008). A molecular model of a putative substrate releasing conformation of multidrug resistance protein 5 (MRP5). *Eur J Med Chem* **43**, 2557–2567.

Reid, G., Wielinga, P., Zelcer, N., de Haas, M., Van Deemter, L., Wijnholds, J., Balzarini, J. and Borst, P. (2003). Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. *Mol Pharmacol* **63**, 1094–1103.

Ricardo, S. and Lehmann, R. (2009). An ABC transporter controls export of a *Drosophila* germ cell attractant. *Science* **323**, 943–946.

Robert, H. S. and Friml, J. (2009). Auxin and other signals on the move in

plants. *Nat Chem Biol* **5**, 325–332.

Ruffins, S. W. and Etensohn, C. A. (1996). A fate map of the vegetal plate of the sea urchin (*Lytechinus variegatus*) mesenchyme blastula. *Development* **122**, 253–263.

Sager, G. and Ravna, A. W. (2009). Cellular efflux of cAMP and cGMP - a question about selectivity. *Mini Rev Med Chem* **9**, 1009–1013.

Sager, G., Ørvoll, E. Ø., Lysaa, R. A., Kufareva, I., Abagyan, R. and Ravna, A. W. (2012). Novel cGMP efflux inhibitors identified by virtual ligand screening (VLS) and confirmed by experimental studies. *J Med Chem* **55**, 3049–3057.

Schulz, T., Schumacher, U. and Prehm, P. (2007). Hyaluronan export by the ABC transporter MRP5 and its modulation by intracellular cGMP. *J Biol Chem* **282**, 20999–21004.

Sherwood, D. R. and McClay, D. R. (1999). LvNotch signaling mediates secondary mesenchyme specification in the sea urchin embryo. *Development* **126**, 1703–1713.

Shipp, L. E. and Hamdoun, A. (2012). ATP-binding cassette (ABC) transporter expression and localization in sea urchin development. *Dev Dyn* **241**, 1111–1124.

Smith, A. B., Pisani, D., Mackenzie-Dodds, J. A., Stockley, B., Webster, B. L. and Littlewood, D. T. J. (2006). Testing the molecular clock: molecular and paleontological estimates of divergence times in the Echinoidea (Echinodermata). *Mol Biol Evol* **23**, 1832–1851.

Solek, C. M., Oliveri, P., Loza-Coll, M., Schrankel, C. S., Ho, E. C., Wang, G. and Rast, J. P. (2013). An ancient role for Gata-1/2/3 and Scl transcription factor homologs in the development of immunocytes. *Dev Biol* **382**, 280–292.

Soliman, S. (1984). Pharmacological control of ciliary activity in the young sea urchin larva: chemical studies on the role of cyclic nucleotides. *Comp Biochem Physiol C* **78**, 175–181.

Strouse, J. J., Ivnitski-Steele, I., Waller, A., Young, S. M., Perez, D., Evangelisti, A. M., Ursu, O., Bologna, C. G., Carter, M. B., Salas, V. M., et al. (2013). Fluorescent substrates for flow cytometric evaluation of efflux inhibition in ABCB1, ABCC1, and ABCG2 transporters. *Anal Biochem* **437**, 77–87.

Sweet, H. C., Gehring, M. and Etensohn, C. A. (2002). LvDelta is a

mesoderm-inducing signal in the sea urchin embryo and can endow blastomeres with organizer-like properties. *Development* **129**, 1945–1955.

