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Education

University of Maryland Baltimore, Founding Campus, School of Pharmacy, Department of Pharmaceutical Sciences, Baltimore, MD

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- Characterized differences recombinant protein K'd using fluorescence quenching
- Evaluated *ex vivo* enzyme activities isolated from tissues
- Histological evaluation of tissues
- Retinoid analysis in several collaborations for various models including cell culture, murine embryo, serum and plasma, nematodes, worm species, mollusk species, murine and NHP lung, murine and NHP heart, and murine and NHP gut.
- Experience working with HIV and NHP infectious samples.
- New lab start up including purchasing basic and complex equipment, validation of instruments and assays, establishing protocols and training new post-docs and students.

- Experienced with HPLC/UPLC instrumentation and software including method development and validation, Agilent 1290 Infinity with Chemstation and Waters UPLC H-Class with Empower
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AstraZeneca, Wilmington, DE

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University of Delaware, Newark, DE

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Peer-Reviewed Publications

Pierzchalski, K., Yu, J. and Kane M.A. (2013) CrbpI regulates mammary retinoic acid homeostasis and the mammary microenvironment. *FASEB J.* 27, 1904-1916.

Pauli, S.A., Session, D.R., Shang, W., Easley, K., Wieser, F., Taylor, R.N., **Pierzchalski, K.**, Napoli, J.L., Kane, M.A. and Sidell, N. (2013) Analysis of Follicular Fluid Retinoids in Women Undergoing In Vitro Fertilization: Retinoic Acid Influences Embryo Quality and Is Reduced in Women With Endometriosis. *Reprod. Sci.* 20(9):1116-2

Billings, S., **Pierzchalski, K.**, Tjaden, N., Trainor, P.A., Kane, M.A. and Moise, A.R. DHRS3 is required for the maintenance of retinoid homeostasis during embryonic development. *FASEB J.* 27(12):4877-4889

Gutierrez-Mazariegos, J., Kumar Nadendla, E., Lima, D., **Pierzchalski, K.**, Jones, J.W., Kane, M.A., Nishikawa, J.I., Hiromonri, Y., Nakanishi, T., Santos, M.M., Castro, L.F.C., Bourguet, W., Schubert, M. and Laudet, V. (2014) A mollusk Retinoic Acid Receptor (RAR) ortholog sheds light on the evolution of ligand binding. *Endocrinology*, 155(11):4275-86

Pierzchalski, K., Taylor, R.N., Nezhat, C., Napoli, J.L., Yang, G., Kane, M.A. and Sidell, N. (2014) Retinoic acid biosynthesis is impaired in endometriosis. *Biol. of Reprod.* 91(4):84

Jones, J.W., **Pierzchalski, K.**, Yu, J. and Kane, M.A. (2015) The use of fast HPLC multiplexing MRM3 for endogenous retinoic acid quantification in complex matrices. *Anal. Chem.* 87(6):3222-3230

Best, M.W., Pauli, S.A., Wu, J., Kane, M.A., **Pierzchalski, K.**, Session, D.R., Shang, W., Taylor, R.N. and Sidell, N. (2015) A role for retinoids in human oogenesis: regulation of connexin 43 by retinoic acid in cumulus granulosa cells. *Mol. Human Reprod.* Accepted.

Byrareddy, S.N., Sidell, N., Zhao, C., Dunbar, P., Little, D., Yang, G.X., **Pierzchalski, K.**, Kane, M.A., Mayne, A., Arthos, J., Cicala, C., Song, B., Soares, M.A., Villinger, F. and Ansari, A.A. (2015) Species-specific differences in the expression and regulation of alpha 4 beta 7 ($\alpha 4 \beta 7$) integrin in various non-human primates. *Journal of Immunology.* Accepted.

Pierzchalski, K., Yu, J., Jones, J.W. and Kane, M.A. Impact of cellular retinol binding protein, type I on retinoic acid homeostasis in murine lung. *FASEB J.* In preparation.

Poster Presentations

Keely Pierzchalski, MT(ASCP), CLS(NCA), Dalal Tonb, PhD, Tracey Nadal, BS, Laura Bolling, BS, Novel Diagnostic Applications of an Available Technology. ASCLS Annual Meeting, Chicago, Illinois, July 2009.

Pierzchalski K, Yu J and Kane MA. Functional Comparisons of mCRBP1 and mCRBP3 and Their Impact on Retinoic Acid Biosynthesis from Vitamin A. University of Maryland, Graduate Research Conference, Baltimore, Maryland, April 5, 2012.

Pierzchalski K, Yu J and Kane MA. Impact of Cellular Retinol-Binding Protein, type I on Mammary Retinoic Acid Homeostasis. FASEB Summer Research Conference, Retinoids, Snowmass Village, Colorado, June 10-15, 2012.

Pierzchalski K, Yu J and Kane MA. mCRBP1 and mCRBP3 Impact on Retinoic Acid Biosynthesis from Vitamin A. American Society for Clinical Laboratory Science Annual Meeting, Los Angeles, California, July 17-21, 2012.

Pierzchalski K, Sidell N and Kane MA. Impact of CrbpI and Retinoic Acid on Endometriosis. School of Pharmacy Research Day, April 11, 2013.

Pierzchalski K, et al. Targeted biomarker discovery by LC-MS/MS for Medical Countermeasures against Radiological Threats. MSBM Summer School, Dubrovnik, Croatia, July 7-13, 2013.

Pierzchalski K, et al. Radiation alters retinoid homeostasis. International Retinoids Meeting, Rende, Italy, September 9-14, 2013.

Pierzchalski K, et al. Retinoic acid biosynthesis is impaired in endometriosis. FASEB SRC, Retinoids, Itasca, Illinois, June 1-6, 2014.

Pierzchalski K, et al. Developmental and Application of Fast HPLC Multiplexing MRM³ for Quantification of Endogenous metabolites in lung tissue. American Society for Mass Spectrometry (ASMS), Baltimore, MD, June 15-19.

Invited Talks

- 2012 FASEB Summer Research Conference, Retinoids, Snowmass Village, Colorado. *Impact of Cellular Retinol-Binding Protein, type I on Mammary Retinoic Acid Homeostasis.*
- 2013 International Retinoids Meeting, Rende, Italy. *Retinoic acid biosynthesis is impaired in endometriosis.*

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2006-2009 Student Advisor
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Abstract:

Title: Impact of Cellular Retinol Binding Protein, Type I

on Retinoic Acid Biosynthesis and Homeostasis

Keely A. Pierzchalski, Doctor of Philosophy, 2015

Dissertation Directed by:

Maureen A. Kane, PhD, assistant professor and co-director of the Mass Spectrometry Core Facility, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Baltimore, MD

Statement: A global *Rbp1* knock out (*Rbp1*^{-/-}) mouse model was used to correlate direct retinoid measurements with vitamin A metabolizing and atRA biosynthesizing enzyme activities, Crbp function and tissue microenvironment for the first time.

Methods: atRA was quantified by LC-MRM³ and ROL/RE/RAL was quantified by HPLC-UV. Enzyme activities were measured from enzymes present in subcellular fractions isolated from WT and *Rbp1*^{-/-} tissues. Mouse CrbpI and CrbpIII were purified from transformed *Escherichia coli* for functional comparative studies. Tissue were formalin fixed for histological examination. Relative gene expression was analyzed using quantitative PCR.

Results: Reduced atRA was consistently quantified in extrahepatic tissues with elevated ROL/RE. Relative gene expression showed altered expression in retinoid pathway proteins and atRA loss preceded expression changes in some cases. Tissue

microenvironments also consistently showed a loss of structure and organization along with accumulation of extracellular matrix and hyperplasia without apparent disease.

Functional studies showed that CrbpIII binds retinol with less affinity than CrbpI and does not function equivalently to CrbpI in regulation of atRA biosynthesis. Also, metabolizing enzymes had altered activities in the *Rbp1*^{-/-} tissues with reduced atRA biosynthesis.

Conclusions: Loss of CrbpI results in altered regulation of enzyme activity and atRA homeostasis cannot be maintained by other Crbp homologs in extrahepatic tissues. Dysfunctional atRA biosynthesis due to loss of CrbpI results in altered tissue microenvironment characteristic of dietary vitamin A deficiency and precancerous dysfunction associated with cancers that are observed to have silenced CrbpI.

Impact of Cellular Retinol Binding Protein, Type I on
Retinoic Acid Biosynthesis and Homeostasis

By
Keely A. Pierzchalski

Dissertation submitted to the faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2015

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Dedication:

To my grandparents, John L. Pierzchalski and Carol R. Pierzchalski, for their love and influence in my life and career. To Mary Ellen d'Urso Hall for her enduring love and support, thank you for being there to share my burdens and joys. Though you may no longer be with us, your love and all you have shared stays with me.

To my father, Steven J. Pierzchalski, who for as long as I can remember, told me I can do anything I set my mind to and be the best at it with hard work and education. This did not fall on deaf ears, Dad, though you may sometimes have thought so. You taught me the value of a nickle penny and two bits. You taught me to be independent, strong willed, strong minded and dedicated. Though these qualities have not always been in your favor, they have made me into the scientist I am today.

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Table of Contents

List of Figures	ix
List of Abbreviations	xi
Chapter 1: Introduction to vitamin A metabolism and function	1
1.1 Vitamin A intake and metabolism	1
1.1.1 <i>Vitamin A absorption, storage and transport</i>	2
1.1.2 <i>Vitamin A metabolism and atRA biosynthesis</i>	5
1.2 Cellular retinol binding proteins	7
1.3 atRA activation of nuclear receptors	12
1.4 <i>In vitro</i> studies of retinoid activity	17
1.5 <i>In vivo</i> studies of retinoid activity	20
1.6 Retinoic acid uses in the clinic	22
1.7 References	24
Chapter 2: CrbpI regulates mammary retinoic acid homeostasis and the mammary microenvironment	33
2.1 Introduction	33
2.2 Experimental Procedures	36
2.2.1 <i>Mice</i>	36
2.2.2 <i>Retinoids and retinoid extraction</i>	36
2.2.3 <i>CrbpI and CrbpIII preparation</i>	37
2.2.4 <i>Fluorescence measurements</i>	38

2.2.5	<i>Subcellular fractionation</i>	39
2.2.6	<i>Enzyme activity</i>	40
2.2.7	<i>Gene expression</i>	41
2.2.8	<i>NAD⁺/NADH determination</i>	41
2.3	Results	42
2.3.1	<i>Endogenous atRA is depleted in mammary tissue of Rbp1^{-/-} mice</i>	42
2.3.2	<i>Expression of atRA biosynthesis enzymes in mammary tissue</i>	44
2.3.3	<i>CrbpIII is not functionally interchangeable with CrbpI</i>	46
2.3.4	<i>Crbp delivery and Rbp1 loss affect atRA biosynthesis</i>	48
2.3.5	<i>atRA-depleted Rbp1^{-/-} mammary tissue exhibits epithelial and stromal hypercellularity</i>	52
2.4	Discussion	54
2.4.1	<i>Degree of atRA depletion in Rbp1^{-/-} mammary tissue is consistent with aberrant physiology</i>	56
2.4.2	<i>atRA homeostasis is disrupted in Rbp1^{-/-} mammary tissue</i>	57
2.4.3	<i>atRA depletion precedes defects in atRA biosynthesis enzyme expression</i>	59
2.4.4	<i>Rdh enzyme activity contributes to depletion of endogenous atRA</i>	60
2.4.5	<i>Loss of Rbp1 and endogenous atRA affects the mammary microenvironment</i>	63
2.5	Conclusion	65
2.6	Acknowledgements	65
2.7	References	66

Chapter 3: Retinoic acid biosynthesis is impaired in human and murine	
endometriosis	75
3.1 Introduction	75
3.2 Experimental Procedures	77
3.2.1 Human endometrial tissue and cell cultures	77
3.2.2 Mouse models of endometriosis	78
3.2.3 Rbp1^{-/-} Mice	79
3.2.4 Determination of retinoid levels	80
3.2.5 Evaluation of Rbp1 mRNA by quantitative real-time (q) PCR	80
3.2.6 Statistical analysis	81
3.3 Results	81
3.3.1 Endogenous atRA is reduced in human endometriotic lesions	81
3.3.2 Reduced atRA production and Crbp1 expression in endometriotic lesions	84
3.3.3 Endogenous atRA is lower in induced murine endometriotic lesions	86
3.3.4 Endogenous atRA is reduced in Rbp1^{-/-} mouse endometrium	88
3.3.5 Rbp1^{-/-} uterus histology exhibits loss of tissue organization and hypercellularity	90
3.4 Discussion	92
3.5 Acknowledgements	96
3.6 References	96

Chapter 4: Impact of CrbpI on atRA biosynthesis and homeostasis in murine lung	100
.....
4.1 Introduction	100
4.2 Experimental Procedures	103
4.2.1 <i>Rbp1</i>^{-/-} Mice	103
4.2.2 <i>Retinoids and retinoid extraction</i>	103
4.2.3 <i>CrpbI and CrpbIII recombinant protein preparation</i>	104
4.2.4 <i>Subcellular fractionation</i>	105
4.2.5 <i>Enzyme activity</i>	105
4.2.6 <i>Apo-Crbp inhibition assays</i>	106
4.2.7 <i>Gene expression</i>	107
4.2.8 <i>NAD⁺/NADH Determination</i>	107
4.2.9 <i>Histology</i>	108
4.3 Results	108
4.3.1 <i>atRA is deficient in the absence of CrbpI expression</i>	108
4.3.2 <i>Retinol dehydrogenase (Rdh) activity is altered in Rbp1</i>^{-/-} <i>lung</i>	110
4.3.3 <i>Retinal reductase (Rrd) activity is not altered in Rbp1</i>^{-/-} <i>lung</i>	114
4.3.4 <i>Retinal dehydrogenase (Raldh) activity is altered in Rbp1</i>^{-/-} <i>lung</i>	116
4.3.5 <i>Rbp1</i>^{-/-} <i>lung microenvironment is characteristic of VAD metaplasia and hyperplasia</i>	118
.....
4.4 Discussion	120
4.4.1 <i>Rbp1</i>^{-/-} <i>lung has altered vitamin A metabolism, atRA biosynthesis and homeostasis in the absence of CrbpI</i>	120

4.4.2	<i>Dysfunctional atRA biosynthesis results in an altered microenvironment that resembles VAD metaplasia and hyperplasia</i>	124
4.5	Acknowledgements	125
4.6	References	126
Chapter 5: Discussion		132
5.1	Retinoic acid quantification	132
5.2	<i>Rbp1</i> ^{-/-} mouse model	133
5.3	The role of vitamin a in health maintenance and the future of retinoids in therapeutics	136
5.4	CrbpI as a potential tissue biomarker for atRA status	138
5.5	Future CrbpI studies	140
5.6	References	141
Appendix A: Chapter 4 Supplemental Figures		148
Comprehensive Reference List		150

List of Figures:

Figure 1.1: Vitamin A absorption, storage and transport4

Figure 1.2: Vitamin A metabolism and atRA biosynthesis6

Figure 1.3: Representative Crbp crystal structures8

Figure 1.4: Human Crbp homolog amino acid sequence alignment10

Figure 1.5: Retinoic acid signaling and RAR activation14

Figure 1.6: atRA modulates targets to maintain cellular homeostasis16

Figure 2.1: Endogenous atRA is depleted in the mammary of *Rbp1*^{-/-} mice43

Figure 2.2: Expression of atRA biosynthesis enzymes in mammary45

Figure 2.3: CrbpIII is not functionally interchangeable with CrbpI47

Figure 2.4: Effects of Crbp delivery and *Rbp1* loss on atRA biosynthesis51

Figure 2.5: atRA depleted mammary exhibits epithelial hyperplasia, stromal hypercellularity, and oxidative stress53

Figure 2.6: Impact of *Rbp1* loss on atRA homeostasis55

Figure 3.1: Altered retinoid levels in ectopic endometrial implants compared with eutopic endometrium83

Figure 3.2: atRA production and *Rbp1* gene expression are reduced in human endometriotic lesions85

Figure 3.3: atRA levels are lower in induced endometriotic mouse lesion87

Figure 3.4: Reduced atRA in *Rbp1*^{-/-} endometrium89

Figure 3.5: Morphological disorganization and hypercellularity of *Rbp1*^{-/-} endometrium91

Figure 4.1: atRA is deficient in the absence of CrbpI expression109

Figure 4.2: atRA biosynthesis enzyme activity workflow110

Figure 4.3: Rdh activity is altered in *Rbp1*^{-/-} lung113

Figure 4.4: Rrd activity is not altered in *Rbp1*^{-/-} lung115

Figure 4.5: Raldh activity is altered in *Rbp1*^{-/-} lung117

Figure 4.6: *Rbp1*^{-/-} lung is characteristic of VAD lung metaplasia and hyperplasia119

Figure 4.7: Altered atRA biosynthesis in *Rbp1*^{-/-} lung121

Supplemental Figure 4.1: Additional enzyme activities148

Supplemental Figure 4.2: Relative gene expression for Stra6, Cyp26 and RAR149

List of Abbreviations:

atRA	all-trans retinoic acid
ROL	retinol (vitamin A)
RE	retinyl esters
RAL	retinal or retinaldehyde
CrbpI	cellular retinol-binding protein, type 1 (protein name)
CrbpII	cellular retinol-binding protein, type 2 (protein name)
CrbpIII	cellular retinol-binding protein, type 3 (protein name for murine homolog of <i>Rbp7</i>)
CrbpIV	cellular retinol-binding protein, type 4 (protein name for human homolog of <i>Rbp7</i>)
CRABP	cellular retinoic acid binding protein (types I and II)
Dhrs	Retinol dehydrogenase (<i>Dhrs9</i>)
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
ESC	endometrial stromal cells
K'd	apparent dissociation constant
LC-MRM³	Fast-LC multiplex multiple reaction monitoring cubed
MMP	matrix metalloproteinase
PPARβ/δ	peroxisome proliferator activated receptor beta/delta
Raldh	retinal dehydrogenase (<i>Raldh1</i> , 2 and 3)
<i>Rbp1</i>	retinol-binding protein, type 1 (gene name for <i>CrbpI</i>)
<i>Rbp2</i>	retinol-binding protein, type 2 (gene name for <i>CrbpII</i>)
<i>Rbp4</i>	circulating retinol binding protein, type 4 (gene name for <i>Rbp4</i>)
<i>Rbp5</i>	retinol-binding protein, type 5 (gene name for human <i>CrpbIII</i>)
<i>Rbp7</i>	retinol-binding protein, type 7 (gene name for murine <i>CrpbIII</i> or human <i>CrpbIV</i>)

Rdh	retinol dehydrogenase (Rdh10/12)
Reh	retinyl ester hydrolase
Rrd	retinal reductase (Dhrs3)
RAR	retinoic acid receptor (isomers alpha, beta and gamma)
RXR	retinoid x receptor (isomers alpha, beta and gamma)
SDR	short chain dehydrogenase/reductase (Rdh/Rrd)
Stra6	stimulated by retinoic acid receptor-6
VAD	vitamin A deficiency
WT	C57BL/6J wild type mouse strain

Chapter 1: Introduction to vitamin A metabolism and function

1.1 Vitamin A intake and metabolism

Vitamin A (retinol) is an essential lipid soluble vitamin that is consumed from plants in the pro-vitamin form of beta-carotene or from animals in the storage form of retinyl esters (RE), with retinyl palmitate being the most abundant.¹ Retinol (ROL) is metabolized to the active metabolite, retinoic acid (RA), which activates nuclear receptors for gene transcription. All-trans RA (atRA) is the primary active isomer. Retinoid structures consist of a β -ionone ring and an isoprenoid tail with a polar end group specific to the metabolite. ROL has a hydroxyl group, RE an ester group, retinal (RAL) an aldehyde group and RA a carboxylic acid group. Retinoids are chaperoned by members of the lipocalin fatty acid binding protein (iLBP) family, which function as carriers for hydrophobic small molecules. iLBP are small molecular weight proteins, ranging from ~15-40 kDa, and share a barrel structure of anti-parallel beta sheets with an alpha helical lid. Cellular retinol binding protein, type II (CrbpII) is the abundant iLBP chaperone protein for vitamin A absorption and metabolism in the small intestine. Cellular retinol binding protein, type I (CrbpI) is ubiquitously expressed and is the predominant chaperone in the rest of the body outside of the eye. Other intracellular retinol binding protein homologs have been identified and studied to a lesser extent than CrbpI, in selective tissues, however, the extent of their functions is still unknown.¹⁻⁵ ROL metabolism and atRA biosynthesis is strictly maintained by a number of mechanisms. Deficiency and excess of retinoids are both detrimental to the cell, tissue and body as a whole. Vitamin A deficiency (VAD) is defined as a whole body deficiency, as a lack of vitamin A ingestion and liver stores. Symptoms of VAD include night blindness, immune

deficiencies and respiratory pathologies.⁶ Vitamin A toxicity (VAT) may be due to multi-vitamin and medication overuse/overdose; and may include symptoms of headaches, nausea, altered vision and skin rash.⁷ VAT during pregnancy can lead to irreversible birth defects. During critical stages of development, VAD and VAT have significant consequences of growth defects and death.⁸ The severity of symptoms are impacted by the extent of acute or chronic VAD/VAT. Retinoid deficiency and excess can also be localized in a tissue or cell, as well as in a retinoid specific manner related to retinoid absorption, transport, storage and metabolism.⁶⁻⁸

1.1.1 Vitamin A absorption, storage and transport

Solubilized in lipid droplets, beta-carotene is absorbed by gut mucosal cells and incorporated into chylomicrons. After absorption, beta-carotene is metabolized to RAL and then to ROL in both the intestine and liver. As RE is consumed, it must be hydrolyzed to ROL for absorption by gut enterocytes.¹ Regulated by CrbpII, ROL is metabolized back to RE to be incorporated into chylomicrons. Chylomicrons are transported to the blood and then transported to tissues, with the liver being the primary destination, as the stellate cells of the liver are the main vitamin A storage site in the body.¹ As extra-hepatic tissues require vitamin A, ROL is secreted by the liver bound to circulating retinol binding protein, type 4 (Rbp4). Holo-Rbp4 is transported through the blood attached to the carrier protein, transthyretin (TTR) to prevent excretion during kidney filtration (**Figure 1.1**).^{1,5,9} Holo-Rbp4 is released from TTR at the cell membrane, where it is recognized by the membrane receptor stimulated by retinoic acid 6 (Stra6). ROL is released from Rbp4, passed through a transmembrane pore to be intracellularly retrieved primarily by apo-CrbpI.⁵ It has also been shown that free ROL

released from holo-Rbp4-TTR can passively diffuse through the cell membrane in addition to transport through the membrane receptor Stra6. The proportion of ROL transport from circulation into cells by either passive diffusion or Stra6 mediated transport is still under debate.^{5,9} By either means, intracellular ROL is bound to CrbpI (holo-CrbpI) in a 1:1 ratio to be directed to vitamin A metabolizing enzymes and protected against nonspecific metabolism. atRA biosynthesis must be elegantly maintained to accommodate the cell and tissue spatiotemporal needs of atRA activity for cell signaling homeostasis.¹⁰⁻¹⁵

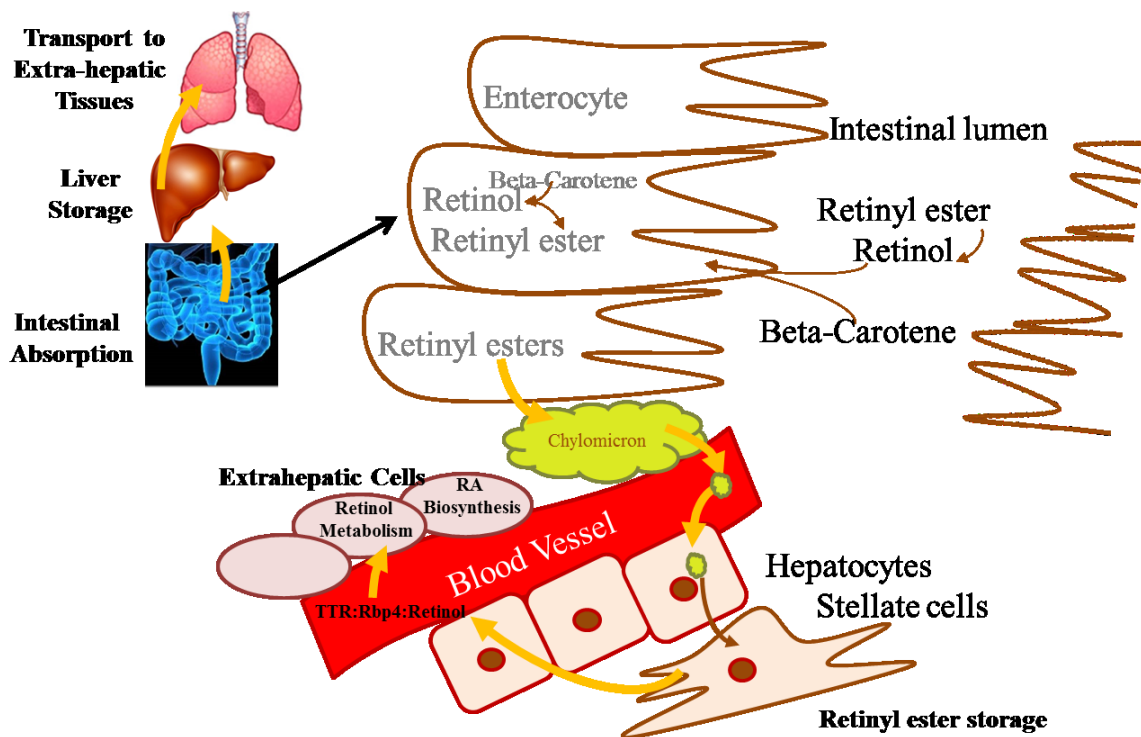


Figure 1.1. Vitamin A absorption, storage and transport. Vitamin A is a lipid soluble essential vitamin that is absorbed in the intestine, stored in the liver and transported to extrahepatic tissue as needed. Beta carotene and retinyl esters are consumed and absorbed, converted to retinol for transport through circulation and re-esterified for liver storage. Retinyl esters are hydrolyzed to retinol and escorted through circulation by carrier proteins to be delivered to extrahepatic tissues.