- Takata, H. and Kominami, T.** (2004). Behavior of pigment cells closely correlates the manner of gastrulation in sea urchin embryos. *Zoolog Sci* **21**, 1025–1035.
- Tresguerres, M., Levin, L. R. and Buck, J.** (2011). Intracellular cAMP signaling by soluble adenylyl cyclase. *Kidney Int* **79**, 1277–1288.
- van de Ven, R., de Groot, J., Reurs, A. W., Wijnands, P. G. J. T. B., van de Wetering, K., Schuetz, J. D., de Gruijl, T. D., Scheper, R. J. and Scheffer, G. L.** (2009). Unimpaired immune functions in the absence of Mrp4 (Abcc4). *Immunol Lett* **124**, 81–87.
- Weekes, M. P., Tan, S. Y. L., Poole, E., Talbot, S., Antrobus, R., Smith, D. L., Montag, C., Gygi, S. P., Sinclair, J. H. and Lehner, P. J.** (2013). Latency-associated degradation of the MRP1 drug transporter during latent human cytomegalovirus infection. *Science* **340**, 199–202.
- Wei, Z., Angerer, R. C. and Angerer, L. M.** (2006). A database of mRNA expression patterns for the sea urchin embryo. *Dev Biol* **300**, 476–484.
- Whalen, K., Reitzel, A. M. and Hamdoun, A.** (2012). Actin polymerization controls the activation of multidrug efflux at fertilization by translocation and fine-scale positioning of ABCB1 on microvilli. *Mol Biol Cell* **23**, 3663–3672.
- Wielinga, P. R., van der Heijden, I., Reid, G., Beijnen, J. H., Wijnholds, J. and Borst, P.** (2003). Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells. *J Biol Chem* **278**, 17664–17671.
- Wijnholds, J., Mol, C. A., van Deemter, L., de Haas, M., Scheffer, G. L., Baas, F., Beijnen, J. H., Scheper, R. J., Hatse, S., De Clercq, E., et al.** (2000). Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. *Proc Natl Acad Sci USA* **97**, 7476–7481.

3.11. CHAPTER ACKNOWLEDGMENTS

Chapter 3, in full, is in review at *Development* and was authored by Shipp L.E., Hill R.Z., Moy G, Gokirmak T, and Hamdoun A. It has been reformatted for this dissertation. The dissertation author was the primary investigator and author of this paper.

CHAPTER 4.

EPILOGUE

4.1. LOOKING FORWARD

In this dissertation, I present a method for identifying potential functions of MDR transporters (Fig. 4.1). MDR transporters are uniquely challenging to study due to the high number of transporters, diverse predicted functions, substrate polyspecificity, varied mechanisms of regulation, and difficulty in solving large, insoluble protein structures. In Chapter 2, I propose the hypothesis that transporters' potential functions can be inferred from a combination of spatial and temporal expression mapping, exogenous protein subcellular localization, and comparison to mammalian homolog functions. In Chapter 3, I validate this hypothesis with one transporter by conducting a functional screen of xenobiotic efflux, optimizing a new antibody developed by our lab, and genetically and chemically manipulating embryos. Together, my work suggests that spatial and temporal mapping of ABC transporter expression can identify candidates for developmental signaling and protective functions. Indeed, for some transporters this seems to be the case. The examples of B1a and C5a have been discussed already, and another example is *Sp*-ABCB4a (B4a), which shows temporal expression, subcellular localization, and efflux activity similar to B1a.

In the pipeline I propose (Fig. 4.1), no single line of expression or localization evidence could reasonably suggest function. For example, *Sp*-*ABCC9a* is temporally expressed similarly to *B1a* and *B4a*, but its subcellular localization (in large apical vesicles) is inconsistent with the protective transporters, as is its ability to efflux chemicals. C9a may represent an

expression profile for a homeostatic transporter, and in fact its mammalian homolog (SUR2) is a potassium channel regulator. As another example, *Sp-ABCC1* is expressed throughout development, and its transcript levels increase steadily through the gastrula stage. The protein localizes basolaterally, yet significantly effluxes a variety of chemicals (Gökirmak et al., 2014). As its mammalian homolog (MRP1) transports both xenobiotics and signaling molecules, C1's seemingly contradictory expression, localization, and efflux activities may indicate that it similarly functions in both protection and signaling in embryogenesis.

There is much to do to learn more about the many unknown functions of MDR transporters in sea urchin development. Further research on the candidate protective transporters would be useful for the field of environmental toxicology, as the major protective efflux transporters of harmful pollutants remain unknown. In addition, this approach - starting with spatial and temporal mapping, and screening with a simple functional assay - may prove useful for uncovering functions of other large protein families potentially related to transporter-mediated signaling, such as the anticipated effectors GPCRs.

It is currently unclear why ABC transport evolved as a mechanism for secreting cellular signals. My discoveries concerning C5a add to the growing body of evidence suggesting ABC transporters play critical signaling roles during morphogenesis. As developmental signaling often involves *bulk* transport of molecules (e.g. exocytosis), the use of *active* transport to move signaling

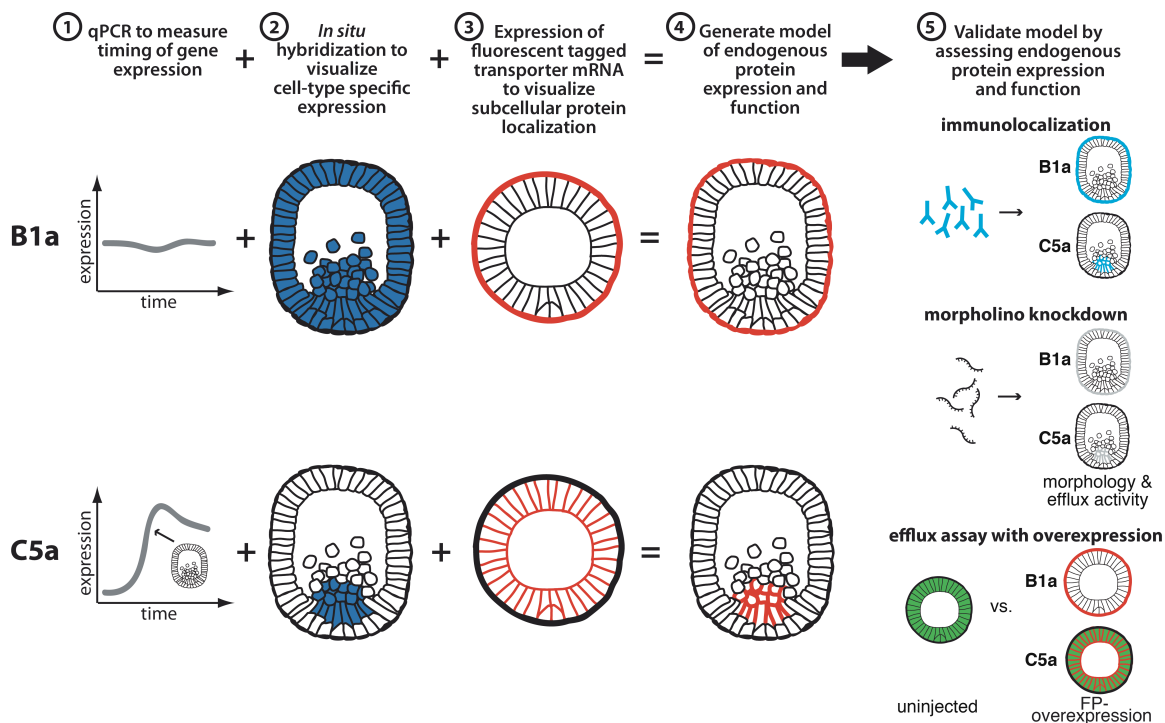


Figure 4.1. Spatio-temporal mapping of transporters. 1: Temporal expression patterns of transporters reveal developmental stages in which transporters are expressed. For example, ABCB1a expression is not restricted to a specific developmental stage, while ABCC5a expression is primarily expressed at and after the mesenchyme blastula stage. 2: Spatial patterns of transporter expression are determined by in situ hybridization. ABCB1a is expressed in all cells of the embryo, while ABCC5a is only expressed in non-skeletogenic mesenchyme cells (blue). 3: Subcellular localization of relevant proteins are determined by the expression of fluorescent protein fusions of a transporter (red) (see also Fig. 2.5). For example, ABCB1a localizes to the apical membrane while ABCC5a localizes to basolateral membranes. 4: Data are merged to model endogenous transporter protein expression and function. 5: Models are tested by assessing endogenous protein expression, developmental function, and xenobiotic efflux activity.