1.1.2 Vitamin A metabolism and atRA biosynthesis

Vitamin A metabolism includes the conversion of ROL to RE storage and atRA biosynthesis (**Figure 1.2**). ROL uptake into cells and RE stores must be maintained to provide substrate for atRA biosynthesis.¹² If ROL uptake is reduced, RE stores are tapped to replenish intracellular ROL substrate for atRA biosynthesis. Apo-CrbpI retrieves ROL from Sta6 to become holo-CrbpI and then holo-CrbpI delivers its ligand to enzymes anchored in the membranes of the endoplasmic reticulum (ER). ROL is shuttled to ER membrane bound lethicin:retinol acyl transferase (Lrat) for esterification and is reversibly hydrolyzed by retinyl ester hydrolase enzymes (Reh). Lrat is the primary ROL esterification enzyme, however, diacylglycerol acyltransferase 1 and 2 (Dgat1/2), additional acyl-coA acyltransferase (ARAT) enzymes, have been identified in select tissues to function in ROL esterification.¹² This flux of retinoid substrate-storage is regulated by the ratio of apo-CrbpI:holo-CrbpI. Greater holo-CrbpI to apo-CrbpI directs ROL to be esterified for storage. Greater apo-CrbpI to holo-CrbpI reflects reduced ROL uptake into the cell and apo-CrbpI directs Reh enzymes to hydrolyze RE to ROL, as well as to inhibit Lrat activity to prevent further esterification.¹² Lrat and Stra6 have also been reported to interact to enhance ROL uptake in addition to apo-CrbpI-Stra6 ROL uptake.⁵

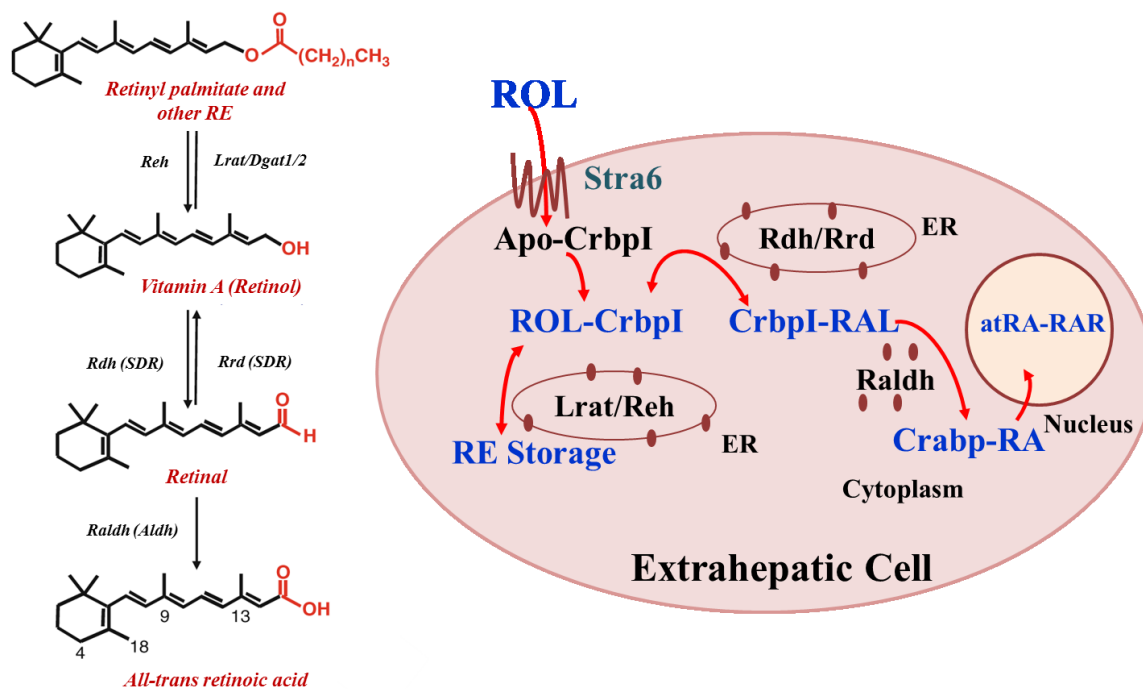


Figure 1.2. Vitamin A metabolism and atRA biosynthesis. Retinoid chemical structures have a beta ionone ring and isoprenoid tail with a polar end group specific to the metabolite. Retinol is taken up into the extrahepatic cell through Stra6 and picked up by apo-CrbpI. CrbpI delivers retinol to vitamin A metabolizing enzymes to be stored as retinyl esters or oxidized into retinoic acid.

atRA biosynthesis is a two-step oxidation reaction to produce atRA from ROL with RAL as an intermediate. The first step is the rate limiting reversible oxidation of ROL to RAL and reduction of RAL to ROL. ER membrane bound short chain dehydrogenase/reductase enzymes (SDR) function as retinol dehydrogenase (Rdh) enzymes for ROL oxidation to RAL and retinal reductase (Rrd) enzymes for RAL reduction to ROL. These reactions are bi-substrate reactions, where Rdh enzymes require NADP^+ and Rrd enzymes require NADPH as well as holo-CrbpI substrate for activity.¹² CrbpI shuttles both ROL and RAL with high binding affinities of 1-60 nM, determined by fluorescence titration experiments to calculate the apparent dissociation constant (K_d) of representative binding sites.¹⁶⁻²⁰ RAL is metabolized quickly to atRA as the aldehyde end group is quickly oxidized to a carboxylic acid. In this second oxidation step, CrbpI chaperones RAL to cytosolic retinal dehydrogenase (Raldh) enzymes for the fast and irreversible oxidation of RAL to atRA with NAD^+ as the cofactor preference. Apo-CrbpI additionally acts upon Raldh as a Raldh inhibitor and therefore a feedback signal to reduce activity until further substrate is available or needed. Alternatively, atRA signals feedback to Lrat, so as atRA is increased, Lrat is signaled to esterify ROL rather than ROL being shuttled to atRA biosynthesis enzymes.^{12, 21}

1.2 Cellular retinol binding proteins

Cellular, or cytosolic, retinol binding proteins, as members of the iLBP family function primarily to chaperone ROL and RAL to vitamin A metabolizing enzymes. These proteins have been identified and partially characterized in a number of species, including human, mice, rat, bovine and zebrafish. According to the NCBI protein databank, CrbpI

and CrbpII have been identified in all of these species and several protein structures have been reported using NMR and protein crystallization (**Figure 1.3**).²²

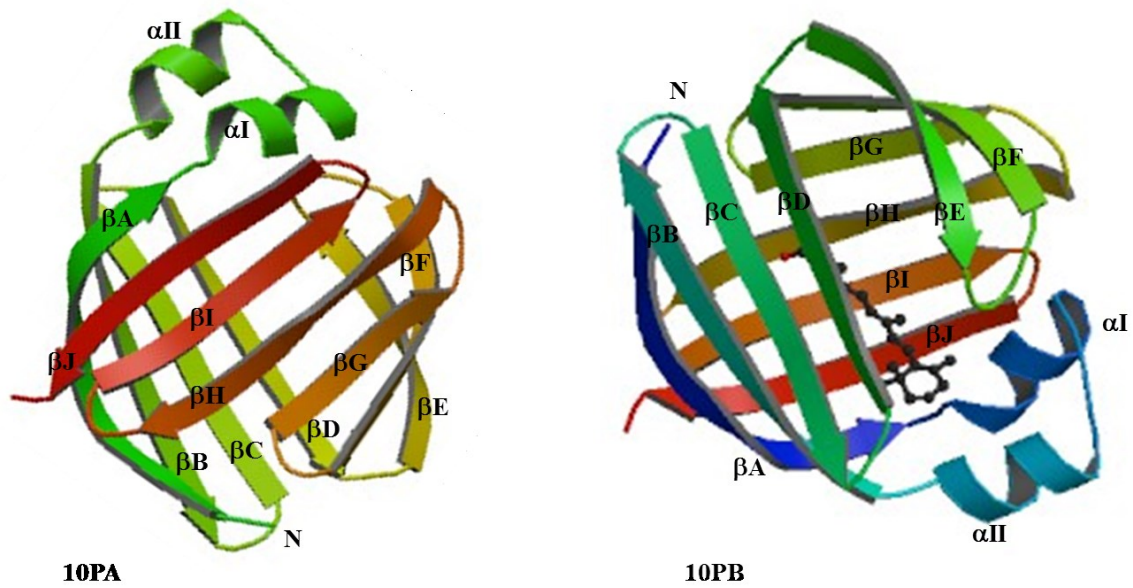


Figure 1.3. Representative Crbp crystal structures. CrbpII crystal structures from the RCSB Protein Data Bank, 10PA and 10PB, show unbound (apo) and retinol ligand bound (holo) CrbpII. 10 antiparallel beta sheets (A-J) form a barrel shape and 2 alpha helices (I-II) form a lid over the portal. Conformational change upon ligand binding protects the ligand during transport.

The gene names for these proteins are *Rbp1* and *Rbp2* respectively. Two additional human homologs have been identified and named CrbpIII and CrbpIV, in which intracellular CrbpIV is distinct from the circulating Rbp4. The Rbp4 gene name is *Rbp4* and CrbpIV gene name is *Rbp7*. The gene for CrbpIII is designated *Rbp5*. The distinction for the gene names is important to distinguish species homologs as they are also identified. *Rbp7* has been identified in mouse and zebrafish, however, the protein name in mouse is CrbpIII. The gene comparisons between species link them, though protein nomenclature has been less consistent. For example, human CrbpIV and mouse CrbpIII share 89% homology versus 35-60% homology to other human homologs.²³ Though there is considerable amino acid sequence conservation between the Crbp homologs, including 4 tryptophan residues which give these proteins intrinsic fluorescence, it is the non-conserved sequences which account for the homologs' variations for their specific conformational changes, ligand binding site affinity and target protein interactions for ligand transfer (**Figure 1.4**).²³

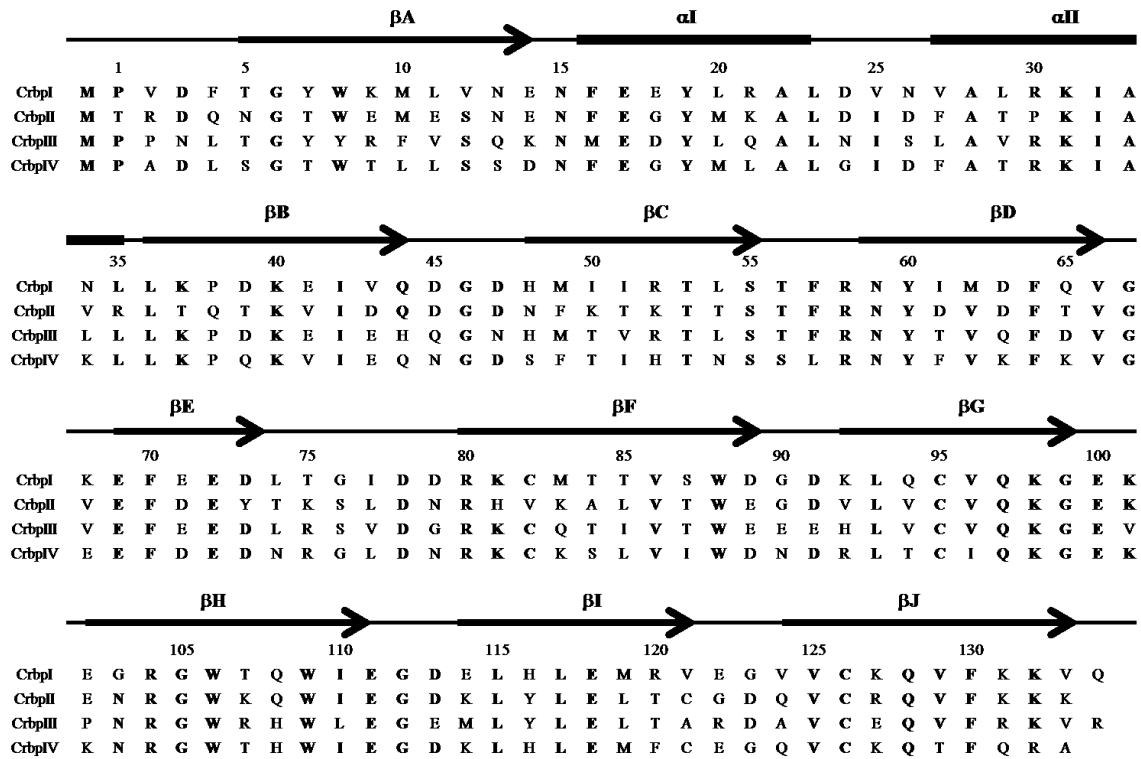


Figure 1.4. Human Crbp homolog amino acid sequence alignment. Human CrbpIV shares 57%, 58% and 49% homology with human CrbpI, CrbpII and CrbpIII respectively.

Conserved sequences are in bold. The 10 antiparallel beta sheet sequences are indicated with arrows and 2 alpha helices are indicated with bars and space between indicates connecting turns.

The protein crystal structures of CrbpI and CrbpII have been well characterized both unbound (apo) and retinoid ligand bound (holo).^{19, 22-27} Human CrbpIII and CrbpIV crystal structures have been reported; however, mouse CrbpIII has not. Comparative functional studies have been done for CrbpI and CrbpII homologs, but not for other homologs, including mouse CrbpIII. Several CrbpI and CrbpII key structural differences may account for observed functional differences. Binding affinities have been evaluated using fluorescence quenching to determine the apparent dissociation constant, K_d ; and protein crystallography to compare ligand binding.¹⁶⁻²⁰ CrbpI and CrbpII binding affinities for ROL have been reported in the range of ~1-15 nM and for RAL in the 5-90 nM range.¹⁶⁻²⁰ In comparing apo and holo structures, upon ligand binding, flexible binding regions become more stable, particularly in CrbpI. Stabilization of the portal region indicates the importance of ligand binding residues and subsequent conformational changes in the alpha helices (αI and αII) and in the turns between the beta sheets βE - βF and βC - βD . Key residue differences between CrbpI and CrbpII result in a loss in hydrophobic side chains, hydrogen bonding and salt bridges in CrbpII that account for holo-CrbpI ligand specificity and protein stability. These structural properties of CrbpI correspond to its functional role in strictly regulating vitamin A metabolism and atRA homeostasis.^{19, 22-27} Besides its role as a carrier protein for retinoids, apo-CrbpI has an important role in maintaining retinoid flux for endogenous atRA homeostasis. Functional studies of apo-CrbpI have reported a regulatory role on inhibition and induction of metabolizing enzyme activity. The apo:holo CrbpI ratio helps direct vitamin A metabolism to maintain proper endogenous retinoid levels. *In vitro* enzyme activity measurements with increasing concentrations of apo-CrbpI have shown inhibition of Lrat

esterification of ROL to RE and induction of Reh for RE hydrolysis to ROL. These *in vitro* enzyme activity assays have also shown inhibition of Rdh and Raldh activity by apo-CrbpI. Apo-CrbpII reportedly did not regulate metabolizing enzyme activity in this manner.^{12, 21, 28-31}

Because atRA activates RAR nuclear receptor transcription, which have hundreds of direct and indirect targets, a small change in activation may result in exponential downstream transcriptional effects. atRA and CrbpI loss has been identified in a number of diseases including reproductive and developmental disorders and a number of cancers at a rate of 7-60%.³²⁻³³ Endogenous atRA homeostasis is complex but the potential benefit of being able to modulate multiple downstream pathways may minimize retinoid side effects and toxicities.

1.3 atRA activation of nuclear receptors

Once atRA has been produced, it is bound to its own iLBP class of proteins, cellular retinoic acid binding proteins (Crabp) or select members of fatty acid binding proteins, FABP to be shuttled across the nucleus membrane to target nuclear receptors. Nuclear receptors are transcription factors which are activated when bound by small molecule ligands. The atRA carrier protein may impart selectivity in receptor activation, where, in some contexts, CrabpII delivers atRA to RAR α , β and γ and alternatively, FABP5 delivers atRA to PPAR β/δ for transcription.³⁴ An additional carrier, CrabpI delivers atRA to Cyp26 catabolizing enzymes, in lieu of nuclear receptor activation, for excretion and prevention of atRA excess. The retinoid X receptor (RXR) is an additional retinoid nuclear receptor, and is known to dimerize with RAR α , β , γ and PPAR β/δ , as well as

multiple other non-retinoid nuclear receptors.³⁵ Nuclear receptors contain multiple domains with specificity in each domain for the activating ligand and target DNA promoter site for distinct gene regulation control. The DNA binding domain (DBD) is centrally located between the highly variable N-terminal domain (NTD) and the C-terminal ligand binding domain (LBD). atRA binds to the ligand binding pocket (LBP) of the LBD to induce conformational changes and activity. The LBD also contains the heterodimerization surface, the co-regulator surface and a phosphorylation site. The DBD binds to specific DNA sequences called retinoic acid response elements (RARE). RARE sequences are composed of two direct repeats of a core hexameric motif that may be separated by 0, 1, 2, 5 or 8 nucleotides. The RARE motifs are therefore distinguished as DR0, DR1, DR2, DR5, DR8 or IR0 (inverted repeat). The DBD also has two zinc-nucleated modules and two alpha helices. Though the NTD are by nature highly variable, there are conserved phosphorylation sites.^{14, 36} Non-genomic activities have been reported by identifying RARs as phosphoproteins which activate a number of kinase cascades with cell type selectivity. p38 MAPK has been shown to be activated by atRA-bound RARs which prompts phosphorylation cascade targets to translocate to the nucleus for subsequent phosphorylation cascade effects, which may include phosphorylation of histones, corepressors, coactivators and RARs themselves at serine residues on the NTD and LBD (**Figure 1.5**).^{14, 36}

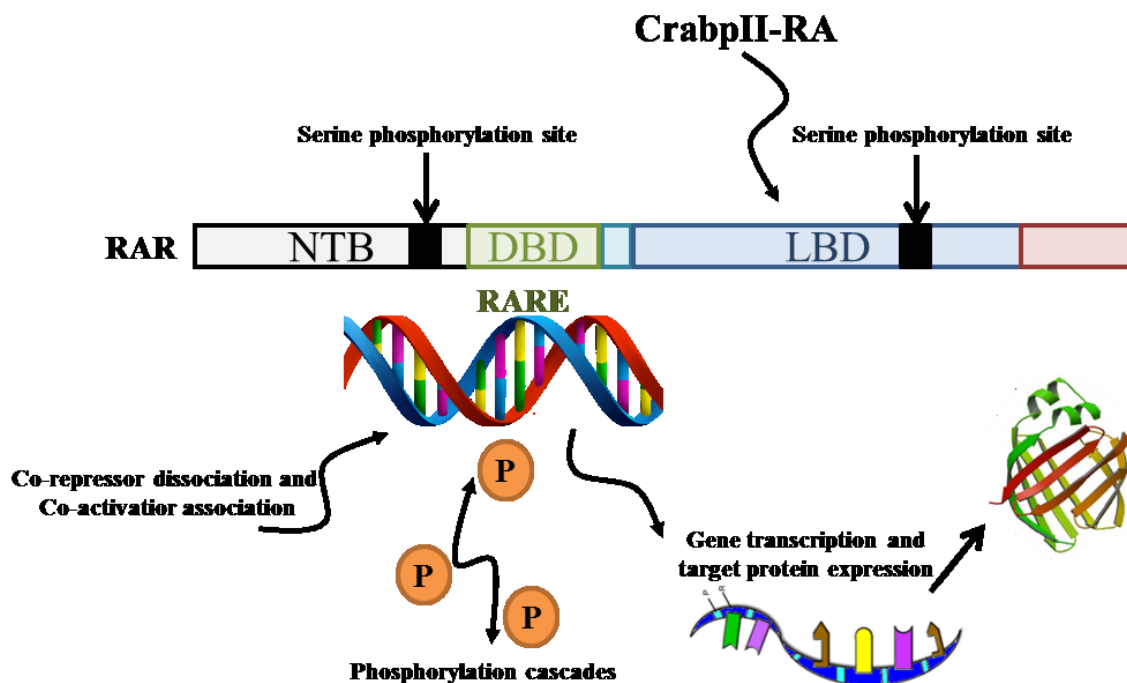


Figure 1.5. Retinoic acid signaling and RAR activation. Representative schematic of RAR binding domains and activated RAR activity. Upon atRA binding at the LBD, RARs undergo conformational changes and the DBD binds to the target gene's RARE on the DNA. Conformational changes upon atRA activation also enable corepressor complexes to dissociate and coactivator complexes to bind. Concomitantly, phosphorylation cascades are initiated for histone phosphorylation, RARE accessibility, phosphorylation of co-regulators and signaling for transcription to end.