molecules across the plasma membrane is unusual. This raises the question of when and why this mechanism of signal secretion evolved. For secreted small molecules that are synthesized in the cytosol (as opposed to peptides progressing through the entire secretory pathway), perhaps ABC transport is the most direct, energetically favorable mechanism of transport. Indeed, despite the need to hydrolyze ATP for every efflux event, ABC transport is relatively

energetically inexpensive, costing less than 0.2% of a cell's ATP usage (Cole et al., 2013). Perhaps this is an energetically favorable mechanism, compared to restructuring of cellular membranes that occurs during exocytosis. Alternatively, as membrane restructuring can also affect transporter activity through rapid and precise membrane insertion and retrieval, and transport can also be modulated allosterically, ABC transport may offer a mechanism to fine-tune the timing, positioning, and quantities of signals secreted. Further investigations into these possibilities would be useful.

Finally, while this dissertation presents novel information about ABCC5 transporters, much remains to be understood. Below are some subjects of interest that could be explored to discover more about this transporter in sea urchin development.

4.1.1. FURTHER STUDIES OF C5a

4.1.1.1. Evolution of ABCC5 transporters and gene duplication

An unresolved issue is the degree to which ABCC5 transporters share substrates and functions across broad phylogenetic spans. In different organisms, transport of different substrates could affect related signaling pathways and processes. For example, transport of either porphyrins or cyclic nucleotides could alter levels of both types of compounds. Heme oxygenase-1 (HO-1), the rate-limiting heme degradation catalyst, is induced by cAMP (Krönke et al., 2003), cGMP (Polte et al., 2000), and heme itself (Ndisang et al., 2003). In

addition, heme stimulates cGMP production (Ndisang et al., 2003). It is unclear if this type of interplay is masking studies of ABCC5, or revealing its physiological niche. More studies are necessary to resolve questions about conservation of ABCC5 substrates and functions.

Exploring a connection between C5a, cAMP, and the extracellular matrix (ECM) during hindgut formation is an interesting area for future studies. Though I show that cAMP is a relevant C5 substrate in C5a-mediated hindgut orientation, a hindgut prolapse similar to that seen in C5a-knockdowns was reported by (Burke et al., 1991), which was caused by antibodies disrupting apical lamina glycoproteins (fibropellins, (Burke et al., 1998)). This suggests that perturbing the ECM can phenocopy the knockdown of C5a, and as part of the ECM, the reported ABCC5 substrate hyaluronan (a glycosaminoglycan (GAG)) could be involved. The ECM is a substratum for cell migration (Kato and Solursh, 1981; Venkatasubramanian and Solursh, 1984) and a regulator of signal transduction (Solursh and Lane, 1988; Werb et al., 1989) during morphogenesis, where it spatially restricts the TGF- β and FGF developmental signaling factors (Müller and Schier, 2011). In sea urchins, GAGs are important for gastrulation and post-gastrular development (Kinoshita and Saiga, 1979; Sugiyama, 1972), and restriction of *Nodal* (a TGF- β protein specifying the oral-aboral axis) is controlled by the ECM (Bergeron et al., 2011). Further work on the role of hyaluronan or other GAGs in hindgut invagination could shed light on a potential connection with C5a.

With 12 paralogs, ABCC5 has been expanded in the *S. purpuratus* genome more than any other transporter, and the evolutionary reason for this is unclear. *C5a* is the only paralog I was able to detect in my gene expression screen, due to other paralogs either being insufficiently expressed for qPCR to detect, or having insufficiently unique stretches of annotated genomic DNA for specific primer design. Based on published microarray data, several *ABCC5* paralogs should be expressed by 60 hpf (Wei et al., 2006). These paralogs could have functional redundancy but be regulated by distinct factors, offering a way to control when and where cyclic nucleotides are transported. Alternatively, they could have slightly modified ligand binding domains that fine-tune unique substrate selectivity, possibly to include heme or hyaluronan. In adult tissues, it is unknown whether *C5a* and its paralogs are expressed in pigment-producing cells, or if transporter activity affects formation of digestive structures. Further investigation is necessary to understand the expression and function of the 12 *ABCC5* paralogs in the embryo and the adult sea urchin.