Murine RAR knock outs have been made to identify phenotypes and study embryonic development. These include single, double and triple knock-outs of RAR and RXR receptors. A number of growth abnormalities have been observed and correlated with spatiotemporal expression changes. The mechanisms by which these defects occur, and subsequent changes in mature tissues and related diseases, is extremely complex.^{14, 37-38} Phosphorylation changes, epigenetics, receptor cross talk, primary target expression changes and secondary target cascades, co-activator/repressor changes and combinations are all possible mechanisms of action. Major pathway targets have been related to nuclear receptors, but more specific mechanisms are still being investigated.^{10, 13-14, 36, 39-43} RAR α and β target pathways to inhibit proliferation and induce apoptosis and differentiation. Some of these targets include induction of caspases, proapoptotic Bcl-2 proteins, and tumor suppressor p53.^{13, 44} Other targets which are inhibited include the Wnt pathway through inhibition of β -catenin to complex to LEF/TCF;⁴⁵ and the hedgehog pathway via downregulation of cyclin D through upregulation of Btg2.⁴⁵⁻⁴⁷ RAR γ and PPAR β/δ target pathways to promote proliferation and development. RAR β induces CrbpI and RAR γ has been shown to downregulate RAR β and CrbpI expression.⁴⁸ Examples of PPAR β/δ targets that are induced include upstream Akt1 kinases and VEGF and a targets that are inhibited includes tumor suppressor PTEN.¹³ **(Figure 1.6)** These pathway alterations are common themes in a number of diseases and have become a focus to understand the role of retinoids and retinoid receptors in maintaining tissue homeostasis.

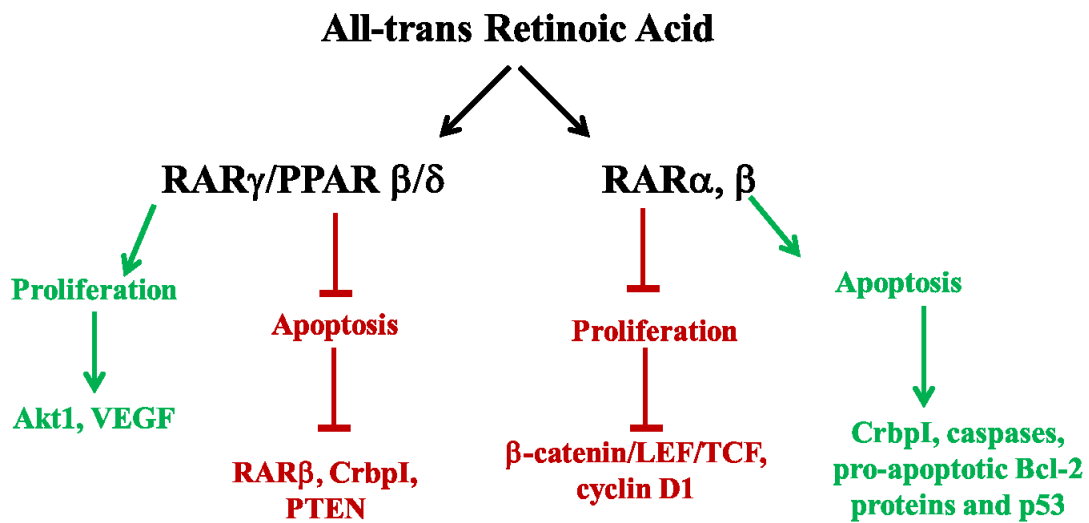


Figure 1.6. atRA modulates targets to maintain cellular homeostasis. RAR γ and PPAR β/δ regulate target cell programs to induce proliferation and development and inhibit apoptosis. RAR α, β target cell programs to inhibit proliferation and induce apoptosis and differentiation.

1.4 *In vitro* studies of retinoid activity

Retinoids have been studied extensively in cell culture models, including naturally occurring and synthetically derived retinoids for nuclear receptor target specific activation.⁴⁹⁻⁵² *In vitro* breast cancer studies screened for gene expression profiles in several breast cancer cell lines. Some of the genes identified in these screens includes atRA biosynthesis pathway proteins, atRA nuclear receptors and their direct targets.⁵³⁻⁵⁷ Additionally, treatment with ROL and atRA have shown induction of cell line differentiation; tumor line apoptosis and inhibition of proliferation; and induced non-adherent, or circulating cell lines to become adherent.⁵⁸ *In vitro* retinoid treatments have been shown to have additive effects on chemotherapy, enabling lower dosages of chemotherapies to be as efficacious as higher doses to inhibit cell proliferation and induce cell death.⁵⁹⁻⁶⁴ MCF-7 cells were used to elucidate possible anti-proliferative effects of atRA actions and showed that altered phosphorylation patterns of tyrosine kinases were consistent with atRA-mediated growth inhibition.⁶⁵ atRA reduced insulin like growth factor, type I (IGF-I) activation of insulin receptor substrate 1 (IRS-1) phosphorylation and therefore PI3-kinase (PI3K) and Akt phosphorylation was also reduced to selectively modulate growth inhibition.⁶⁵ In MTSV1-7 cells PI3K/Akt signaling was inhibited by CrbpI in a RAR α dependent manner to inhibit p85-p110 heterodimerization.⁶⁶ Similar results were reported in an ovarian cancer cell line studies, in which downregulation of STAT1, STAT5, and JUN were observed with restored CrbpI signaling.³² Non-genomic interactions between Stra6, holo-Rbp4 and the JAK/STAT phosphorylation cascade have been linked to induced oncogenic transformations in cell

lines overexpressing Stra6.⁹ These studies did not report CrbpI or atRA levels, so the impact of CrbpI on these interactions is of great interest for CrbpI function.

atRA has been previously shown to enhance gap junction intracellular communication (GJIC), pores that are formed by connexin (Cx) proteins, to connect cells for metabolite exchange and cell-cell communication, which is often inhibited in tumors and infertility.⁶⁷⁻⁶⁸ Cx43 phosphorylation inhibits GJIC and the mechanism by which atRA enhances GJIC has been shown to be through dephosphorylation of Cx43.⁶⁸ atRA showed dephosphorylation specificity for Cx43, the primary Cx in the endometrium, by activating PPA2, the primary phosphatase of Cx43 in human endometrial stromal cells. Increased PPA2 dephosphorylation of Cx43 subsequently enhanced GJIC.⁶⁸

Mechanisms of atRA action have been further investigated in human primary cells isolated from endometrial biopsies and endometriosis lesions collected during surgeries. Endometriosis is a proliferative disorder of chronic inflammation where endometrial cells translocate outside of the uterus, deposit and continue to proliferate, forming lesions. atRA production has been reported to originate primarily in the stromal cells of the endometrium and CrbpI expression is also localized to stromal cells of biopsies and lesions.⁶⁹⁻⁷² Other mechanisms of endometriosis modulated by atRA and RAR investigated involve proliferative and inflammatory genes and pathways. A RAR α knockdown significantly increased proliferative genes; while RAR α activated by agonist AM580 significantly inhibited proliferative genes of malignant endometrial cells including proliferating cell nuclear antigen (PCNA), TNF α , Smad3 and anti-apoptotic Bcl-2 genes. KEGG pathway analysis identified dozens of atRA targets modulated by AM580 that were categorized into main pathways including apoptosis, MAPK signaling,

focal adhesion and ECM communication, and Wnt signaling.⁷³ An endometrial cell line was correlated with primary endometrial stromal and epithelial cells to investigate the impact of atRA on immune activating interleukin 6 (IL-6). atRA was reported to suppress IL-6 expression and secretion in a time and dose-dependent manner.⁷⁴

atRA measurements by HPLC-UV were low to undetectable in breast cancer cell lines compared to normal or immortalized cell lines. When treated with atRA, proliferation was inhibited in a dose dependent manner. CrbpI was also not detectable in several of the breast cancer cell lines but was detectable in the normal breast cell lines.⁵⁰ Another malignant cell line of pleural mesothelioma also demonstrated dose dependent cell proliferation inhibition when treated with atRA. TGF- β 1, TGF- β 1 receptors and platelet derived growth factor beta (PDGF- β) were downregulated in response to atRA as well.⁷⁵ These studies show how important atRA and nuclear receptor availability are for cellular homeostasis.

Investigation into the mechanism of CrbpI and nuclear receptor loss of expression identified dysregulation in epigenetic expression. Hypermethylation of genes results in downregulation or silencing of protein expression. Several proteins have been identified to be hypermethylated in breast cancer cell lines including RAR β and CrbpI.^{33, 76-78}

Studies reported demethylation of DNA encoding for CrbpI restored CrbpI protein expression and inhibited tumor cell survival. Additional *in vitro* cancer cell studies evaluated the effects of histone deacetylase inhibition on epigenetic modification, where histone acetylation relaxes chromatin to enhance chromatin exposure for transcription and histone deacetylation condenses chromatin to limit transcription. Inhibition of histone deacetylase enzymes therefore promotes transcription activity.⁷⁹⁻⁸⁴ Histone deacetylase

inhibitors (HDACI) have been shown to selectively inhibit proliferation. Genetics screens with HDACIs identified the RA pathway as a target. Co-treatment of select HDACIs with atRA had synergistic effects on atRA signaling to inhibit tumor growth.⁸⁰ Lung cancer cell lines showed that co-treatment with select HDACIs and atRA also had additive effects on proliferation inhibition and induced cell death.⁸¹ Studies with breast cancer cell lines reported that when histone deacetylation is left unchecked, RAR α is suppressed, and upon inhibition of histone deacetylase, RAR α expression is restored.⁸³ These epigenetic modifications to enhance transcription activity are reprogramming cells to be responsive to atRA signaling to direct cell programs. Furthermore, RAR γ has been reported to suppress CrbpI and RAR β , so epigenetic mechanisms to enhance RAR α activity and suppress RAR γ activity are viable strategies to inhibit proliferation and enhance apoptosis and differentiation of tumor cells.^{48, 85}

1.5 *In vivo* studies of retinoid activity

Several murine models have been developed to study retinoid signaling and its role in growth and development, as well as progression and treatment of cancer.^{6, 11, 14, 31, 37-38, 86}

Patient endometrium has also been evaluated for CrbpI expression and clinical relevance in normal tissue, stages of menses and disease progression.^{69, 87} Spatiotemporal studies have been important to identify when and where retinoid signaling occurs to understand how alterations in retinoid signaling effects tissue homeostasis.^{6, 11, 69, 86-87}

A number of retinoid protein knock-out mouse strains have been developed for these studies, including a *Dhrs3*^{-/-} model to investigate embryonic development, which reported embryonic growth defects with elevated atRA levels and major expression

changes of atRA targets resulting in embryonic lethality.⁸ An *Rdh10*^{-/-} mouse study also reported embryonic lethality with reduced RARE-lacZ signal.⁸⁶ RAR and RXR knock-outs showed specific expression profiles during embryogenesis and development which when knocked out resulted in a number of growth defects, tissue abnormalities and VAD syndromes.^{14, 37-38} *CrbpI* knock-out mice do not show growth abnormalities unless challenged with vitamin A deficient diets, which also results in depleted vitamin A stores, dysregulated retinoid homeostasis and vitamin A deficiency syndromes.³¹ Retinol was shown to be elevated in the *CrbpI* knock-out pancreas. This study also reported changes to glucose homeostasis and fatty acid oxidation, to indicate one important role *CrbpI* has in retinoid and tissue homeostasis.⁸⁸

A transgenic mouse model was used to induce endometriosis lesions. These studies showed that atRA was reduced in these lesions and had increased IL-6, MCP-1 and vascular formation. atRA treatment reduced the number and size of the lesions formed, as well as inflammatory markers. There was also an increase in macrophage differentiation.^{71, 89} Complementary studies of human endometriosis lesions also showed reduced atRA and *CrbpI* expression which were confirmed to be reduced in endometrial stromal cells (ESC) isolated from the patients' lesions.⁷¹ One group performed extensive *CrbpI* expression studies in human populations to identify spatiotemporal expression in endometrium throughout menses and in endometrium related disease to determine if *CrbpI* could be utilized as a biomarker for ESC.^{69, 87} *CrbpI* loss in stromal cells increased with tumor grade and in less differentiated areas. *CrbpI* was determined to corresponded with more specificity for eutopic and ectopic ESC than the currently used biomarker, CD10.^{69, 71, 87}

An additional focus of *in vivo* studies has been on the RAR nuclear receptors, RAR α , RAR β , and RAR γ .^{13, 37-38, 89-92} Xenograft mouse models have shown that synthetic retinoid agonists selective for RAR α and RAR β 2 significantly reduce tumor formation, size and metastasis.^{51, 85, 92-93} Inducing CrabpII, to direct atRA to RAR α and RAR β 2, also reduces tumor formation, size and metastasis.⁹⁴ Another xenograft mouse model treated with atRA also showed a decrease in tumor volumes, modulated induced cell migration, and reduced expression of TGF- β 1 and PGFR- β .⁷⁵ CrbpI and RAR α are induced by RAR α agonist AM580.⁹² Human breast tumors analyzed for Crbp and RAR expression showed approximately 25% loss of CrbpI and 37% loss of RAR β 2 due to epigenetic silencing by DNA hypermethylation. Additionally, gene expression profile screens of human breast tumors identified atRA nuclear receptor targets.⁹⁵⁻⁹⁶ *In vivo* studies have been successful in demonstrating that modulating the retinoid pathway impacts clinically desirable outcomes including reduced tumor volumes, slowed tumor progression, inhibition of proliferation and induction of differentiation and apoptosis.

1.6 Retinoic acid uses in the clinic

FDA approved retinoic acid isomers, as cited by the FDA drug database, are indicated for topical and oral treatments of skin conditions including severe acne, melisma and solar lentigo. atRA (tretinoin) and 13-cis RA (isotretinoin), which are active ingredients in several treatment brands, are prescribed alone or in combination with antibiotics as skin conditions require. Kaposi's sarcoma is a more severe skin condition, which is treated with topical 9-cis RA (alitretinoin). atRA under the brand name Vesanoid, is an oral therapy used to treat acute promyelocytic leukemia (APL) to induce RAR α activity for

differentiation and apoptosis. Isotretinoin is used to treat neuroblastoma in the maintenance phase of treatment, after chemotherapy, to also induce RAR α activity for cell differentiation. Isotretinoin treatment has been successful to prolong patient survival. Bexarotene/targretin is a FDA approved synthetic RXR ligand, which induces cell differentiation and apoptosis, is indicated for skin conditions and cutaneous t-cell lymphoma.⁹⁷⁻⁹⁸ Because of the successful uses described, several phase I and II trials have been implemented to investigate additional chemo-preventative and chemotherapy co-treatment applications of current FDA approved retinoic acid therapies for solid tumors.^{44, 98-101} Though these trials have not yet produced new FDA approvals, the effort for new retinoic acid therapeutic applications is still ongoing.

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Chapter 2: CrbpI regulates mammary retinoic acid homeostasis and the mammary microenvironment¹

2.1 Introduction

Breast cancer is the second leading cause of cancer death in women in the United States.¹ The development of breast cancer involves progression through several stages starting with abnormal proliferation, atypical hyperplasia, and then carcinoma *in situ* before becoming invasive.²⁻³ All-*trans*-retinoic acid (atRA), an active metabolite of vitamin A, regulates key physiological processes that become defective during breast cancer disease progression, including, cell proliferation, differentiation, migration, and apoptosis.⁴ Cellular retinol-binding protein, type 1 (*Rbp1*), delivers vitamin A to enzymes for metabolism to produce atRA while protecting retinol and retinal from undesired reactions.⁴ Epigenetic disruption of *CrbpI* that results in silencing of *CrbpI* expression is a common alteration in more than 10 different types of human cancer, with the prevalence of this alteration ranging from 7% in head and neck cancer to 60% in lymphoma.⁵⁻⁸ *CrbpI* down-regulation in ~25% of breast cancers represents an epithelial cell loss of function that resembles atRA deficiency.⁴⁻⁶ Both *CrbpI* and atRA have been shown to be anti-tumorigenic in breast epithelial cells, and the tumor-suppressive effect of *CrbpI* has been attributed to its ability to regulate atRA signaling.⁵ atRA homeostasis is essential to proper regulation of atRA signaling, and while the importance of atRA in maintaining epithelial tissue has long been recognized,⁹ how endogenous atRA homeostasis is altered and the functional effects of altered atRA homeostasis in mammary tissue as a consequence of *CrbpI* loss are unclear.

¹ Adapted from the publication: Pierzchalski, K., Yu, J., Norman, V. and Kane, M.A. (2013) *CrbpI* regulates mammary retinoic acid homeostasis and the mammary microenvironment. *FASEB J.* 27, 1904-1916

atRA homeostasis is maintained through dietary intake of vitamin A (retinol), and its storage, mobilization, transport, and biosynthesis into atRA.⁴ atRA production consists of a reversible and rate-limiting dehydrogenation of retinol to retinal catalyzed by membrane-bound, short-chain retinol dehydrogenase/reductases (Rdhs). Subsequently, retinal undergoes an irreversible dehydrogenation to form atRA by cytosolic retinal dehydrogenases (Raldhs). Storage of retinol is catalyzed by lecithin:retinol acyltransferase (Lrat) into retinyl esters (RE), and retinol can be mobilized by stimulating RE hydrolases (Rehs) to hydrolyze RE. atRA is catabolized mainly by a number of cytochrome (Cyp) P450 enzymes found in the microsomal membrane.⁴ atRA affects physiological processes through several nuclear receptors (RARs) and peroxisome proliferator-activated receptors, type β/δ (PPAR β/δ). Nongenomic actions of atRA have also been reported.¹²⁻¹³

Because atRA is a potent signaling molecule, atRA biosynthesis and homeostasis are tightly regulated by a number of mechanisms. CrbpI participates in atRA regulation both when ligand bound and ligand free. Ligand-bound CrbpI (holo-CrbpI) chaperones vitamin A to atRA-biosynthesizing enzymes while sequestering retinol (and retinal) from nonspecific oxidation.^{4, 14-15} The CrbpI chaperone model has been discussed in detail and postulates that CrbpI ensures efficient retinol metabolism, but also posits that CrbpI is not obligatory for retinol metabolism.^{4, 46} As such, oxidation of retinol (and retinal) can proceed in the presence or absence of chaperone; however, reactions that are normally restricted by CrbpI may occur in the absence of CrbpI chaperone.⁴ Unbound CrbpI (apo-CrbpI) has additional regulatory influence on atRA homeostasis, as it is able to inhibit and stimulate specific enzymes in order to control flux through the vitamin A

pathway.^{4,14-17} Retinol oxidation by Rdh enzymes is the rate-limiting step in atRA biosynthesis.^{4, 14} According to current reports, CrbpI loss affects atRA homeostasis variably in *Rbp1*^{-/-} mice depending on tissue context, with some tissues having altered atRA levels and some tissues possessing compensation mechanisms to maintain atRA levels.¹⁸⁻²⁰ The up-regulation of homologous Crbp proteins (CrbpII and/or CrbpIII) contributes to maintaining atRA in the absence of CrbpI.²⁰⁻²¹ However, because CrbpI and its homologues (CrbpII, CrbpIII) normally have distinct functions, compensatory up-regulation does not usually result in full replacement of CrbpI functionality.²⁰⁻²⁴ For example, in regard to atRA homeostasis, when CrbpII is up-regulated in the absence of CrbpI, rates of atRA biosynthesis are altered.²⁰ CrbpIII has been shown to be up-regulated in the absence of CrbpI in mammary tissue;²¹ however, the effect on atRA homeostasis has not been investigated. As such, the effect of CrbpI loss and the respective roles of CrbpI and CrbpIII in terms of atRA homeostasis require further examination for several reasons: CrbpI regulates atRA biosynthesis and atRA homeostasis in mammary tissue;^{4, 14, 19-20} atRA is critical to normal mammary morphology;⁹ and in *Rbp1*^{-/-} mammary tissue, CrbpIII is up-regulated in the absence of CrbpI and represents the only Crbp that is expressed.²¹ In addition, because CrbpI loss is an early event in breast cancer progression and is associated with poor prognosis,⁶ understanding the molecular mechanisms of the CrbpI phenotype will help characterize a significant breast tumor subset and aid in the development and/or targeting of therapy based on genetic signature. Here, we determine the effect of CrbpI loss on mammary atRA homeostasis and mammary tissue morphology.

2.2 Experimental Procedures

2.2.1 Mice

Female, virgin, C57BL/6 wild-type (WT) or *Rbp1*^{-/-} mice aged 1.5-4 mo were used according to institutional guidelines of the University of Maryland, Baltimore. WT mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), and *Rbp1*^{-/-} mice were bred in-house from breeders obtained from Pierre Chambon and Norbert Ghyselinck (Institute de Genetique et de Biologie Moleculaire et Cellulaire, Institut National de la Sante et de la Recherche Medicale, Illkirch, France). Mice were fed a chow diet ad libitum (Harlan Teklad Global; 18% protein extruded rodent diet no. 2018SX with the equivalent of 30 IU/g vitamin A; Harlan Laboratories, Indianapolis, IN, USA). Mammary tissue was collected from the left and right abdominal and thoracic glands.

2.2.2 Retinoids and retinoid extraction

Retinoids were purchased from Sigma-Aldrich (St. Louis, MO, USA) and handled under yellow light. Tissue samples were harvested from mice under yellow light, frozen immediately in liquid nitrogen, and kept at -80°C until extraction. Retinoids were extracted from homogenized tissue, subcellular fractions, or lysed cells by a 2-step liquid-liquid extraction, as described previously.^{18, 25-27} Retinoids were quantified in extracted samples within ~1 d by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for RA isomers or by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) for neutral retinoids (retinol, REs).^{18, 25-27} RA isomers had the following LCMS/MS retention times: atRA, 7.1 min; 9-cis-RA (9cRA), 6.8 min; 9,13-di-cis-RA (9,13dcRA), 6.6 min; and 13-cis-RA (13cRA), 6.3 min (Figure 1.1 D). Neutral retinoids

had the following HPLC-UV retention times: retinol, 4.1 min; retinyl acetate (internal standard), 7.2 min; retinyl palmitate (RE), 15.9 min (Figure 1.1 E). Serum retinoids were normalized per milliliter, and liver retinoids were normalized per gram of tissue. Mammary retinoids were normalized per gram of protein to adjust for the hypercellularity of the *Rbp1*^{-/-} mammary tissue and because of the fibrous nature of the tissue and the difficulty in achieving a complete homogenization. Protein content in the mammary homogenate was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Mammary retinoid content was also determined per gram of tissue (data not shown), and on the basis of the data, the approximate conversion factor for retinoid per gram protein to retinoid per gram of tissue in mouse mammary tissue was 233 ± 40 (mean \pm sd):1 (protein:tissue).

2.2.3 *CrbpI and CrbpIII preparation*

Mouse CrbpI and CrbpIII were expressed in BL 21 *E. coli* purchased from Sigma-Aldrich with plasmids purchased from Genecopia (Rockville, MD, USA), according to manufacturer's instructions. Purification was performed using a GE Healthcare GST bulk kit (GE Healthcare, Pittsburgh, PA, USA). The GST tag was cleaved with Promega ProTEV protease (Promega, Madison, WI, USA), after which the protease was removed with GE Healthcare Ni resin. The GST tag was separated from the purified protein by running the protein solution through the GST column a second time. Purified protein was dialyzed and stored in 20 mM KH₂PO₄ and 100 mM KCl at -80°C. CrbpI and CrbpIII concentrations were determined from absorbance at 280 nm using published ϵ values: apo-CrbpI, $\epsilon = 28,080 \text{ M}^{-1} \text{ cm}^{-1}$; apo-CrbpIII; $\epsilon = 25,800 \text{ M}^{-1} \text{ cm}^{-1}$.²⁸⁻²⁹ Holo-CrbpI/holo-CrbpIII have a molar absorptivity of $\epsilon = 50,200 \text{ M}^{-1} \text{ cm}^{-1}$ at 350 nm. The A₃₄₀/A₂₈₀ ratios,

used to assess purity, were between 1.4 and 1.6. Stock and binding assay solutions of CrbpI and CrbpIII were prepared in 20 mM KH₂PO₄ and 100 mM KCl (pH 7.4). K^{'d} value determinations were performed with 150 nM CrbpI or CrbpIII. CrbpI and CrbpIII have amino acid sequences that are highly conserved between human and murine species (>95% for CrbpI and 89% for CrbpIII) and share 57% amino acid sequence identity.^{22, 30-31} CrbpI and CrbpIII tryptophan residues (responsible for the intrinsic protein fluorescence) are located at positions 8, 88, 106 and 109.

2.2.4 Fluorescence measurements

Data were generated on an ISS PC1 fluorometer with a 300-W Xe lamp (ISS, Champaign, IL, USA). Measurements were made at 25°C. CrbpI and CrbpIII were excited at 280nm, and emission was monitored at 340 nm with spectral bandpass of 2 nM. Retinoid solutions were added with a glass Hamilton syringe (Sigma-Aldrich), gently mixed, and equilibrated for 5 min, before measuring the solution fluorescence. Quenching of intrinsic tryptophan fluorescence was measured as a function of added retinoid for retinol, retinal, and RA. All retinoids were of the all-trans configuration. For titrations with retinol, increasing retinol fluorescence was also monitored at 490 nm with excitation at 350 nm, as a function of added retinol. Retinal and RA do not fluoresce; therefore, only quenching of Crbp fluorescence was monitored when evaluating these retinoids. The excitation shutter was closed between measurements, and retinoid solutions (kept shielded from light) were added in the dark. Inner filter effects were negligible at the protein and retinoid concentrations used and were not corrected. Excitation and emission spectra were corrected using the appropriate blank. Blank

contributions were < 2% of total intensity. Data were analyzed by nonlinear least squares fit, according to **Equation 1**:

$$\text{Equation (1): } \frac{F}{F_f} = 1 + \left(\frac{F_b}{F_f} - 1 \right) \times \left(\frac{P_t + R_t + K'_d - \sqrt{(P_t + R_t + K'_d)^2 - 4P_t R_t}}{2P_t} \right)$$

In **Equation 1** F is the observed fluorescence, F_f is the fluorescence of the free protein, F_b is the fluorescence of the bound protein, P_t is the total protein concentration, R_t is the total retinoid concentration, and K'_d is the apparent dissociation constant. Data were fit to **Equation 1** using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Before adding retinoids, apo-CrbpI and apo-CrbpIII exhibited not detectable emission at wavelengths > 400 nm, indicating the absence of retinol. Binding experiments were performed at least 3 times.

2.2.5 Subcellular fractionation

Tissue was collected and placed in 10% sucrose, 10 mM Tris HCl, 1 mM EDTA, and 1.5 mM DTT (pH 7.4). DTT was added on the day of use. Tissue was homogenized in the same buffer on ice with a motorized homogenizer at ~1260 rpm for 6-8 strokes (Eberbach ConTorque no. 7265; Eberbach Corp., Ann Arbor, MI, USA). Microsomal and cytosolic fractions were isolated by centrifugation at 4°C: 100g for 10 minutes, 10,000 g for 15 minutes, 17,000 g for 15 minutes, and 100,000 g for 1-2 hours. The 100,000 g supernatant (cytosol) was diluted with homogenization buffer to 1.0 µg protein/µl. The 100,000 g pellet (microsomes) was resuspended in homogenization buffer to 4.0 µg protein/µl solution. Protein content was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Fractions were frozen in a methanol dry-ice and stored at -80°C.

2.2.6 Enzyme activity

Enzyme activity for Rdh oxidation of Crbp-bound retinol was assayed with a combination of mammary microsomes (300 μg protein) and cytosol (10 μg protein).^{14, 20,}
³⁴ Lrat activity to convert Crbp-bound retinol to RE was measured concurrently during the Rdh oxidation assay. Raldh activity to oxidize Crbp-bound retinal was assayed with mammary cytosol (10 μg protein). Rdh activity to reduce Crbp-bound retinal to retinol was assayed with mammary microsomes (300 μg protein). All enzyme activity was assayed at 37°C under initial velocity conditions in the linear ranges of time and protein in 50 mM HEPES, 150 mM KCl, 1 mM EDTA, and 2 mM DTT (pH 8.0) in the presence of 4 mM NAD^+ and 2 mM NADP^+ with a total reaction volume of 250 μl and 65 rpm shaking. DTT was added the day of use. Rdh/Lrat and Rrd activity assays also included a NADPH regenerating system added in 30 μl as a 1:1:1 solution of glucose-6-phosphate dehydrogenase (2.5 U in 5 mM sodium citrate, pH 7.5), 50 mM glucose-6-phosphate, and 50 mM NADP^+ in assay buffer. The activity of the glucose-6-phosphate dehydrogenase was confirmed spectrophotometrically before assay by monitoring production of NADPH. In all assays, microsomal and/or cytosolic protein was reacted with 1.0 μM CrbpI-bound retinoid (retinol or retinal) or CrbpIII-bound retinoid (retinol or retinal) for 60 minutes. Reactions were initiated by adding 10 μl Crbp-bound retinoid in buffer via glass syringe. Control reactions were performed with Crbp-bound retinol in the absence of subcellular fractions (microsomes/cytosol that contain enzymes). Each reaction condition was performed in triplicate, and each experiment was performed twice.

2.2.7 Gene expression

Total RNA was isolated with a Purelink miniRNA plus kit (Invitrogen; Life Technologies, Grand Island, NY, USA). RNA was quantified by a NanoDrop 2000 spectrophotometer (Thermo, Wilmington, DE, USA). RNA was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems; Life Technologies). Quantitative polymerase chain reaction (qPCR) was done with predesigned and optimized primers (Applied Biosystems). mRNA gene expression was measured with a Step-One Plus real-time PCR (Applied Biosystems). mRNA gene expression was calculated by the comparative C_t method with beta-actin as the reference gene.

2.2.8 NAD^+ /NADH Determination

Mammary tissue from female, 2-mo-old C57BL/6 or *Rbp1*^{-/-} mice were used for analysis. Tissue was processed and the NAD^+ /NADH ratio was determined using an EnzyChrom NAD^+ /NADH assay kit (BioAssay Systems, Haywood, CA, USA) according to manufacturer's instructions. Changes in absorbance were measured at 565 nm using a Bio-Tek Eon plate reader (Bio-Tek, Winooski, VT, USA). NAD^+ and NADH concentrations were quantified using an NAD^+ standard curve, per kit recommendations, normalized per milligram of tissue, and the NAD^+ /NADH ratio was calculated from those values.

2.3 Results

2.3.1 Endogenous atRA is depleted in mammary tissue of *Rbp1*^{-/-} mice

We quantified multiple retinoids in mammary tissue to determine the extent of disrupted retinoid homeostasis in *Rbp1*^{-/-} mice using LC-MS/MS and HPLC-UV (**Figure 2.1 D and E**).^{18-20, 25-27} Relative to WT, *Rbp1*^{-/-} mice had 40% depleted atRA in mammary tissue (**Figure 2.1 A**). 9,13dcRA, an RA isomer without known biological activity, was also detected in mammary tissue but was unchanged between WT and *Rbp1*^{-/-} mice (**Figure 2.1 D and data not shown**). No 9cRA or 13cRA was detected in mammary tissue above the LC-MS/MS assay limit of detection in biological matrices (**Figure 2.1 D**).^{25, 27} In contrast, mammary retinol and RE were elevated 89 and 69%, respectively (**Figure 2.1 A**). Liver had similar atRA levels and 56% reduced retinol and 80% reduced RE (**Figure 2.1 B**). Circulating retinoids in serum were similar, with the exception of retinol, which was elevated 20% (**Figure 2.1 C**).

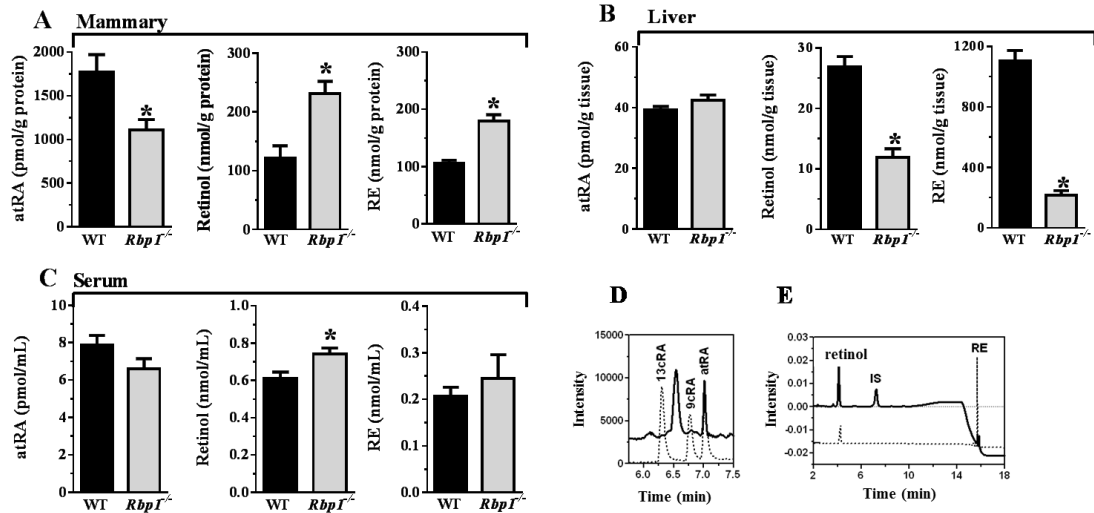


Figure 2.1. Endogenous atRA is depleted in the mammary of *Rbp1*^{-/-} mice. A, depleted atRA, elevated retinol, and elevated RE in mammary. (*) $p < 0.0051$ vs. WT. **B**, similar atRA, depleted retinol, and depleted RE in liver. (*) $p < 0.0001$ vs. WT. **C**, similar atRA, elevated retinol, and similar RE in serum. (*) $p = 0.007$ vs. WT. **A-C**, atRA was quantified by LC-MS/MS and retinol and RE were quantified by HPLC-UV. Values are means of three experiments with 10 mice/group/experiment. All data are \pm SEM. **D**, representative LC-MS/MS chromatogram of atRA in mouse mammary. **E**, representative HPLC-UV chromatogram of retinol and RE produced by mouse mammary microsomes treated with CrbpIII-retinal. **D and E**, dotted overlays are chromatograms of authentic retinoid standards.

2.3.2 Expression of atRA biosynthesis enzymes in mammary tissue

Because atRA is depleted in *Rbp1*^{-/-} mouse mammary tissue, we assayed the expression of atRA biosynthesis enzymes by qPCR. Rdhs catalyze the first and rate limiting dehydrogenation step in atRA biosynthesis. Rdh1, Rdh8, Rdh10, Rdh12, Dhhs3, and Dhhs9 have all been confirmed to participate in vitamin A homeostasis through genetic models.³⁵⁻⁴⁰ Mammary tissue expressed *Rdh1*, *Rdh10*, *Rdh12*, *Dhhs3*, and *Dhhs9*; *Rdh8* was not expressed (**Figure 2.2**). mRNA expression of *Rdh1*, *Rdh10*, *Rdh12*, *Dhhs3* and *Dhhs9* was similar in WT and *Rbp1*^{-/-} (**Figure 2.2**). Raldh catalyze the second dehydrogenation step of atRA biosynthesis. Raldh1, Raldh2, and Raldh3 have all been confirmed by genetic models to participate in retinoid metabolism.⁴¹⁻⁴⁴ Mammary expressed *Raldh1*, *Raldh2*, and *Raldh3* mRNA expression for *Raldh1* was reduced 45%, *Raldh2* was reduced 30%, and *Raldh3* was similar between WT and *Rbp1*^{-/-} (**Figure 2.2**). We also assayed expression of *Stra6*, the membrane receptor for Rbp4 that facilitates uptake of cellular retinol. mRNA expression of *Stra6* was 4-fold higher in *Rbp1*^{-/-} mammary tissue (**Figure 2.2**).

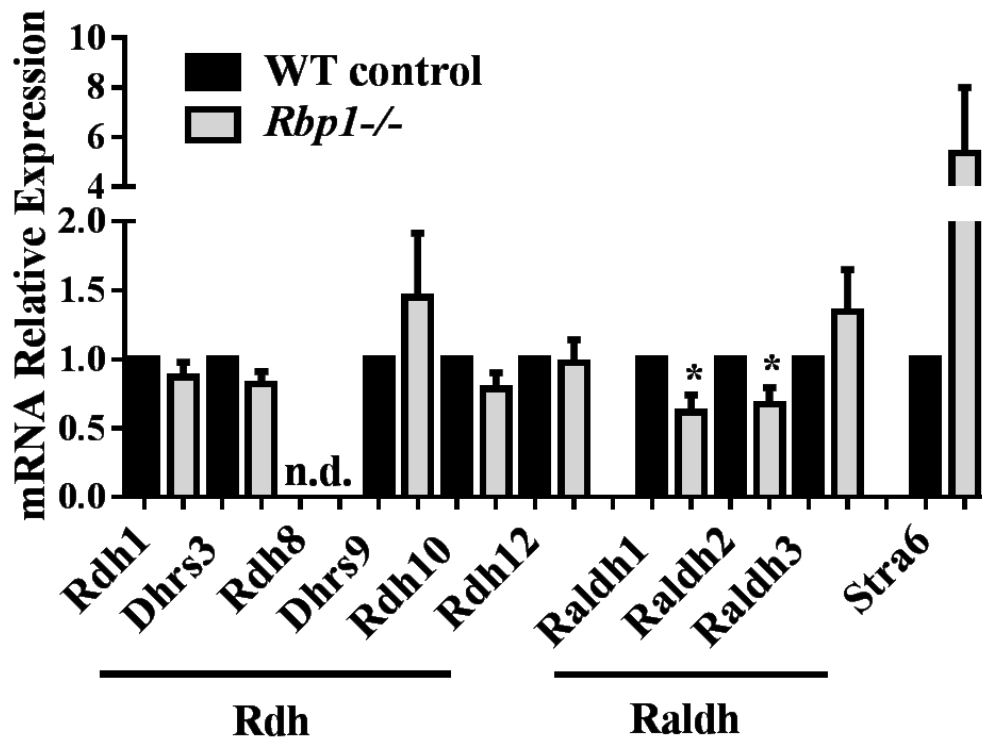


Figure 2.2. Expression of atRA biosynthesis enzymes in mammary. Rdh (*Rdh1*, *Rdh8*, *Rdh10*, *Rdh12*, *dhrs3*, *dhrs9*) mRNA expression, Raldh (*Raldh1*, *Raldh2*, *Raldh3*) mRNA expression and *Stra6* mRNA expression (membrane receptor for Rbp4 that facilitates uptake of vitamin A). mRNA expression determined in WT and *Rbp1*^{-/-} mammary by q-PCR analysis normalized to β -actin mRNA. Data represented as mean relative expression \pm SEM, n=10-12. (*) p=0.0208.

2.3.3 *CrbpIII is not functionally interchangeable with CrbpI*

Because, in the absence of CrbpI, compensatory upregulation of other Crbp proteins (CrbpII, CrbpIII) has been observed,²⁰⁻²² we assayed for expression of homologous Crbp proteins. CrbpIII and CrbpI are similarly expressed in WT mammary tissue. In the absence of *Rbp1* (which encodes CrbpI), *Rbp7* (which encodes CrbpIII) is slightly, but not significantly, elevated compared to WT *Rbp7* (**Figure 2.3 A**). CrbpII is not expressed in mammary tissue.⁴⁵ Because Crbp retinoid-binding affinity has been reported to influence Crbp function,^{20, 22, 30} we determined the binding affinity of CrbpI and CrbpIII for retinoid ligands. To determine whether CrbpI and CrbpIII have differences in binding affinity for retinoids, we used a fluorescence method modified from Cogan et al.³² that we used previously to compare CrbpI and CrbpII.³³ In this assay, ligand binding quenches the intrinsic fluorescence emission of the apo-protein by energy transfer from the tryptophan residues (**Figure 2.3 B and F**). Excitation and emission maxima for CrbpI and CrbpIII were identical at 280 and 340 nm, respectively (**Figure 2.3 G and H**). Data for binding of retinol, retinal, and atRA to CrbpI and CrbpIII were determined by calculating the apparent dissociation constant K_d using nonlinear least squares fit to **Equation 1**. The K_d for retinol was 15 nM for binding to CrbpI and 113 nM for binding to CrbpIII, indicating that CrbpI has ~7 fold stronger affinity for retinol than CrbpIII (**Figure 2.3 C**). K_d values were similar for retinal binding to CrbpIII, with values of 52 nM and 53 nM, respectively (**Figure 2.3 D**). atRA did not exhibit significant binding to CrbpI or CrbpIII, as expected from previous binding and structural analysis (**Figure 2.3 E**).³⁰⁻³³ Both CrbpI and CrbpIII show 1:1 stoichiometry for retinol and retinal binding (**Figure 2.3 C, D**).

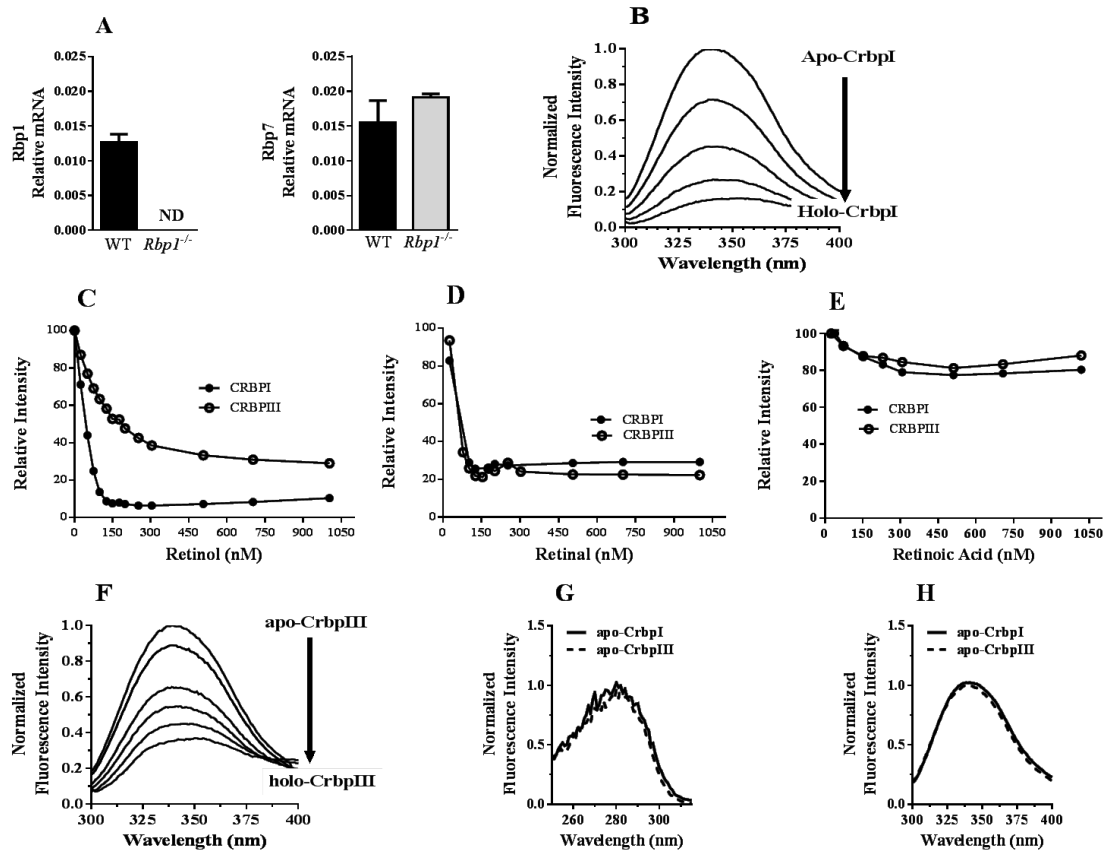


Figure 2.3. CrbpIII is not functionally interchangeable with CrbpI. **A**, mRNA expression of *Rbp7*, which encodes CrbpIII, is maintained in *Rbp1*^{-/-} mice. Data is represented as mean relative expression \pm SEM, n=14-16. **B**, representative normalized fluorescence emission from CrbpI protein demonstrating fluorescence quenching of intrinsic CrbpI tryptophan emission upon binding to increasing amounts of ligand. **F**, Similar data was obtained for CrbpIII. **C-E**, Fluorescence quenching of intrinsic CrbpI/CrbpIII tryptophan emission upon binding of retinoid ligand. **C**, CrbpIII binds to retinol with less affinity than CrbpI. **D**, CrbpI and CrbpIII bind to retinal with similar affinity. **E**, Neither CrbpI nor CrbpIII bind to atRA. **G**, Normalized excitation spectra of apo-CrbpI and apo-CrbpIII. **H**, Normalized emission spectra of apo-CrbpI and apo-CrbpIII.