4.1.1.2. Regulation and trafficking of C5a

Much remains to be learned about the expression, processing, trafficking, and membrane dynamics governing *ABCC5a* activity.

4.1.1.2.1. Gene regulation

While I have shown that expression of C5a is downstream of D/N signaling from the SM, and also downstream of *gcm*, it would be interesting to identify the direct activating factor(s) responsible for C5a expression in pigment cells. *Hs-ABCC5* is transcriptionally regulated by the epithelial-mesenchymal transition (EMT) inducing transcription factors Snail, Twist, and FOXC2 (Saxena et al., 2011). It would also be interesting to study whether there is a role for microRNAs (miRNAs) in C5a transcriptional and post-transcriptional regulation, as miRNAs are known to regulate other ABC transporters (Toscano-Garibay and Aquino-Jarquin, 2012).

4.1.1.2.2. Post-translational processing

C5a has two N-linked glycosylation sites that may be important for its membrane targeting and activity. I found that on Western blots, C5a-mCherry ran as a doublet, while endogenous C5a did not, possibly indicating that post-translational modifications are altered by addition of the mCherry tag. It would be interesting to determine if this is the case, and if so, whether this affects the protein's activity, substrate specificity, stability, or trafficking. Mammalian ABCB1 must be fully glycosylated in order to function as an efflux transporter, and immature, core-glycosylated ABCB1 is nonfunctional (Loo and Clarke, 1999). ABCB1 function also decreases with increased ubiquitination, which, interestingly, stabilizes the less functional ABCB1 protein (Zhang et al., 2004). More knowledge of the post-translational modification of C5a is needed.

4.1.1.2.3. Membrane trafficking

It is unknown which portion of the C5a sequence is responsible for its basolateral membrane localization. Residues in ABC transporter non-catalytic CLs between TMHs are important for routing both apical (CFTR/ABCC7) (Xie et al., 1995) and basolateral (ABCC1) (Iram and Cole, 2011) transporters to the membrane. No membrane routing signal has been identified for any ABCC5 transporter, but because it lacks MSD0 and routes to the basolateral membrane, it is possible the signal is located on the COOH-terminal. This hypothesis is based on ABCC1, which is a “long” transporter that contains an extra N-terminal MSD (MSD0). Basolateral routing of ABCC1 can be mediated by either of two redundant trafficking signals, one on MSD0 and another on the COOH-terminal region (Westlake et al., 2005). A more detailed characterization of C5a subcellular localization coupled with targeted mutagenesis could identify the basolateral routing sequence(s).

4.1.1.2.4. Membrane dynamics

Beyond synthesis and processing, C5a activity is likely affected by membrane dynamics that remain to be explored, but could include Rab-mediated cycling, endocytosis, and alterations in membrane potential. The role of Rab endosomal recycling organelles should be probed, as mouse ABCC5 colocalizes with Rab4, Rab5, Rab9, and Rab11 (Korolnek et al., 2014), and in sea urchins

ABCB1a activity depends on actin and Rab11 mediated localization to microvillar tips (Whalen et al., 2012). Also in sea urchins, constitutive endocytosis causes reduction in ABC transporter efflux activity (Campanale and Hamdoun, 2012). Other regulatory mechanisms could include a host of macromolecular complexes including kinases, signaling molecules, and SNAREs that may associate, for example, with ABCC7 to modify transporter trafficking and activity (Guggino and Stanton, 2006). Finally, as membrane electrical potential controls human ABCC5-mediated cGMP transport (Kucka et al., 2010), this is yet another possible factor that could be studied for C5a.