2.3.4 *Crbp* delivery and *Rbp1* loss affect atRA biosynthesis

Because both the K_d values for retinol binding to CrbpI and CrbpIII (nanomolar) are well below retinol concentrations in vivo (micromolar), retinol would exist mainly bound to CrbpI and/or CrbpIII (**Figures 2.1 and 2.3**). In addition, Crbp has been reported to be in excess of retinol by up to ~40%.⁴⁶⁻⁵⁰ Thus, the differences in CrbpI and CrbpIII binding affinity for retinol might foster different outcomes for retinol metabolism. To investigate whether the nature of the Crbp affects the amount of atRA produced from retinol, we quantified the atRA biosynthesis capacity of subcellular fractions containing atRA biosynthesizing enzymes as a function the Crbp delivering the retinoid (**Figure 2.4**). We used these data to make 3 comparisons: CrbpI vs. CrbpIII delivery of retinoid to enzymes of a given genotype; total WT vs. *Rbp1*^{-/-} enzyme activity for a given substrate; and a mimic of the *in vivo* condition through comparison of WT mammary tissue that utilizes CrbpI vs. *Rbp1*^{-/-} mammary tissue that utilizes CrbpIII (when CrbpI is missing).

Rdh enzymes are localized to the ER (present in the microsomal subcellular fraction) and catalyze the first and rate-limiting dehydrogenation step in atRA biosynthesis. Raldh enzymes are cytosolic and catalyze the second dehydrogenation step of atRA biosynthesis.⁴ We used a combination of microsomes and cytosol to measure Rdh enzyme activity based on previous atRA biosynthesis assays.^{14, 20, 34} Using a combination of microsomes and cytosol in our atRA biosynthesis assay also allowed us to use LC-MS/MS detection of RA, which is a much more sensitive detection scheme (by ~1000-fold) than HPLC-UV detection of retinal and allowed us to use physiological levels of Crbp-retinol during assay. Because Rdh activity is 200 to 400-fold lower than Raldh activity, the Rdh assay reflects the rate-limiting activity of the Rdh enzymes (**Figure 2.4**).

To then isolate the effect of Crbp on the conversion of retinal to atRA by Raldh enzymes, we quantified the amount of atRA produced from CrbpI-retinal or CrbpIII-retinal in WT and *Rbp1*^{-/-} mouse mammary cytosol (**Figure 2.4 B**).

In the Rdh enzyme activity assay, to examine CrbpI vs. CrbpIII delivery, we provided a combination of isolated microsomes and cytosol with either CrbpI-retinol or CrbpIII-retinol. When WT and *Rbp1*^{-/-} subcellular fraction enzyme activity is compared, CrbpIII-retinol generated atRA at a faster rate than CrbpI-retinol in both WT (17%) and *Rbp1*^{-/-} (54%) subcellular fractions (**Figure 2.4 A**). However, metabolism of retinol was less in *Rbp1*^{-/-} mice regardless of the nature of Crbp. CrbpI and CrbpIII-bound retinol produced 42 and 24% less atRA, respectively, in the *Rbp1*^{-/-} subcellular fractions (**Figure 2.4 A**).

To mimic the *in vivo* condition, we compared atRA production from CrbpI-retinol by WT subcellular fractions with atRA production from CrbpIII-retinol by *Rbp1*^{-/-} subcellular fractions. We found that atRA production from CrbpI-retinol in WT subcellular fractions was 13% greater than atRA production from CrbpIII-retinol in *Rbp1*^{-/-} subcellular fractions. In the Raldh enzyme activity assay, the rate of metabolism of retinal did not depend on the specific Crbp and was similar in WT and *Rbp1*^{-/-} cytosol (**Figure 2.4 B**).

In all enzyme assays shown in figure 1.4, there were multiple cofactors present, but atRA biosynthesis enzymes generally have distinct cofactor preferences for reaction with Crbp-bound substrate: Rdhs (oxidative) prefer NADP⁺,⁵¹⁻⁵³ Raldhs (oxidative) prefer NAD⁺,^{52, 54} and Rdhs (reductase) prefer NADPH.⁵⁵ Identical experiments performed with single cofactors, including Raldh activity to convert Crbp-retinal to atRA in cytosol only in the presence of NAD⁺ and Rdh (reductase) activity to reduce Crbp-retinal to retinol only in the presence of NADPH, returned similar results to figures 1.4 B and D. Nonspecific

oxidation in the absence of subcellular fractions was <10%, and an all-trans substrate resulted in an all-trans product, indicating that there was no isomerization during assay (either enzymatic or artifactual).

We concurrently measured the RE produced during the Rdh oxidation assay that uses microsomes and cytosol. Previous work has shown that there is sufficient endogenous fatty acid in the microsomal fraction to support retinol esterification and that cytosol can provide an additional source of fatty acid.⁵⁶⁻⁵⁸ From these data, we observed that the nature of the Crbp did not influence esterification of retinol by Lrat; however, *Rbp1*^{-/-} subcellular fractions produced RE at a 28% greater rate than WT (**Figure 2.4 C**). To study the ability of Rdh enzymes to reduce CrbpI-retinal and CrbpIII-retinal to retinol, we determined retinal reductase activity in the microsomal fraction (**Figure 2.4 D**). CrbpIII-retinal resulted in 30 and 24% less retinol production as compared to CrbpI-retinal for both WT and *Rbp1*^{-/-}, respectively. *Rbp1*^{-/-} microsomes yielded 16–17% increase in retinol production as compared to WT for both CrbpI-retinal and CrbpIII-retinal, respectively. Retinol produced from CrbpI-retinal by WT microsomes was 18% greater as compared to retinol produced from CrbpIII-retinal by *Rbp1*^{-/-} microsomes to mimic the *in vivo* condition.

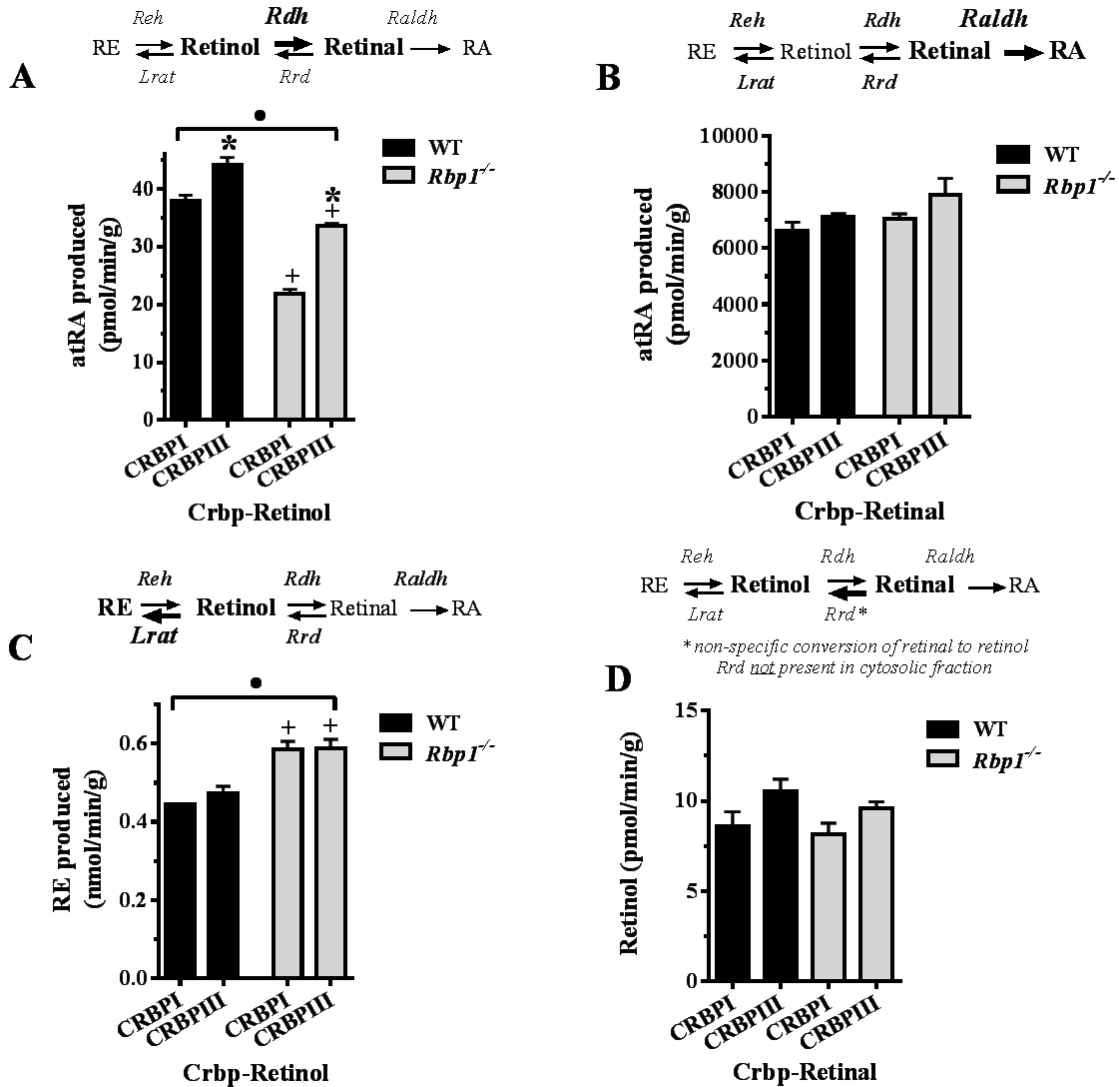
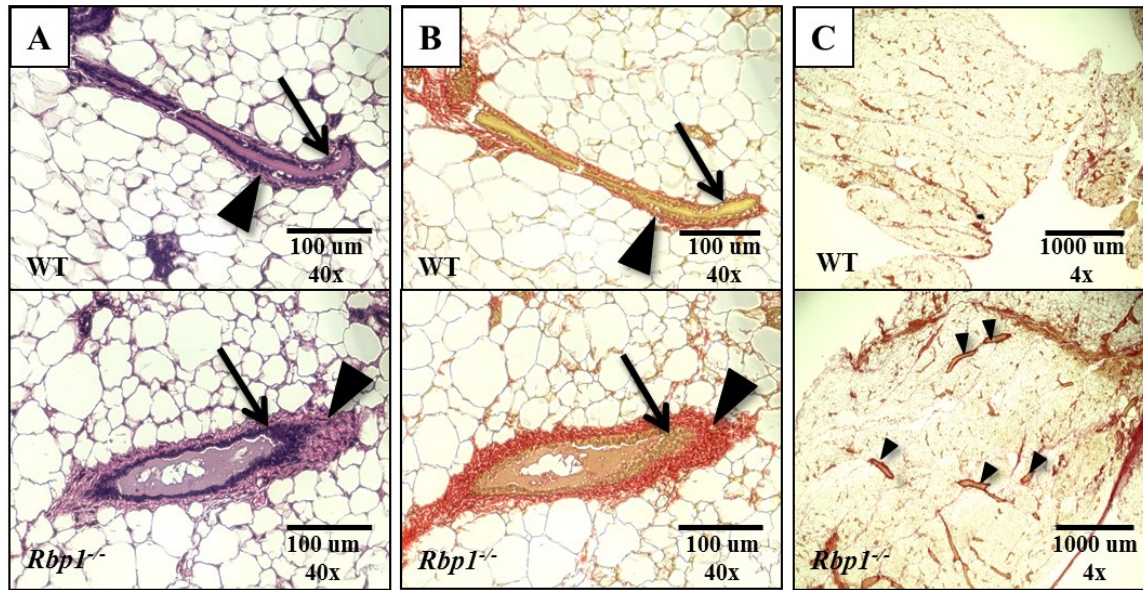


Figure 2.4. Effects of Crbp delivery and *Rbp1* loss on atRA biosynthesis. **A**, Biosynthesis of atRA from retinol bound to CrbpI or CrbpIII by a combination of microsomes and cytosol to quantify Rdh activity. **B**, Biosynthesis of atRA from retinal bound to CrbpI or CrbpIII by cytosol to quantify Raldh activity. **C**, Conversion to RE from retinol bound to CrbpI or CrbpIII by a combination of microsomes and cytosol to quantify Lrat activity. **D**, Conversion to retinol from retinal bound to CrbpI or CrbpIII by microsomes to quantify Rdh reductase activity. Representative data produced by 1 μ M Crbp-retinoid in 1 hour. Data expressed as mean \pm SEM, n=6 per condition. (*) p<0.043 vs. CrbpI delivery for respective genotype. (+) p<0.045 vs. WT for respective Crbp-retinoid substrate. (●) p<0.0312 for comparison of WT, CrbpI-retinoid to *Rbp1*^{-/-}, CrbpIII-retinoid representing a mimic of the *in vivo* condition.

2.3.5 *atRA-depleted Rbp1^{-/-} mammary tissue exhibits epithelial and stromal hypercellularity*

Because atRA is essential to the maintenance of epithelial cells and because *Rbp1^{-/-}*-tissue has depleted atRA, we stained for hematoxylin and eosin to determine whether the morphology of *Rbp1^{-/-}* mammary tissue was altered (**Figure 2.5 A**). Hematoxylin is a basic dye that stains acidic features of the cell purple, e.g., nucleic acids in the nuclei. Eosin is an acidic dye that stains basic features pink, e.g., cytoplasm and extracellular matrix. A typical WT mammary duct has a uniform layer of epithelial cells that appear purple surrounded by a structurally supportive stromal layer rich in extracellular matrix proteins that appear pink (**Figure 2.5 A**). *Rbp1^{-/-}* mammary tissue exhibits epithelial cell hyperplasia and hypercellularity of the stromal layer. We observed generally uniform appearance of ducts throughout a tissue for a given genotype. Because we observed expansion of the eosinophilic stromal layer, we also stained with Sirius red to assess the presence of collagen, a major component of the extracellular matrix that is secreted by fibroblasts (**Figure 2.5 B and C**).³ Collagen appears red and epithelial cells appear tan after Sirius red staining. *Rbp1^{-/-}* mammary tissue exhibits a significant increase in the density of collagen in the expanded stromal layer as compared to WT (**Figure 2.5 B**). Figure 1.5 C indicates that multiple hyperplastic regions exist in a typical *Rbp1^{-/-}* mammary section. Because atRA deficiency has been shown to cause oxidative stress⁵⁹ and because *Rbp1^{-/-}* mammary tissue has depleted atRA, we quantified the NAD⁺/NADH ratio as a marker of oxidative stress.⁶⁰ WT mammary tissue displayed a 65% higher NAD⁺/NADH ratio, indicating that *Rbp1^{-/-}* mammary tissue displays greater oxidative stress as compared to WT (**Figure 2.5 D**).



D

Figure 2.5. atRA depleted mammary exhibits epithelial hyperplasia, stromal hypercellularity, and oxidative stress. **A**, representative images of mammary ducts stained with hematoxylin and eosin captured at 40X. Epithelial layers are purple (indicated by arrow) and stromal layer is pink (indicated by triangle). WT mammary ductal epithelial cells uniformly surround the lumen, whereas, *Rbp1*^{-/-} mammary ducts show thickening of the epithelial cell layer surrounding the lumen. **B-C**, representative images of mammary ducts stained with Sirius red for collagen. Epithelial layers are tan (indicated by arrow) and collagen is red (indicated by triangle). WT ducts show typical stromal layers containing collagen at 40X (**B**) and 4X (**C**). *Rbp1*^{-/-} mammary exhibits hypercellularity and expansion of the stromal layer as indicated by increased collagen staining. **C**, *Rbp1*^{-/-} mammary has multiple hyperplastic regions within a given tissue as indicated by the triangles. **D**, the NAD⁺/NADH ratio in mammary tissue is reduced in *Rbp1*^{-/-} mice. Data expressed as mean ± SEM, n=20. (*) p=0.0459.

2.4 Discussion

CrbpI loss occurs in ~25% of breast cancers and is associated with poor prognosis.⁶⁻⁷

Because CrbpI participates in regulating atRA biosynthesis, it was previously postulated that loss of CrbpI expression results in loss of atRA biosynthesis capacity, which disrupts essential atRA signaling.⁵ However, endogenous atRA has not previously been reported in *Rbp1*^{-/-} mouse mammary tissue. Previous studies that showed absence of *Rbp1* (which encodes CrbpI) results in phenotypes characteristic of atRA-deficiency in xenograft mammary models did not directly quantify endogenous atRA either.⁵⁰ We undertook this study to determine the effect of *Rbp1* loss on atRA biosynthesis and homeostasis in the mammary gland; our findings are summarized in **Figure 2.6**.

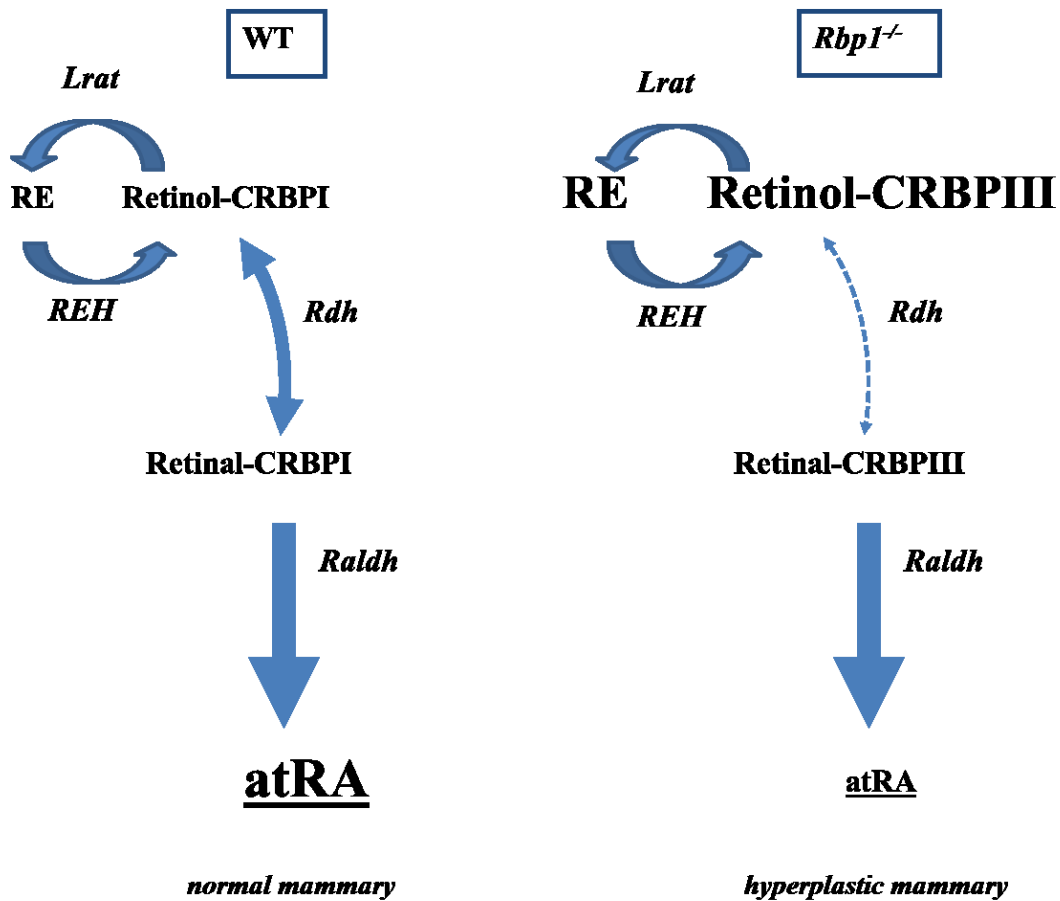


Figure 2.6. Impact of *Rbp1* loss on atRA homeostasis. atRA is produced in a two-step oxidative process consisting of a reversible and rate-limiting conversion of retinol to retinal by Rdh followed by an irreversible conversion of retinal to retinoic acid by Raldh. In the absence of CrbpI, CrbpIII chaperones retinol and retinal in the *Rbp1*^{-/-} mammary. The main defect in *Rbp1*^{-/-} mammary is decreased Rdh enzyme activity that results in reduced atRA levels *in vivo* despite an excess substrate pool of vitamin A (retinol) and vitamin A storage (RE). It should be noted that the first step of atRA biosynthesis labeled “Rdh” consists of multiple enzymes and includes both dehydrogenases and reductases.

2.4.1 Degree of atRA depletion in Rbp1^{-/-} mammary tissue is consistent with aberrant physiology

Here, we report the direct quantification of atRA in vivo in WT and *Rbp1* mouse mammary tissue using sensitive and analytically rigorous methodology.^{18-20, 25-27} We found that *Rbp1* loss depleted endogenous atRA 40% in *Rbp1* mammary tissue due to a loss of local atRA biosynthesis capacity. This magnitude of change in endogenous atRA levels observed in *Rbp1* mammary tissue is similar to changes in atRA that have previously been associated with local biosynthesis defects and aberrant physiology. In fact, the magnitude of disruption in endogenous atRA levels shows similarity across diverse models. In these previous reports, altering atRA from 20 to 67% in diverse tissue systems (brain, bone, and skin) all yielded aberrant physiological outcomes.^{19-20, 61-64} For example, atRA is an essential meninges derived cue for cortical neuron generation, and a 20% decrease in meningeal atRA accompanied by a 50% decrease in cortical atRA in the *Foxc1* mutant resulted in severe defects in forebrain development and corticogenesis.⁶¹ Because meninges contain at least ~3-fold greater levels of atRA than cortex, the absolute loss of atRA in meninges is actually greater than the loss in cortex. Local meningeal atRA biosynthesis in *Foxc1* mutants was compromised by markedly reduced levels of atRA biosynthesis enzymes in meningeal cell and a reduction in the number of cells available to biosynthesize atRA.⁶¹ In a pair of studies by Williams et al.,⁶²⁻⁶³ skeletal growth, matrix homeostasis, and growth plate function were shown to require retinoid signaling where availability of endogenous atRA dictates physiological differences between the retinoid signaling-restricted upper growth plate and the retinoid signaling-rich lower growth plate. The restricted availability of atRA in the upper growth plate is

attributed to lower local anabolic capacity that results in a 67% lower level of atRA. As a result, the endogenous level of atRA in the immature upper growth plate is below the threshold for activation of retinoid signaling, which allows chondrocyte proliferation, aggrecan gene expression, and matrix accumulation. The lower growth plate, in contrast, produces an endogenous level of atRA sufficient to activate atRA signaling, which initiates terminal chondrocyte maturation, and stimulates functions in hypertrophic chondrocytes required for replacement of chondrocytes with bone cells and marrow, including expression of MMP13 and Runx2, matrix degradation, and blood vessel invasion.⁶²⁻⁶³ An additional report by Shih et al.⁶⁴ showed that *Dgat1*^{-/-} is essential for retinoid homeostasis in murine skin. Local changes in retinol metabolism yielded a 40% increase in endogenous skin atRA levels in *Dgat1*^{-/-} mice, as compared to WT, which was sufficient to increase the mRNA expression of atRA target genes and cause excess skin retinoid activity that results in adult onset alopecia. Deprivation of vitamin A in the diet reduced endogenous atRA in the *Dgat1*^{-/-} skin to levels similar to WT and prevented the onset of alopecia in *Dgat1*^{-/-} mice.⁶⁴ Thus, the fold change in active atRA isomer levels in diverse tissue systems that is required to affect physiology is quite similar and consistent with the magnitude of dysregulation observed in *Rbp1*^{-/-} mammary atRA homeostasis.

2.4.2 atRA homeostasis is disrupted in *Rbp1*^{-/-} mammary tissue

Maintaining atRA homeostasis depends on rates of retinol metabolism. Rates of retinol metabolism into atRA or RE depend, in part, on retinol concentration.⁶⁵ Quantification of retinol in mammary tissue revealed that reduced endogenous atRA levels are not due to a deficiency of retinol available for atRA or RE biosynthesis. Increased retinol in

mammary tissue is due, at least in part, to a 4-fold elevation in Stra6 (**Figure 2.2**). Stra6 is the membrane receptor for Rbp4 that facilitates uptake of vitamin A into the cell.⁶⁶ Retinol is elevated 89% in *Rbp1*^{-/-} mammary tissue, as compared to WT, and RE is proportionally elevated 69%. The lack of an increase in endogenous mammary atRA proportional to the increase in mammary retinol and RE is consistent with a defect in atRA biosynthesis. Our data from isolated subcellular fractions identify reduced Rdh activity as the defect in atRA biosynthesis (**Figures 2.4 and 2.6**). This finding is important because retinol oxidation by Rdh enzymes is the rate-limiting step in atRA biosynthesis.^{4, 14} Elevated RE levels in mammary tissue are consistent with retinol concentration-dependent RE synthesis and indicate that storage of vitamin A is actually increased in *Rbp1*^{-/-} mammary tissue, providing excess reserves of vitamin A. Thus, there is not a deficiency in the potential pool of mammary vitamin A that could limit atRA production. Excess retinol and RE in mammary tissue is consistent with reports that *Rbp1*^{-/-} extrahepatic tissues (adipose, testis, pancreas) exhibit elevated retinol and RE.¹⁹⁻²⁰ An increase in extrahepatic retinoids is also indicative of increased mobilization of retinoid stores from liver in *Rbp1*^{-/-} mice.²⁴ Consistent with previous retinoid measurements and with the observations of increased mobilization, liver retinol and liver RE are lower in *Rbp1*^{-/-} mice, and liver atRA is similar in *Rbp1*^{-/-} as compared to WT mice.^{19-20, 24} Elevated circulating retinoids are also consistent with increased mobilization of retinoid stores from liver. Female *Rbp1*^{-/-} serum retinol was 20% elevated in *Rbp1*^{-/-} mice, whereas serum atRA and serum RE were similar. Previous observations of male *Rbp1*^{-/-} mice indicated that serum atRA was elevated by 2.4-fold, and serum retinol was elevated by 40%.²⁰

2.4.3 *atRA depletion precedes defects in atRA biosynthesis enzyme expression*

A number of previous reports implicate loss of particular atRA biosynthesizing enzyme expression in tumor development.⁶⁷⁻⁷¹ Dhhs3 has been observed to be down-regulated in neuroblastoma and melanoma, and Dhhs9 has been reported to be down-regulated in colon cancer.⁶⁷⁻⁶⁹ Raldh1 was reported to be significantly lower in grade 3 ovarian cancers as compared to normal ovarian tissue.⁷⁰ Raldh3 has been reported to be absent in breast cancer cells.⁷¹ We observed expression of *Rdh1*, *Rdh10*, *Rdh12*, *Dhhs3*, *Dhhs9*, *Raldh1*, *Raldh2*, and *Raldh3* in both WT and *Rbp1*^{-/-} mouse mammary tissue. *Rbp1*^{-/-} mice did not exhibit any changes in expression of *Rdh1*, *Rdh10*, *Rdh12*, *Dhhs3*, *Dhhs9*, and *Raldh3*, but *Rbp1*^{-/-} mammary tissue had reduced *Raldh1* and *Raldh2*. The loss of *Raldh1/Raldh2* expression, however, was not sufficient to alter the overall capacity for *Rbp1*^{-/-} cytosol to convert retinal into atRA, indicating that a greater loss in *Raldh1/Raldh2* expression would be necessary to reduce atRA production **Figure 2.4**. It has been reported previously that CrbpI loss is an early event in cancer,^{6,8} and, from these data, it seems that *Rbp1* loss could precede defects in other atRA-biosynthesizing enzyme expression (*Rdh1*, *Rdh10*, *Rdh12*, *Dhhs3*, *Dhhs9* and *Raldh3*) that has been associated with carcinogenesis.⁶⁷⁻⁷¹ Because *Rbp1* loss alone is not sufficient to alter expression of *Dhhs3*, *Dhhs9*, or *Raldh3*, or sufficient to alter *Raldh1/Raldh2* expression enough to alter atRA biosynthesis, the loss of expression of these enzymes may represent later events in cancer progression. *Raldh1* and *Raldh3* expression has been previously reported to correlate with breast cancer progression.⁷²⁻⁷³

2.4.4 *Rdh enzyme activity contributes to depletion of endogenous atRA*

In the *Rbp1*^{-/-} mouse mammary tissue that lacks CrbpI, CrbpIII is the only Crbp expressed (**Figures 2.3 and 2.6**).^{21, 45} Because expression of Rdh enzymes was unchanged and because the decrease in expression of *Raldh1* in *Rbp1*^{-/-} mammary tissue was not sufficient to affect conversion of retinal to atRA, we investigated the possibility that replacement of CrbpI with CrbpIII as the main Crbp chaperone was influencing atRA production. The nature of the Crbp protein to which retinol is bound influences rates of retinol metabolism by Rdh enzymes where Crbp protein with lower affinity for retinol allows greater rates of atRA biosynthesis.²⁰ Previous studies of the ligand-binding properties of Crbp proteins found that lower affinity for retinol was also associated with less specificity of ligand release.³⁰ Lower affinity of Crbp for retinol or retinal would allow a larger portion of retinoid to be unprotected or “free” and, thus, available to other enzymes that have demonstrated activity toward retinoids (i.e., Rdh, as well as ADH and/or AKR). Here, we determined that the K[']d for CrbpI binding to retinol is 15 nM and for CrbpIII binding to retinol is 113 nM. The recovered K[']d was in good agreement with previous determinations of ~3–10 nM for CrbpI binding to retinol and 109 nM for CrbpIII binding to retinol.^{22, 33} CrbpIII has ~7-fold less affinity for retinol than CrbpI and CrbpIII allows a ~17–54% faster rate of retinol dehydrogenation than CrbpI. This finding is consistent with previous work that found that CrbpII has ~6-fold lower affinity for 9c-retinol than CrbpI and that CrbpII allowed for ~2.5-fold increased rate of 9c-retinol dehydrogenation as compared to CrbpI.²⁰ Even though CrbpIII-bound retinol produced more atRA in both WT and *Rbp1*^{-/-} mammary subcellular fractions, *Rbp1*^{-/-} subcellular fractions produced less atRA for both CrbpI- and CrbpIII-bound retinol. Thus, in the

Rbp1^{-/-} mammary tissue, even though CrbpIII is the major retinol chaperone, there is lower atRA production due to a lower net enzymatic activity of Rdh enzymes.

Defects in retinol dehydrogenation are significant for atRA production because retinol dehydrogenation by Rdh enzymes is the first and rate-limiting step in atRA biosynthesis. Confirming that Rdh enzyme activity is rate limiting, we observed that Rdh activity in mammary fractions was ~200-fold less than Raldh activity for atRA production (**Figure 2.4**). Similar assays in pancreas subcellular fractions and in primary astrocytes had ~1.3- to 100-fold less Rdh activity as compared to Raldh activity for atRA production.^{62, 75} Enzymatic dehydrogenation of retinol by Rdh is cofactor (NAD⁺, NADP⁺) dependent and reversible.^{4, 76} The balance between the oxidative and reductive activity of multiple Rdh enzymes controls flux through the atRA pathway.⁷⁶ atRA deficiency causes oxidative stress, which increases NADH and NADPH synthesis.^{58,77} Shifting of the ratio of oxidized/reduced cofactor (i.e., NAD⁺/NADH and NADP⁺/NADPH) shifts the equilibrium of Rdh activity by reducing the activity oxidative Rdh, while increasing the activity of reductive Rdh, thus influencing the flux that Rdh enzymes allow toward atRA biosynthesis.^{15, 76, 78} Indeed, *Rbp1*^{-/-} mammary tissue exhibits atRA deficiency (**Figure 2.1**) and a decreased NAD⁺/NADH ratio. Our enzyme activity data in *Rbp1*^{-/-} mammary subcellular fractions is consistent with Rdh enzymes allowing less flux toward atRA biosynthesis (**Figure 2.4**). Thus, it is plausible that lower atRA biosynthesis capacity in *Rbp1*^{-/-} mammary tissue creates a state of atRA deficiency that favors oxidative stress and tumor progression. In fact, absence of CrbpI increases the tumorigenicity of epithelial breast cancer cells.⁵⁰ In addition, these epithelial breast cancer cells that are CrbpI-null and that have less capacity to produce atRA also display

evidence of oxidative stress.⁷⁹⁻⁸⁰ Oxidative stress is regarded as a potentiator of tumor progression.⁷⁹⁻⁸⁰ In addition to influencing Rdh activity, it is also possible that changes to the metabolic state of the cells influences Rdh function. Dhhrs9 has been reported to undertake moonlighting functions additional to Rdh activity based on the metabolic state of the cell.⁸¹ Although the effect on atRA biosynthesis was not investigated in the moonlighting studies, it is possible that partitioning of the protein to other functions could detract from its functions as an Rdh.

CrpbIII and CrpbI bound to retinal with equal affinity and allowed for equivalent rates of retinal dehydrogenation (**Figures 2.3 and 2.4**). Raldh activity was similar between WT and *Rbp1*^{-/-} mammary subcellular fractions (**Figure 2.4**). Our finding that rates of dehydrogenation by Raldh were unaffected by the nature of the Crbp protein (CrpbI or CrpbIII) or by the *Rbp1*^{-/-} genotype is consistent with previous data that showed that Raldh activity was the same for both CrpbI-retinal and CrpbII-retinal substrates.²⁰ The recovered K_d values for CrpbI and CrpbIII binding to retinal (52 and 53 nM, respectively) are similar to those recovered previously for CrpbI and CrpbII, which range between ~5 and 90 nM. Our result for CrpbIII binding affinity differed from a previous result that indicated that CrpbIII was unable to bind retinal.²² However, in that study, instead of measuring the quenching of intrinsic tryptophan emission on binding, their assay relied on measuring an increase in retinoid fluorescence on binding to Crbp. Whereas monitoring the increase in retinoid fluorescence on binding to Crbp shows very good agreement with quenching data for retinol binding to Crbp,³²⁻³³ retinal and RA do not fluoresce under these experimental conditions, and thus one must monitor the protein quenching to determine binding affinity. The result that CrpbIII does not bind atRA is

consistent with previous determinations of the binding affinities of CrbpI and CrbpII for RA.^{30, 32-33} atRA is chaperoned by a separate class of proteins, the cellular RA-binding proteins (Crabps), which do not bind retinol or retinal.^{4,30}

We found retinol delivery via CrbpI or CrbpIII to Lrat allowed the same rate of RE formation (**Figure 2.4 C**). This is in agreement with previous reports that holo-CrbpI and holo-CrbpIII are equally good substrates for Lrat.²¹ apo-CrbpI can inhibit Lrat activity, CrbpIII cannot.²¹ The inability of apo-CrbpIII to inhibit RE synthesis by Lrat may contribute to the increased levels of RE observed in *Rbp1*^{-/-} mammary gland (**Figure 2.1 and Figure 2.4 C**). Adipose tissue also expresses CrbpIII, and we previously observed a similar excess level of RE in adipose tissue in the absence of Rbp1.²⁰

2.4.5 Loss of Rbp1 and endogenous atRA affects the mammary microenvironment

Mammary gland morphology is regulated by atRA, and levels of atRA vary according across morphological stages.^{9, 25} Normally, atRA is important to regulating epithelial cell proliferation and apoptosis, which are key events in changing morphology.⁹ Aberrant proliferation of epithelial cells is a characteristic early event in mammary dysfunction that can progress to cancer.³ Hypercellularity of the epithelial layer observed in *Rbp1*^{-/-} mammary tissue is consistent with dysregulated proliferative programs and atRA deficiency.⁸²⁻⁸³ Understanding the effect of CrbpI loss is significant because epigenetic silencing of CrbpI represents a significant subset of human breast cancers (~25%).^{6, 8} Our data show that CrbpI loss results in depleted endogenous mammary atRA to a degree that other previous studies have shown to disrupt essential atRA signaling.^{19-20, 61-64} Consistent with a loss of atRA signaling, *Rbp1*^{-/-} mammary tissue displays epithelial and stromal

hyperplasia (**Figure 2.5**). In mouse mammary tissue, loss of atRA signaling, as shown by a dominant-negative RAR α mutant mouse model, initiated tumors and exacerbated tumor progression.⁸⁴ Thus, reduced atRA biosynthesis capacity may be an important epithelial cell loss of function.

The growth and function of mammary epithelial cells relies on interactions with supportive stroma.⁸⁵ The importance of epithelial-stromal interactions has been shown in culture and *in vivo*.^{79, 86} Normally, epithelial cells are surrounded by stroma composed of mainly quiescent fibroblasts that secrete extracellular matrix components and provide a normal barrier to tumor formation.⁸⁵⁻⁸⁶ Epithelial cell dysfunction has been shown to be an instigating factor in conversion of normal fibroblasts to cancer-associated fibroblasts, which secrete significantly more collagen.⁸⁷ Accordingly, breast cancer stroma exhibits accumulation of fibroblasts and a collagenized extracellular matrix.⁸⁵⁻⁸⁷ *Rbp1*^{-/-} mammary tissue displays increased collagen and hypercellularity consistent other mouse models of dysregulated mammary tissue homeostasis and oncogenesis that display an aberrant microenvironment (**Figure 2.5**).⁸⁸⁻⁸⁹ Excess secretion of collagen contributes to the abnormal growth of fibrotic tissue around tumors. The abnormal growth of fibrotic tissue around tumors is a well-defined stromal phenotype, referred to as the desmoplastic reaction, that is associated with poor prognosis in breast cancer.⁸⁷ These fibrotic defects result in increased breast density, which is linked to a >4-fold increased risk of breast cancer, with highly dense breast regions being associated with cancer progression, invasion, and recurrence.⁹⁰⁻⁹²

2.5 Conclusion

Loss of CrbpI disrupts atRA homeostasis, resulting in mammary defects similar to those observed in the early stages of tumorigenesis. These data support the role of CrbpI as a critical regulator of atRA homeostasis and provide mechanistic insight into the source of the atRA deficiency seen in the absence of CrbpI. These results also underscore the essential role of atRA in maintaining proper mammary morphology and the need for tight regulation of the levels of vitamin A's active metabolite. As such, careful characterization of the CrbpI phenotype will assist with developing new therapeutic strategies for the CrbpI-deficient breast cancer subset.

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Chapter 3: Retinoic acid biosynthesis is impaired in human and murine endometriosis²

3.1 Introduction

Endometriosis is an enigmatic disease, in which endometrial cells translocate outside of the uterine cavity. This disease affects more than 8 million women in North America alone and gives rise to a variety of symptoms, the most common being chronic pelvic pain and infertility.¹⁻² Although endometriosis is not considered a premalignant condition, it has mixed traits of benign disease and malignancy; its pathogenesis involves loss of control of cell proliferation and is associated with local invasion and distant metastasis. It is well known that normal endometrium is responsive to physiologic fluctuations of steroid hormones with highly orchestrated proliferative, secretory, and inflammatory changes.³⁻⁴ However, in cases of endometriosis, numerous reports have demonstrated aberrant hormonal regulation of the eutopic (intrauterine) endometrial tissue as well as the ectopic implants.⁵⁻⁶ In addition to regulation by steroid hormones, studies have shown that retinoids also play fundamental roles in the normal maintenance of the endometrium.⁷⁻⁹ To this end, the action of all-trans retinoic acid (atRA) produced from metabolic conversion of retinol has long been recognized as being necessary for normal endometrial cell differentiation and function.¹⁰⁻¹¹ This activity is mediated by the expression of nuclear and cytoplasmic retinoid receptors and localized atRA synthesis in endometrial stromal cells.¹²⁻¹³ During the human menstrual cycle, expression of retinoid receptors and synthesis of atRA are influenced by the changing pattern of steroid exposure. Among the numerous aspects of endometrial behavior regulated by local atRA

² Adapted from publication: Pierzchalski, K., Taylor, R.N., Nezhat, C., Jones, J.W., Napoli, J.L., Yang, G., Kane, M.A. and Sidell, N. (2014) Retinoic acid biosynthesis is impaired in human and murine endometriosis. *Biol. Reprod.* 90(4):84

production are matrix metalloproteinase (MMP) secretion, gap junctional intracellular communication, and the expression of a variety of cytokines involved in stromal cell growth, adhesion, and differentiation.¹⁴⁻¹⁵ Some examples of atRA-regulated genes are IL-6, MCP-1, TNF α , VEGF, connexin43, various integrins, and fas ligand,¹⁵⁻¹⁹ genes which are also known to be aberrantly expressed in endometriotic lesions.²⁰ Thus, a number of seemingly discordant features of endometriosis including decreased cell death, increased growth and migration, and enhanced invasive properties of intraperitoneally seeded endometrial cells could be accounted for by dysregulation of atRA synthesis. This contention was recently supported in a mouse model of endometriosis where treatment with atRA suppressed the establishment and growth of peritoneal implants, promoted macrophage differentiation, and inhibited peritoneal fluid accumulation of IL-6 and MCP-1.²¹ Together, these studies suggest that defective retinoid metabolism and atRA production in the endometrium predisposes to the pathophysiology of endometriosis.^{16, 21-23} However, this contention has not been confirmed since direct quantitation of atRA in this tissue has heretofore not been reported.

Cellular retinol-binding protein, type I (CrbpI), encoded by *Rbp1*, is a retinol chaperone protein and CrbpI-bound retinol is the preferred substrate for retinol dehydrogenase, the rate-limiting enzyme in atRA biosynthesis.²⁴ Reduced CrbpI results in significantly less efficient metabolism of retinol to retinal and subsequent oxidation of retinal to atRA. Thus, because of the regulatory influence of CrbpI on atRA production, loss of CrbpI consequentially results in defective atRA biosynthesis and signaling in a variety of tissue.²⁴⁻²⁵ CrbpI and other genes involved in retinol uptake and metabolism have been shown to be aberrantly expressed in endometriosis²²⁻²³ as well as in various cancers²⁵⁻²⁷

and some developmental diseases of the brain, bone, and skin.^{24,28} These studies, along with the demonstrated ability of atRA to regulate endometrial cell differentiation and proliferation, suggest that defects in *CrbpI* gene expression results in abnormal retinoid biosynthesis and may play a role in the etiology and/or progression of endometriosis. To further address this question, we directly measured atRA levels and *CrbpI* expression in endometrial tissue and lesions from endometriosis patients. In addition, we have utilized animal model systems of endometriosis and *CrbpI* deficiency to support conclusions reached using these human tissues.

3.2 Experimental Procedures

3.2.1 Human endometrial tissue and cell cultures

Tissues were obtained from patients undergoing surgery for infertility or pelvic pain, according to protocols approved by the Institutional Review Boards, Emory University School of Medicine and Northside Hospital, Atlanta, Georgia. Women who had been on estrogen- or progesterone-containing medications or other forms of pituitary suppression in the previous 3 months were excluded. At surgery (laparoscopy or laparotomy), a full visual inspection of the pelvic cavity was performed by senior gynecologic surgeons with extensive experience in the recognition and treatment of typical and atypical endometriotic lesions.²⁹ Following surgery, women were classified as having endometriosis and entered into the study if their surgeon noted laparoscopic evidence of endometriosis lesions that were histopathologically confirmed to contain endometrial-type glands and stroma.

Biopsies of eutopic endometrium and endometriosis implants were flash frozen at -80°C for extraction and quantitation of retinoids. In some cases, fresh tissue was used to isolate endometrial biopsy (-B) and endometriosis lesion (-L) cells. Separation of epithelial and stromal cells from either eutopic or ectopic endometrial tissue was performed using the procedure originally developed by Ryan et al.³⁰ and Hornung et al.³¹ Primary endometrial stromal cell (ESC) cultures were prepared from biopsies of the eutopic endometrium (ESC-B) or ectopic lesions (ESC-L) according to our published procedures.¹⁶ All cultures were grown in complete medium: DMEM/F12 (Cellgro, Manassas, VA) containing 10% fetal bovine serum, 100 U/ml of penicillin, 100 $\mu\text{g/ml}$ of streptomycin, 2 mM L-glutamine, and 1 mM HEPES. For treatment, cultures were washed with phosphate-buffered saline and then cultured in serum-free medium in the presence of 2 μM retinol (ROL, Sigma Chemical Co., St. Louis, MO) or solvent control (DMSO) for 16 hours. The final concentration of DMSO as solvent was always less than 0.1%.

3.2.2 Mouse models of endometriosis

The immunocompetent mouse endometriosis model used in these experiments has been previously described.²¹ This study was approved by the Emory Institutional Animal Care and Use Committee and performed according to the NIH Guidelines for Care and Use of Laboratory Animals. Briefly, endometrial tissue from 2 to 4 month-old C57BL/6-Tg (Tg-GFP) donor mice, which express enhanced green fluorescent protein (GFP) under the direction of the human ubiquitin C promoter, was minced and injected into the peritoneal cavity of syngeneic recipient mice as described.²¹ The mice were purchased from Jackson

Laboratories and fed a chow diet ad libitum (Harlan Teklad Global 18% protein extruded rodent diet #2018SX with the equivalent of 30 IU/g vitamin A). The Tg-GFP donor mice were treated subcutaneously (SC) with 100 µg/kg estradiol valerate (dissolved in corn oil) 1 week before being sacrificed to stimulate proliferation of their endometrial tissue for transplantation. Recipient mice also received 100 µg/kg per week estradiol valerate SC in corn oil each week, starting 1 week before IP inoculation of endometrial tissue, to synchronize their estrus cycles. 2-3 weeks after uterine fragment inoculation, we sacrificed the recipient mice and examined their peritoneum under 488 nm light (excitation wavelength peak of GFP) by use of interference filter eyeglasses to observe the GFP+ endometrial implants (emission wavelength peak: 502 nm). The implants were then carefully excised and processed for retinoid determination. As in our previous study, we confirmed the authenticity of the implants by verifying the histopathologic criteria for endometriosis.²¹

3.2.3 *Rbp1*^{-/-} Mice

CrbpI global knock-out mice (*Rbp1*^{-/-}) on a C57BL/6 background³² and wild type (C57BL/6) mice aged 2 to 4 months were used according to institutional guidelines. The *Rbp1*^{-/-} mice were bred in-house from breeders obtained from Pierre Chambon and Norbert Ghyselinck. Female WT or *Rbp1*^{-/-} mice were treated SC with 100 µg/kg estradiol valerate (dissolved in corn oil) each week for 2 weeks before being sacrificed to stimulate proliferation of their endometrial tissue. After this time period, whole uterus, endometrium and myometrium tissues were dissected from the uteri and immediately flash-frozen and stored at -80°C until retinoid analysis. For histological analysis, uteri from 2 month old wild-type and *Rbp1*^{-/-} mice were preserved in formalin upon collection

until tissue was embedded in paraffin, sliced, and stained by the University of Maryland Core Facility for Histology. Hematoxylin and Eosin (H & E) and Masson's Trichrome (MTC) stain images were captured using an EVOS XL digital microscope (AMG, Bothell, WA).