4.1.2. HINDGUT MOVEMENT AND THE GRN

Here I demonstrate that *C5a* is activated by the pigment cell GRN, but an outstanding question is which downstream effectors are modulated by C5a activity. I have proposed that GPCRs in the hindgut may mediate cAMP reception and subsequent movements, and future studies should address this. It would be interesting to determine if cellular movements are affected through misregulation of *Brachyury (bra)*. *Bra* is dynamically expressed in two invaginating regions: (1) the posterior-most regions of the gut, and (2) the oral ectoderm/stomodeum. In both locations, it controls cytoskeletal modulators that mediate morphogenetic movements (Gross and McClay, 2001; Rast et al., 2002). In the endoderm, it is first expressed in *veg2* cells (18-20 hpf in *S. purpuratus*), then turns off in *veg2* and on in *veg1* cells, becoming dynamically expressed at the base of the

archenteron throughout gastrulation. It helps define the endoderm-ectoderm boundary and is ultimately expressed in the larval hindgut/anus (Annunziata et al., 2014; Gross and McClay, 2001; Peter and Davidson, 2010; Peter and Davidson, 2011; Rast et al., 2002). *Bra* expression is under the control of Hox11/13b, which is thought to control an unknown diffusible signal from veg2 to veg1 cells. It acts as an auto-repressor in veg2 cells, clearing its own transcripts as well as those of *bra*, and it also controls expression of *bra* in veg1 descendants (Peter and Davidson, 2010; Peter and Davidson, 2011). *Bra*-knockdown embryos do not gastrulate (Rast et al., 2002), and it would be interesting if *bra* expression was perturbed in a minor way by C5a-knockdown, perhaps being reduced in midgut cells such that they do not invaginate completely, leading the hindgut to be pushed outward. In pigment cells, the cAMP-dependent PKA (*CAPK*) is a *bra* target (Rast et al., 2002), and interestingly, I found that embryos exposed to pCPT-cAMP hyperinvaginate in both *bra*-expressing regions: the stomodeum and the blastopore. Further links between cAMP, C5a, and *bra* remain to be explored.

Finally, in C5a-knockdown embryos, it is unknown whether hindgut perturbation is caused by a defect in movement alone or in cell specification as well. It is possible that a specification problem would cause more severe gut defects than is observed in C5a-knockdowns. For example, Wnt1 knockdown causes ectopic *nodal* and *bra* expression and a shift in the dorsal/ventral axis by gastrulation, resulting in a severely deformed gut with no contribution from ventral

endoderm (Wei et al., 2012). However, the timing of larval gut specification, C5a expression, and C5a-mediated prolapse overlap such that hindgut differentiation could be perturbed. The larval gut is specified throughout the late gastrula and prism stages (Annunziata et al., 2014), when C5a expression is highest and C5a-knockdown mediated prolapse becomes evident. This specification depends largely on *ParaHox* gene control, which is synergistically activated by earlier endodermal transcription factors including Hox11/13b and Bra (Annunziata and Arnone, 2014). Assessment of the expression and localization of midgut- and hindgut-specific factors in C5a-knockdowns would begin to address the question of whether hindgut specification is controlled by C5a-mediated cAMP signaling.

4.2. CONCLUSIONS

This dissertation demonstrates how proteins of interest to biomedical research, for example ABC transporters, can reveal novel functions when studied in developmental systems. It also contributes to our understanding of development by highlighting that unlikely players – such as ABCC5a – can play important roles in developmental signaling. Using an integrative approach to both transporter biology and development has proved informative for studying mechanisms of morphogenesis and transporter activity. This work demonstrates that we can learn a lot about ABC transporters by studying them in whole embryos, and we can learn a lot about embryos by studying elusive proteins like ABC transporters.