3.2.4 Determination of retinoid levels

Biopsy specimens, cultured cell pellets, or culture supernatant were prepared for retinoid analysis under yellow lights. Processing and extraction of samples have been described in detail.³³⁻³⁵ Total protein concentrations were determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Retinoic acid was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with atmospheric pressure chemical ionization in positive-ion mode on an API-4000 or 5500 Qtrap instrument (AB Sciex, Foster City, California). Retinol and total retinyl ester (RE) were quantified by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) on an alliance 2690 or Aquity H-Class apparatus (Waters, Milford, MA).

3.2.5 Evaluation of Rbp1 mRNA by quantitative real-time (q)PCR

Total RNA from tissue or cultured ESC was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. For *Rbp1* mRNA quantitation, reverse transcription was used to synthesize cDNA from RNA template as described previously.³⁶ For real-time PCR, a total of 20 µl reaction mix was prepared using iQ™ SYBR® Green Supermix (Bio-Rad) and specific primer sets (0.3 µM each). Primer sequences used were as follows: *Rbp1*, sense (5'-AATGTGGCCTTGCGCAAAT-3'), antisense (5'-CAGCTCATCACCCCTCGATCC-3');

RPL17, sense (5'-TGAACAAAGCACCTAAGATGCGCC-3'), antisense (5'-TGGGCAACCTCCTCTTCTGGTTTA-3'. The PCR was set for 40 cycles in a Opticon 2 real-time thermocycler (Bio-Rad) under the following conditions: one denaturation cycle of 95°C for 30 seconds followed by amplification cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The data were analyzed after normalization with *RPL17* mRNA levels using the formula $2^{-\Delta\Delta CT}$, where ct is the cycle threshold.

3.2.6 Statistical analysis

Statistical analysis was performed using GraphPad Software. Data are presented as mean \pm standard error of the mean (SEM). Differences between groups as indicated were analyzed by *t* test (two-tailed), where $p < 0.05$ was considered statistically significant. Each experiment was replicated a minimum of three times.

3.3 Results

3.3.1 Endogenous atRA is reduced in human endometriotic lesions

Direct quantitation was performed on human eutopic endometrial biopsies (EB) and ectopic endometriosis lesions (EL) after tissue extraction using LC-MS/MS and HPLC-UV (**Figure 3.1**). Mean atRA levels in EL were 42% less than those detected in EB ($p < 0.005$) (**Figure 3.1 A**). 13-cis and 9, 13-di-cis RA, RA isomers without known biological activity, were also detected, but did not differ based on the source. No other RA isomers, including 9-cis RA, were detected above the LC-MS/MS assay limit of detection in biological matrices (~ 12 pmol/g protein). Retinol (ROL), and retinyl esters

(RE) were both higher in EL compared to EB by 67% and 64%, respectively ($p < 0.0001$) **(Figure 3.1 B and C)**. When comparing the concentrations of these retinoids in the ectopic and eutopic tissues from the same patient (matched samples), atRA levels were lower in EL versus EB while ROL and RE were higher in EL versus EB in most cases **(Figure 3.1 D)**.

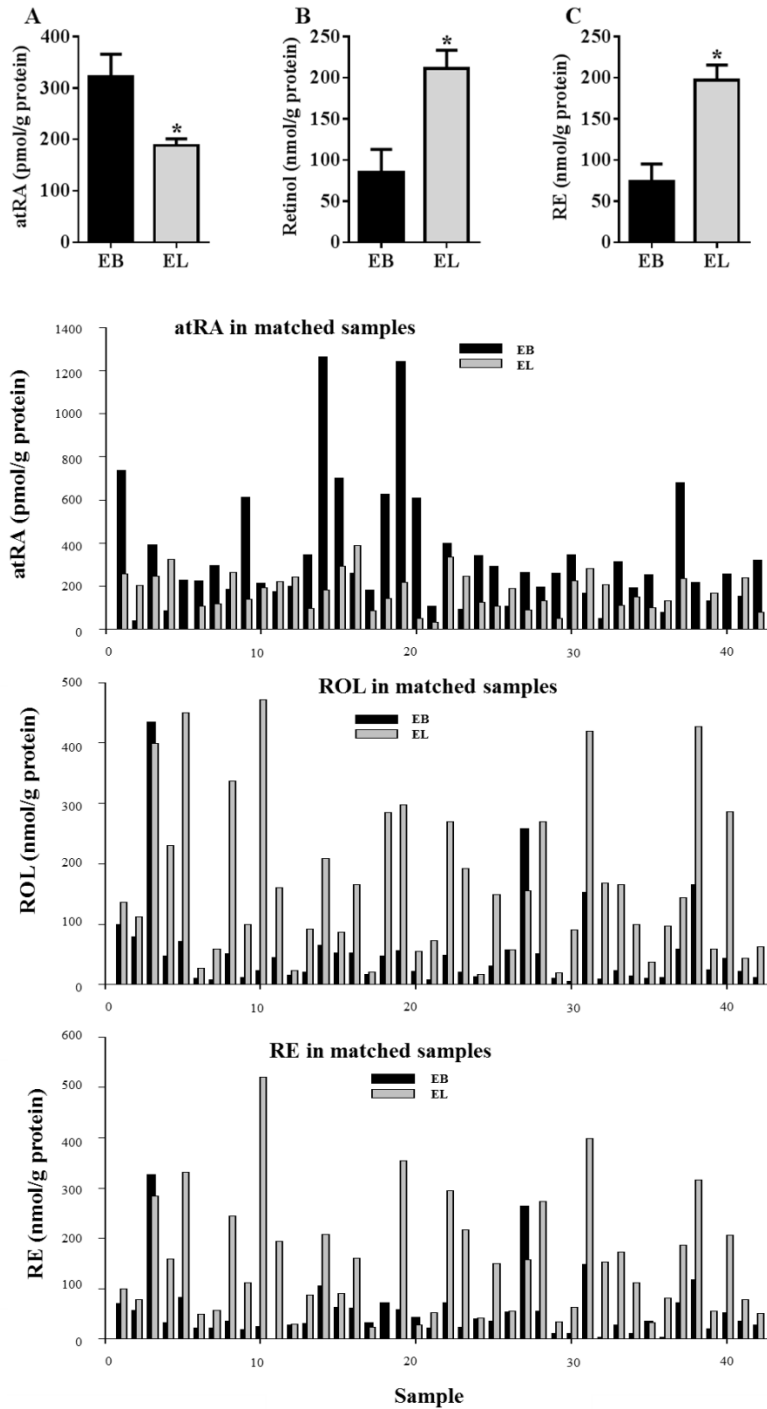


Figure 3.1. Altered retinoid levels in ectopic endometrial implants compared with eutopic endometrium. Direct quantitation of **A**, all-trans retinoic acid (atRA), **B**, retinol (ROL) and **C**, retinyl ester (RE) levels was performed on extracted human eutopic endometrial biopsies (EB) and endometriotic lesions (EL) from endometriosis patients (n=36). Values represent \pm SEM of retinoid concentrations. (*) $p < 0.005$; (+) $p < 0.0001$ when compared with EB.

3.3.2 Reduced atRA production and CrbpI expression in endometriotic lesions

We compared CrbpI mRNA expression in EB versus EL from three endometriosis patients and the possible association of this expression with atRA production in the tissue and in cultured endometrial stromal cells (ESC) derived from EB and EL. **Figure 3.2 A** shows that in the primary tissue, a >80% reduction of atRA concentrations in EL versus EB was associated with an approximate 50% reduction in CrbpI mRNA.

Correspondingly, endometrial stromal cultures derived from EL (ESC-L) showed a >75% reduction in CrbpI expression and an impaired ability to synthesize atRA from retinol compared with cultures derived from matched EB (ESC-B) (**Figure 3.2 B**).

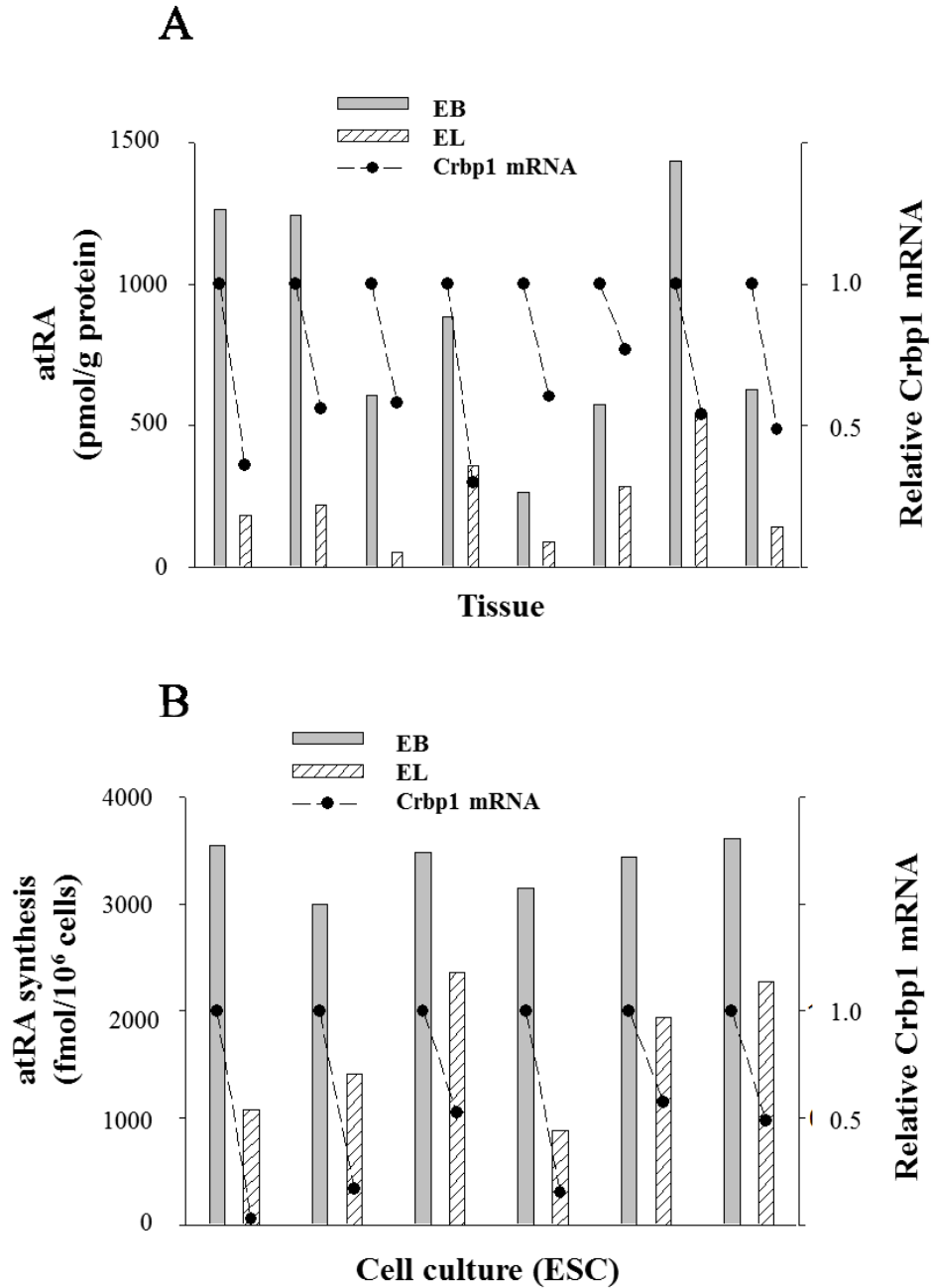


Figure 3.2. atRA production and *Rbp1* gene expression are reduced in human endometriotic lesions. **A**, Relative *Rbp1* mRNA levels (●) and atRA concentrations (bars) in matched eutopic endometrial biopsy (EB) and endometriotic lesion (EL) tissue from 8 endometriosis patients. **B**, *Rbp1* mRNA levels (●) and atRA production (bars) in cultured endometrial stromal cells (ESC) derived from endometriotic lesions (ESC-L) relative to those derived from patient-matched eutopic biopsies (ESC-B). Total atRA production in the cultures was assessed by LC-MS/MS analysis of atRA in cell pellets plus supernatant following addition of 2 μ M ROL for 16 hr. Results were normalized to 10^6 cells. In the absence of ROL, atRA values were <27 fmol/ 10^6 cells in all cases.

3.3.3 Endogenous atRA is lower in induced murine endometriotic lesions

Previous work demonstrated the ability of atRA to inhibit endometriotic lesion development in the immunocompetent mouse model of endometriosis.²¹ Having found that atRA levels were reduced in EL versus EB in humans, we questioned whether endometriotic lesions induced in our mouse model also showed a reduction in atRA compared with eutopic endometrial tissue. Our previous studies showed that 100% of the animals develop prominent peritoneal lesions in this time period with many containing well-formed vasculature, characteristic of invasive endometriotic lesions in humans. We then compared retinoid levels in the lesions with those in the eutopic tissue from which the lesions were derived (i.e. the endometrium of the GFP+ donor mice). **Figure 3.3 A** shows a 37% reduction in mean atRA levels in the mouse lesions compared with eutopic endometrium. HPLC-UV detection indicated that ROL was 40% higher in the lesions, but no significant changes in RE were noted (**Figures 3.3 B and C**). There was no significant difference between atRA levels in endometrial tissue from the GFP+ donor mice and that found in the eutopic endometrium of wild-type recipient mice in which the lesions developed (data not shown).

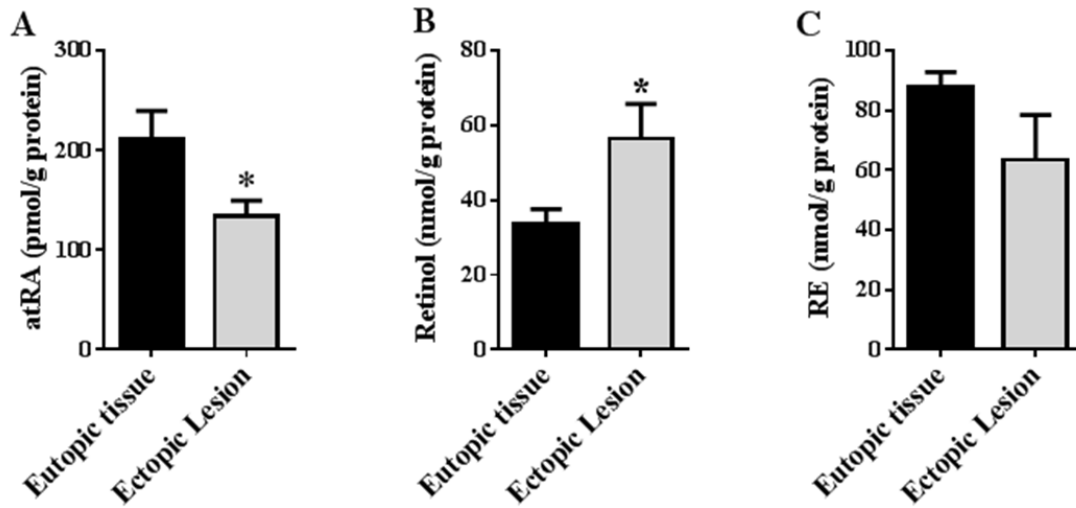
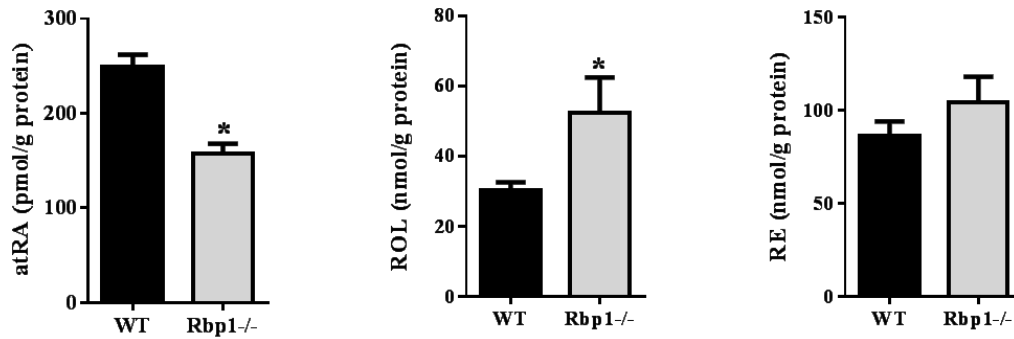


Figure 3.3. atRA levels are lower in induced endometriotic mouse lesions. Quantitation of **A**, atRA, **B**, Retinol, and **C**, RE was performed on peritoneal implants that developed in recipient mice 14-21 days after challenge with uterine fragments from GFP+ syngeneic donors. Results are compared with retinoid levels in the original GFP+ tissue inoculum. n=8 for all data sets and represent mean ± SEM. (*) p<0.05.

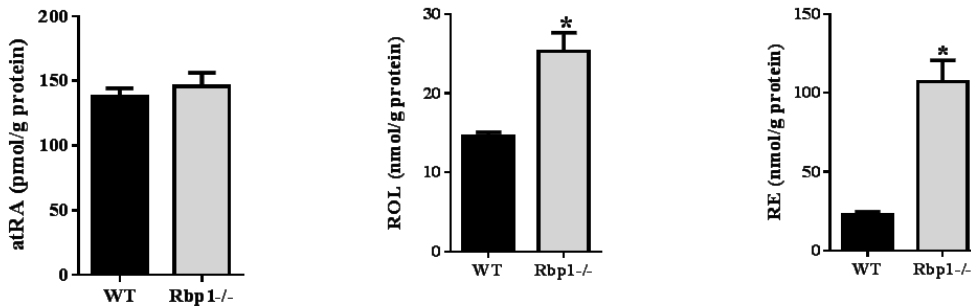
3.3.4 Endogenous atRA is reduced in *Rbp1*^{-/-} mouse endometrium

Loss of CrbpI has been associated with defective retinoid metabolism and atRA synthesis in a variety of human cancers.²⁵⁻²⁷ This fact, along with our finding of a similar association of CrbpI and atRA levels in clinical endometriotic implants, supports the contention that reduced expression of CrbpI in this tissue plays a causative role leading to reduced atRA production. To test this hypothesis, we quantified retinoid levels in the dissected endometrial tissue of a previously established CrbpI global knock-out (*Rbp1*^{-/-}) mouse model.³² As shown in **Figure 3.4 A**, endogenous atRA levels in the endometrium of *Rbp1*^{-/-} mice is reduced by 37% compared to wild-type. Quantitation of endogenous ROL and RE in the *Rbp1*^{-/-} endometrium shows an increase of 42% and no change, respectively (**Figures 3.4 B and C**). Myometrium and whole uterus were also analyzed with data shown in **Figure 3.4 D-I**. In both myometrium and uterus, atRA was not changed (**Figure 2.4 D and G**) while ROL (**Figure 3.4 E and H**) and RE (**Figure 3.4 F and I**) were both significantly elevated.

Endometrium



Myometrium



Whole Uterus

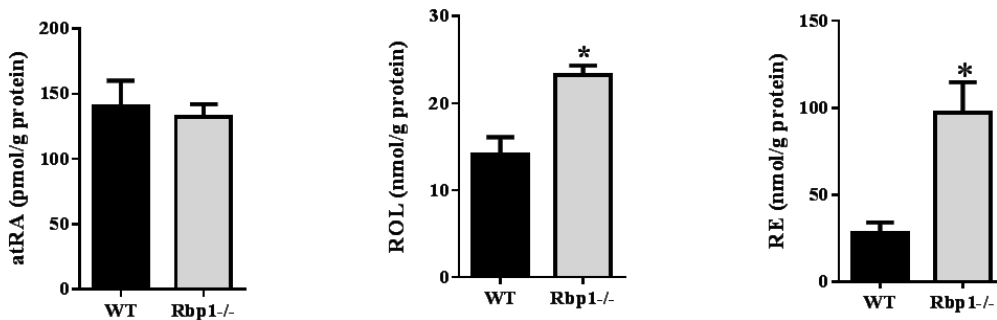


Figure 3.4. Reduced atRA in *Rbp1*^{-/-} endometrium. Quantitation of **A**, atRA, **B**, ROL, and **C**, RE was performed on the dissected endometrium of *Rbp*^{-/-} mice and compared to wild-type (WT) endometrium. Retinoid levels in the myometrium and whole uterus of *Rbp1*^{-/-} compared to WT mice. atRA concentrations are not different (**D**, **G**) while ROL (**E**, **H**) and RE (**F**, **I**) are elevated in *Rbp1*^{-/-} myometrium and uterus compared to WT. Values represent mean \pm SEM of retinoid concentrations for endometrium and myometrium (n=15) and whole uterus (n=5) data sets. *, p<0.0002; +, p<0.006.

3.3.5 Rbp1^{-/-} uterus histology exhibits loss of tissue organization and hypercellularity

Because atRA biosynthesis was found to be deficient in *Rbp1^{-/-}* mouse endometrium, we analyzed tissue histology to determine if the morphology of the *Rbp1^{-/-}* uterus and/or endometrium was altered. For this analysis hematoxylin & eosin (HE) and Masson's Trichrome (MTC) stains were applied to evaluate cellular and extracellular matrix morphology of the endometrium, respectively (**Figure 3.5**). In wild-type uterus, the endometrial/myometrial layers of the uterus are clearly defined and organized (**Figure 3.5 A**). However, the uteri of *Rbp1^{-/-}* were seen to lack the clear separation of the endometrium and myometrium while displaying numerous regions of hypercellularity. Higher magnification also indicated a loss of order in the epithelial layer of the *Rbp1^{-/-}* endometrium as well as hypercellularity of the stroma. Stromal cell counts provided statistical evidence to confirm hypercellularity in *Rbp1^{-/-}* endometrium (**Figure 3.5 I**). In addition, MTC staining indicated excess collagen accumulation in the stroma of *Rbp1^{-/-}* uteri (**Figures 3.5 D and H**).