4.3. REFERENCES

- Annunziata, R. and Arnone, M. I.** (2014). A dynamic regulatory network explains ParaHox gene control of gut patterning in the sea urchin. *Development* **141**, 2462-2472.
- Annunziata, R., Perillo, M., Andrikou, C., Cole, A. G., Martinez, P. and Arnone, M. I.** (2014). Pattern and process during sea urchin gut morphogenesis: The regulatory landscape. *Genesis* **52**, 251–268.
- Bergeron, K.-F., Xu, X. and Brandhorst, B. P.** (2011). Oral-aboral patterning and gastrulation of sea urchin embryos depend on sulfated glycosaminoglycans. *Mech Dev* **128**, 71–89.
- Burke, R. D., Lail, M. and Nakajima, Y.** (1998). The apical lamina and its role in cell adhesion in sea urchin embryos. *Cell Adhes Commun.* **5**, 97–108.
- Burke, R. D., Myers, R. L., Sexton, T. L. and Jackson, C.** (1991). Cell movements during the initial phase of gastrulation in the sea urchin embryo. *Dev Biol* **146**, 542–557.
- Campanale, J. P. and Hamdoun, A.** (2012). Programmed reduction of ABC transporter activity in sea urchin germline progenitors. *Development* **139**, 783–792.
- Cole, B. J., Hamdoun, A. and Epel, D.** (2013). Cost, effectiveness and environmental relevance of multidrug transporters in sea urchin embryos. *J Exp Biol* **216**, 3896–3905.
- Gökirmak, T., Shipp, L. E., Campanale, J. P., Nicklisch, S. C. T. and Hamdoun, A.** (2014). Transport in technicolor: mapping ATP-binding cassette transporters in sea urchin embryos. *Mol Reprod Dev* **81**, 778–793.
- Gross, J. M. and McClay, D. R.** (2001). The role of Brachyury (T) during gastrulation movements in the sea urchin *Lytechinus variegatus*. *Dev Biol* **239**, 132–147.
- Guggino, W. B. and Stanton, B. A.** (2006). New insights into cystic fibrosis: molecular switches that regulate CFTR. *Nat Rev Mol Cell Biol* **7**, 426–436.
- Iram, S. H. and Cole, S. P. C.** (2011). Expression and function of human MRP1 (ABCC1) is dependent on amino acids in cytoplasmic loop 5 and its interface with nucleotide binding domain 2. *J Biol Chem* **286**, 7202–7213.

- Katow, H. and Solursh, M.** (1981). Ultrastructural and time-lapse studies of primary mesenchyme cell behavior in normal and sulfate-deprived sea urchin embryos. *Exp Cell Res* **136**, 233–245.
- Kinoshita, S. and Saiga, H.** (1979). The role of proteoglycan in the development of sea urchins. I. Abnormal development of sea urchin embryos caused by the disturbance of proteoglycan synthesis. *Exp Cell Res* **123**, 229–236.
- Korolnek, T., Zhang, J., Beardsley, S., Scheffer, G. L. and Hamza, I.** (2014). Control of metazoan heme homeostasis by a conserved multidrug resistance protein. *Cell Metab* **19**, 1008–1019.
- Krönke, G., Bochkov, V. N., Huber, J., Gruber, F., Blüml, S., Fürnkranz, A., Kadl, A., Binder, B. R. and Leitinger, N.** (2003). Oxidized phospholipids induce expression of human heme oxygenase-1 involving activation of cAMP-responsive element-binding protein. *J Biol Chem* **278**, 51006–51014.
- Kucka, M., Kretschmannova, K., Murano, T., Wu, C.-P., Zemkova, H., Ambudkar, S. V. and Stojilkovic, S. S.** (2010). Dependence of multidrug resistance protein-mediated cyclic nucleotide efflux on the background sodium conductance. *Mol Pharmacol* **77**, 270–279.
- Loo, T. W. and Clarke, D. M.** (1999). The human multidrug resistance P-glycoprotein is inactive when its maturation is inhibited: potential for a role in cancer chemotherapy. *Faseb J* **13**, 1724–1732.
- Müller, P. and Schier, A. F.** (2011). Extracellular movement of signaling molecules. *Dev Cell* **21**, 145–158.
- Ndisang, J. F., Wu, L., Zhao, W. and Wang, R.** (2003). Induction of heme oxygenase-1 and stimulation of cGMP production by hemin in aortic tissues from hypertensive rats. *Blood* **101**, 3893–3900.
- Peter, I. S. and Davidson, E. H.** (2010). The endoderm gene regulatory network in sea urchin embryos up to mid-blastula stage. *Dev Biol* **340**, 188–199.
- Peter, I. S. and Davidson, E. H.** (2011). A gene regulatory network controlling the embryonic specification of endoderm. *Nature* **474**, 635–639.
- Polte, T., Abate, A., Dennery, P. A. and Schroder, H.** (2000). Heme oxygenase-1 is a cGMP-inducible endothelial protein and mediates the cytoprotective action of nitric oxide. *Arterioscler Thromb Vasc Biol* **20**, 1209–1215.
- Rast, J., Cameron, R., Poustka, A. and Davidson, E.** (2002). Brachyury target

genes in the early sea urchin embryo isolated by differential macroarray screening. *Dev Biol* **246**, 191–208.

- Saxena, M., Stephens, M. A., Pathak, H. and Rangarajan, A.** (2011). Transcription factors that mediate epithelial-mesenchymal transition lead to multidrug resistance by upregulating ABC transporters. *Cell Death Dis* **2**, e179.
- Solursh, M. and Lane, M. C.** (1988). Extracellular matrix triggers a directed cell migratory response in sea urchin primary mesenchyme cells. *Dev Biol* **130**, 397–401.
- Sugiyama, K.** (1972). Occurrence of mucopolysaccharides in the early development of the sea urchin embryo and its role in gastrulation. *Dev Growth Differ* **14**, 63–74.
- Toscano-Garibay, J. D. and Aquino-Jarquín, G.** (2012). Regulation exerted by miRNAs in the promoter and UTR sequences: MDR1/P-gp expression as a particular case. *DNA Cell Biol.* **31**, 1358–1364.
- Venkatasubramanian, K. and Solursh, M.** (1984). Adhesive and migratory behavior of normal and sulfate-deficient sea urchin cells in vitro. *Exp Cell Res* **154**, 421–431.
- Wei, Z., Angerer, R. C. and Angerer, L. M.** (2006). A database of mRNA expression patterns for the sea urchin embryo. *Dev Biol* **300**, 476–484.
- Wei, Z., Range, R., Angerer, R. and Angerer, L.** (2012). Axial patterning interactions in the sea urchin embryo: suppression of nodal by Wnt1 signaling. *Development* **139**, 1662–1669.
- Werb, Z., Tremble, P. M., Behrendtsen, O., Crowley, E. and Damsky, C. H.** (1989). Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J Cell Biol* **109**, 877–889.
- Westlake, C. J., Cole, S. P. C. and Deeley, R. G.** (2005). Role of the NH₂-terminal membrane spanning domain of multidrug resistance protein 1/ABCC1 in protein processing and trafficking. *Mol Biol Cell* **16**, 2483–2492.
- Whalen, K., Reitzel, A. M. and Hamdoun, A.** (2012). Actin polymerization controls the activation of multidrug efflux at fertilization by translocation and fine-scale positioning of ABCB1 on microvilli. *Mol Biol Cell* **23**, 3663–3672.
- Xie, J., Drumm, M. L., Ma, J. and Davis, P. B.** (1995). Intracellular loop between transmembrane segments IV and V of cystic fibrosis transmembrane

conductance regulator is involved in regulation of chloride channel conductance state. *J Biol Chem* **270**, 28084–28091.

Zhang, Z., Wu, J.-Y., Hait, W. N. and Yang, J.-M. (2004). Regulation of the stability of P-glycoprotein by ubiquitination. *Mol Pharmacol* **66**, 395–403.

4.4. CHAPTER ACKNOWLEDGMENTS

Figure 4.1 and legend are published in Gökırmak T, Shipp LE, Campanale JP, Nicklisch SC, Hamdoun A. Transport in technicolor: Mapping ATP-binding cassette transporters in sea urchin embryos. *Molecular Reproduction and Development*. 2014 Sep; 81 (9): 778-793. The dissertation author made the figure and legend.