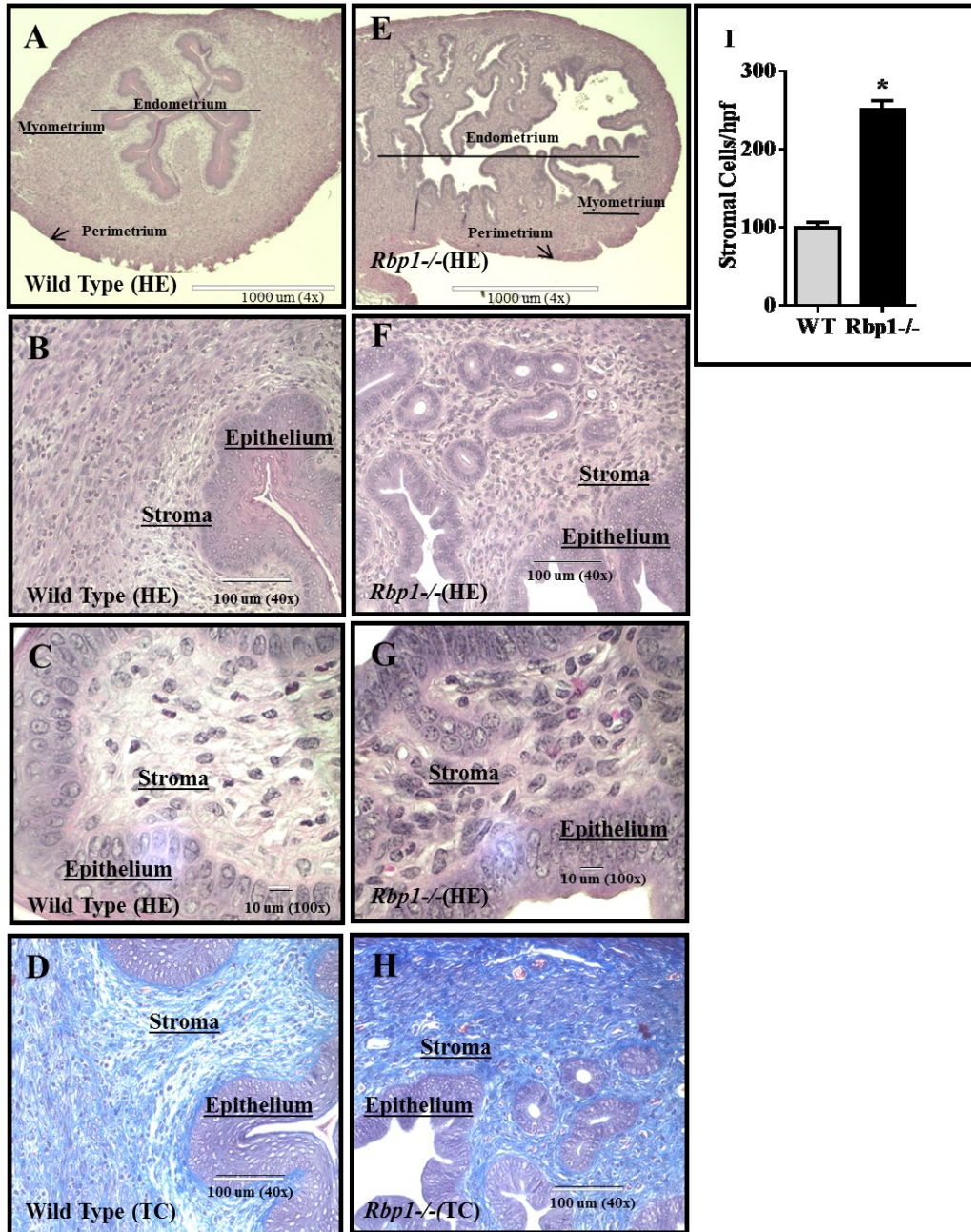


Figure 3.5. Morphological disorganization and hypercellularity of *Rbp1*^{-/-} endometrium. C57BL/6 wild-type (panels A-D) and *Rbp1*^{-/-} uterus (panels E-H) were stained with hematoxyline and eosin (HE, panels A-C, E-G) or Masson's Trichrome (MTC, panels D and H). Low power magnification (4x) shows a distinct endometrial-myometrial interface (lighter-darker areas, respectively) in the uterus of WT mice (A). In contrast, *Rbp1*^{-/-} uterus lacks a clear delineation of these regions (E). Higher magnifications (40x, 100x) show hypercellularity of both epithelial and stromal layers of the endometrium (F, G) compared with the WT (B, C). The blue intensity in the MTC-stained images (40x) indicates excess stromal collagen accumulation in the *Rbp1*^{-/-} uterus (H) compared with the WT (D). **Panel I** represents mean \pm SEM stromal cell counts of the endometrium per high power field (hpf) at 100x with n=10 total fields from 3 mice in each group. (*) p<0.0001.

3.4 Discussion

Originally proposed by Sampson in the 1920's,³⁷ retrograde menstruation is the most accepted theory to explain the migration of endometrial tissue outside the uterine cavity. This occurs in most women throughout their reproductive years,³⁸ but the reason that only ~10% of women develop endometriosis is not known. Investigators from a number of laboratories have demonstrated the presence of retinoid binding proteins and metabolic enzymes in endometrial tissue and have established that synthesis of atRA is essential for normal cellular differentiation and function.^{7-9, 39} To this end, studies have shown that the ability of endometrial stromal cells to synthesize atRA from retinol correlates well with their degree of decidualization and that most of the requirement for atRA in this process is met by local synthesis.¹³ This biosynthesis of atRA is mediated by two sequential oxidations, first producing retinal from retinol by retinol dehydrogenases utilizing CrbpI-retinol complexes as the preferred substrate. Retinal is then oxidized to atRA by retinal dehydrogenases of the aldehyde dehydrogenase family.²⁴ While numerous reports have elaborated on altered hormone responses (progesterone and estrogen) within ectopic endometrial implants compared to eutopic tissue,³⁻⁶ few studies have investigated potential retinoid metabolic defects in these lesions. Pavone and colleagues showed altered expression of genes involved in retinoid signaling, including CrbpI, in lesion cells from endometriosis patients compared with normal eutopic endometrium from patients without endometriosis.²²⁻²³ Although their study did not measure atRA levels, the results were consistent with decreased retinoid uptake, metabolism, and action. In contrast, the present work utilizes matched samples from endometriosis patients (i.e. lesions vs eutopic tissue from the same subject) and has directly quantified atRA levels and

metabolic conversion of retinol to atRA in ESC, the primary source of atRA biosynthesis in endometrial tissue.²² Our results demonstrate reduced atRA levels in EL versus EB from endometriosis patients both on a population basis and in a matched sample comparison (**Figure 3.1**). In contrast, relative tissue concentrations of ROL and RE were reversed such that EL showed higher concentrations of these retinoids than EB. This finding of elevated ROL in EL is consistent with a “roadblock” in metabolic conversion of retinol to retinal and is supported by our data demonstrating reduced CrbpI in EL and in ESC derived from this tissue (**Figure 3.2**). These results also support the contention that dysfunctional retinoid biosynthesis is responsible for the suppressed atRA levels in EL, rather than reduced ROL transport or uptake into ectopic sites. Impaired atRA synthesis was directly confirmed by treating primary ESC cultures with ROL; results showed a significant reduction of atRA production in lesion-derived cultures compared with those derived from patient matched eutopic tissue. Reduced atRA biosynthesis in ESC-L cultures was associated with a significant decrease in mRNA levels of CrbpI. Importantly, reduced atRA levels were also found in peritoneal lesions that developed in a mouse model of endometriosis in which recipient mice were inoculated intraperitoneally with syngeneic endometrial tissue to mimic a massive retrograde menses (**Figure 3.3**). As in the human samples, the mouse endometriotic implants showed an increase in ROL concentration consistent with a decrease in atRA biosynthesis from retinol. These human and animal data suggest two possible scenarios to account for aberrant retinoid metabolism in ectopically growing endometrial cells: 1) cells reaching the peritoneal cavity via retrograde menstruation, with intrinsically defective atRA synthesis, preferentially populate the ectopic sites due to properties that result from their

reduced atRA levels (e.g. increased MMP and proinflammatory cytokine secretion); or 2) the peritoneal milieu provides environmental interactions that induce defects in atRA synthesis in some shed endometrial cells, as opposed to impaired retinoid metabolism being an intrinsic property of the cells. Evidence from studies showing alterations in cytokine and MMP profiles of eutopic endometrium from some women with endometriosis support the former possibility.⁴⁰⁻⁴¹ Our animal studies demonstrated that eutopic endometrial cells from normal healthy donors can implant and grow at ectopic sites following intraperitoneal injection into ostensibly “normal” recipients.²¹ Extrapolated to humans, this finding suggests that the endometrium in all women inherently contains cells capable of forming ectopic lesions. As such, the predisposition to develop endometriosis may depend on the frequency of such cells within the eutopic population and/or the mass of cells that are shed into the peritoneal cavity during menses. Support for the latter hypothesis is provided by reports indicating that women with “heavy” menstrual cycles and those with obstructed menstrual outflow resulting in increased menstrual reflux are at increased risk for developing endometriosis.⁴² Consistent with the associative relationship in human tissue and cells (**Figure 3.2**), a direct causal relationship between reduced *CrbpI* expression and depleted atRA levels in endometrial tissue was established by analysis of *Rbp1*^{-/-} mice. Results demonstrated a similar reduction of atRA and an increase of ROL in the endometrium of the *Rbp1*^{-/-} mice compared with wild-type animals. The myometrium and whole uteri were also evaluated in this model, showing significantly elevated ROL and RE but no differences in atRA between *Rbp1*^{-/-} and wild-type (**Figure 3.4**). These analyses show that a primary role for *CrbpI* in the biosynthesis of atRA has tissue specificity. Based on a comparison

of absolute retinoid levels in the endometrium versus the myometrium (**Figure 3.4**), the former appears to be the primary source of atRA production in the uterus in the wild-type animals. To address the question of whether reduced CrbpI and atRA production plays a causative role in inducing phenotypic and functional changes in endometrial tissue consistent with endometriotic implants, histochemical comparisons of the uteri of *Rbp1*^{-/-} versus wild-type mice were performed. HE stained tissues showed an overall lack of tissue organization in the *Rbp1*^{-/-} mice and numerous regions of hypercellularity. There was a loss of definitive borders between endometrium and myometrium, and stromal hypercellularity was observed in *Rbp1*^{-/-} endometrium. In addition, in comparison to wild-type, the *Rbp1*^{-/-} endometrial stroma showed gross accumulation of extracellular matrix, a feature which is characteristic of endometriotic lesions in women.

In summary, these studies provide the first direct quantitative measurements of atRA levels in human endometrium and have demonstrated reduced atRA concentrations in ectopic versus eutopic tissue and in corresponding ESC cultures. Our findings showed a direct association between atRA biosynthesis and CrbpI expression. Quantitation of retinoids in a mouse model of endometriosis and in *Rbp1*^{-/-} endometrium supports the contention that impaired atRA synthesis caused by reduced CrbpI promotes an “endometriosis phenotype” that is able to implant and grow at ectopic sites. While it is unlikely that atRA itself can be used as a drug to treat endometriosis, the present findings may lead to a new appreciation of the cause(s) of endometriosis, and the search for new diagnostic and treatment modalities that target the retinoid metabolic pathway.

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Chapter 4: Impact of CrbpI on atRA biosynthesis and homeostasis in murine lung³

4.1 Introduction

There are several diseases that affect the lung function, the most prevalent include: asthma, cystic fibrosis, chronic obstructive pulmonary disorder (COPD), influenza, pneumonia, tuberculosis and lung cancer.¹ Lung damage caused by these diseases is often irreversible which makes early detection, treatment and disease control extremely important for patient outcomes and survival. Lung diseases can display some common organ injuries including pneumonitis, fluid accumulation in the lungs, and fibrosis, the scarring of lung tissue. Fibrosis is irreversible lung damage, often resulting from chronic inflammation.¹⁻²

Retinoic acid (RA) is the active metabolite of vitamin A, which functions through activating nuclear receptors to regulate cellular programs for development, proliferation, differentiation and apoptosis to maintain cellular and tissue homeostasis.³ RA is necessary in lung function, development, regeneration and repair. Lung morphogenesis and RA regulation have been likened to a symphony, as exquisite regulation of spatiotemporal patterns is imperative for proper lung morphogenesis and development to achieve mature, uncomplicated lung function.⁴⁻⁶ Lung development progresses to form terminal branching and budding, angiogenesis and vascularization to establish gas exchange for mature lung function.⁴⁻⁵ RA has both direct and indirect regulation through a number of RAR target pathways. all-trans RA (atRA) specifically is involved with initiating lung morphogenesis, cell differentiation and regulation of surfactant proteins.⁷⁻

³ Pierzchalski, K., Yu, J., Jones, J.W. and Kane, M.A. Impact of CrbpI on atRA biosynthesis and homeostasis in murine lung. In preparation for submission to FASEB J.

¹¹ atRA is linked directly and indirectly to a number of lung morphogenesis and regeneration pathways including growth factors (FGF, TGF β , IGF, EGF and VEGF), Wnt, sonic-hedgehog, Notch, smooth muscle differentiation, Fox, Bmp2, MMP/TIMP, collagen, vimentin, laminin, and Hox, which in turn impact cytokine activation and inflammatory responses.^{8-9, 11-20} *in vivo* studies using mouse models have shown that vitamin A deficiency (VAD) results in lung morphology defects including budding, branching and alveolar defects; and microenvironmental hyperplasia and metaplasia.^{9, 21-25} Additional mouse models to investigate reduced alveoli surface area in lung disease are improved with atRA administration, showing a potentially important role in lung regeneration.^{9, 12, 23-24, 26} Because VAD has been associated with changes in lung morphology, vitamin A and atRA are being investigated for potential therapeutic use in lung disease. Understanding these morphological changes within the lung tissue and how atRA homeostasis impacts lung morphology will improve treatments to address lung regeneration and repair to prevent permanent lung damage.

Vitamin A (retinol) is an essential vitamin that is stored in the liver. As extra-hepatic tissues require retinol (ROL), ROL is transported through the blood by the chaperone protein, circulating retinol binding protein 4 (Rbp4).³ Upon uptake into cells, ROL is retrieved by cellular retinol binding protein, type I (CrbpI) to be delivered to lethicin:retinol-acyltransferase (Lrat) for esterification for storage as retinyl esters (RE); or to short chain dehydrogenase/reductase (SDR) enzymes for atRA biosynthesis.³ atRA biosynthesis is a two-step oxidation reaction, with the first oxidation reaction being the rate limiting step. CrbpI delivers ROL to retinol dehydrogenase (Rdh) enzymes to be reversibly oxidized to retinal (RAL), where RAL may be reduced by retinal reductase

(Rrd) enzymes. CrbpI then delivers RAL to retinal dehydrogenase (Raldh) enzymes to be irreversibly oxidized to atRA.³ atRA is delivered by unique chaperone proteins, cellular retinoic acid binding protein, type II (CrabpII) or fatty acid binding protein 5 (FABP5) to the target nuclear receptors, retinoic acid receptors (RARs) and peroxisome proliferative activated receptor β/δ (PPAR β/δ), respectively, to initiate gene transcription. CrabpI delivers atRA to Cyp26 enzymes to be metabolized for excretion and prevent excess atRA accumulation.^{3, 21, 27-31} RAR α,β and γ and PPAR β/δ mediate transcription of genes in pathways that maintain cellular programs, in which hundreds of direct and indirect targets have been identified.^{7-8, 32}

CrbpI is the primary chaperone protein for ROL and also has regulatory functions for overall atRA biosynthesis.³ Additional Crbp proteins have been identified, but little is known about their expression patterns and functional roles in lung.^{3, 28, 33-38} Previous studies show that CrbpI loss through DNA hypermethylation has been identified in several diseases, including up to 60% of lymphomas, 25% of breast cancer and 15% of lung cancer.^{35, 39-41} The effect of this loss has been postulated to alter atRA biosynthesis; however, technical challenges have limited the analytical ability to directly quantify atRA.^{28, 39-45} Advances in atRA quantification methodology has made measuring endogenous atRA levels possible.⁴²⁻⁴⁶ Reduced atRA with deficient CrbpI gene expression, as quantified in human breast tumors, human endometriosis lesions and related murine models have now been reported, however, endogenous atRA levels with CrbpI gene expression has yet to be reported in lung.⁴⁷⁻⁴⁸ Using a global *RbpI* knock-out (*RbpI*^{-/-}) mouse model, which codes for CrbpI protein expression, we have quantified endogenous atRA levels in the lung and probed the regulatory role of CrbpI. In addition,

we have investigated the functional impact of cellular retinol binding protein, type III (CrbpIII), a CrbpI homolog that is present in the absence of CrbpI expression. Finally, histological evaluation was performed to relate any microenvironmental changes in the *Rbp1*^{-/-} lung.

4.2 Experimental Procedures

4.2.1 *Rbp1*^{-/-} Mice

Female, virgin, C57BL/6 wild-type (WT) or *Rbp1*^{-/-} mice aged 1.5 to 4 mo were used according to institutional guidelines of the University of Maryland, Baltimore. WT mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), and *Rbp1*^{-/-} mice were bred in-house from breeders obtained from Pierre Chambon and Norbert Ghyselinck (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Institut National de la Santé et de la Recherche Medicale, Illkirch, France). Mice were fed a chow diet ad libitum (Harlan Teklad Global; 18% protein extruded rodent diet no. 2018SX with the equivalent of 30 IU/g vitamin A; Harlan Laboratories, Indianapolis, IN, USA). Mice were euthanized with isofluorane followed by cardiac puncture and cervical dislocation.

4.2.2 *Retinoids and retinoid extraction*

Retinoids were purchased from Sigma-Aldrich (St. Louis, MO, USA) and handled under yellow light. Lung samples were harvested from mice under yellow light, frozen immediately and kept at -80°C until extraction.⁴² Retinoids were extracted from lung tissue homogenate (in normal 0.9% saline) or enzyme activity reactions with subcellular fractions by a 2-step liquid-liquid extraction that allows for RA, ROL and RE analysis.⁴²

RAL extractions were first reacted with 0.1 M o-ethylhydroxylamine in 0.1M HEPES, pH 8.0 (oxime) and methanol then extracted. Extraction procedures are describe in detail references provided.⁴² RA isomers were quantified by fast high performance liquid chromatography (HPLC) with multiplexeing multiple reaction monitoring cubed (MRM³) and retention times are as follows: atRA, 4.0 minutes, 9-cis RA, 3.8 minutes, and 13-cis RA, 3.3 minutes.⁴³ ROL, RE and RAL were quantified by HPLC-UV.^{42, 49} ROL and RE measured simultaneously had retention times of 4.1 minutes and 15.9 minutes respectively, and 7.2 minutes for the internal standard, retinyl acetate. Lung homogenate retinoids were normalized per gram of tissue. Oxime-reacted RAL, producing anti-RAL and syn-RAL isomers had retention times of 5.9 and 9.9 minutes respectively at 368 nm. Peak areas are added together for total RAL-oxime quantification. ROL can also be detected by this method at 325 nm with the retention time of 6.2 minutes.⁴²

4.2.3 CrbpI and CrbpIII recombinant protein preparation

Mouse CrbpI and CrbpIII were expressed in BL21 *E. coli* purchased from Sigma-Aldrich (St. Louis, MO, USA) with plasmids purchased from Genecopia (Rockville, MD, USA), according to manufacturer's instructions. Purification was performed using a GE Healthcare GST bulk kit (GE Healthcare, Pittsburgh, PA, USA). The GST tag was cleaved with Promega ProTEV protease (Promega, Madison, WI, USA), after which the protease was removed with GE Healthcare Ni resin. The GST tag was separated from the purified protein by running the protein solution through the GST column a second time. Purified protein was dialyzed and stored in 20 mM KH₂PO₄ and 100 mM KCl, pH 7.4, at -80°C. CrbpI and CrbpIII concentrations were determined from absorbance at 280 nm using published ϵ values: apo-CrbpI, ϵ 28,080 M⁻¹ cm⁻¹; apo-CrbpIII, ϵ 25,800 M⁻¹ cm⁻¹.^{42, 50}

Holo-CrbpI/holo-CrbpIII have a molar absorptivity of $\epsilon_{50,200}^{M^{-1} cm^{-1}}$ at 350 nm. The A340/A280 ratios, used to assess purity, were between 1.4 and 1.6. Stock and assay solutions of CrbpI and CrbpIII were prepared in 20 mM KH_2PO_4 and 100 mM KCl (pH 7.4).

4.2.4 Subcellular fractionation

Whole lung was placed in 10% sucrose, 10 mM Tris HCl, 1 mM EDTA, and 1.5 mM DTT (pH 7.4) on ice upon collection. DTT was added on day of use. Lungs were pooled from approximately 20 mice per collection. Lung was homogenized in the same buffer on ice with a motorized homogenizer at 1260 rpm (Eberbach ConTorque no. 7265; Eberbach Corp., Ann Arbor, MI, USA). Microsomal and cytosolic fractions were isolated by centrifugation at 4°C: 1000 g for 10 min, 10,000 g for 15 min, 17,000 g for 15 min, and 100,000 g for 1 hour. The aliquots of the 100,000 g supernatant (cytosol) were prepared and flash frozen. The 100,000 g pellet (microsomes) was resuspended in homogenization buffer and flash frozen. Aliquots were stored at -80°C until use and were not re-frozen. Protein content was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Approximately 7.5 mg of microsomes in 1.5 mL resuspension (~5 mg/mL) and ~10-15 mL of cytosol (2-5 mg/mL) were recovered.

4.2.5 Enzyme activity

Enzyme activity for Rdh oxidation of Crbp-bound retinol was assayed with a combination of lung microsomes (200 μ g protein) and cytosol (10 μ g protein). Rrat activity to convert Crbp-bound ROL to RE was measured concurrently during the Rdh oxidation assay. Raldh activity to oxidize Crbp-bound retinal was assayed with mammary cytosol (10 μ g protein). Rrd activity to reduce Crbp-bound retinal to retinol was assayed

with lung microsomes (200 µg protein). All enzyme activity was assayed at 37°C under initial velocity conditions in the linear range in 50 mM HEPES, 150 mM KCl, 1 mM EDTA, and 2 mM DTT (pH 8.0) in the presence of 4 mM NAD⁺ and 2 mM NADP⁺ with a total reaction volume of 250 µl and 65-rpm shaking. DTT was added the day of use. Rdh/Lrat and Rrd activity assays also included a NADPH regenerating system added in 30 µl as a 1:1:1 solution of glucose-6-phosphate dehydrogenase (2.5 U in 5 mM sodium citrate, pH 7.5), 50 mM glucose-6-phosphate, and 50 mM NADP⁺ in assay buffer. The activity of the glucose-6-phosphate dehydrogenase was confirmed spectrophotometrically before assay by monitoring production of NADPH. In all assays, microsomal and/or cytosolic protein was reacted with 1.0 µM CrbpI-bound retinoid (retinol or retinal) or 1.0 µM CrbpIII-bound retinoid (retinol or retinal) for 60 min. Reactions were initiated by adding 10 µl Crbp-bound retinoid in buffer via glass syringe. Control reactions were performed with Crbp-bound retinol in the absence of subcellular fractions (microsomes/cytosol that contain enzymes). Each reaction condition was performed in triplicate, and each experiment was performed twice.

4.2.6 Apo-Crbp inhibition assays

Apo-inhibition assays of Raldh enzyme activity combined the cytosolic fraction only (10 µg) with 1 µM holo(RAL)-Crbp and quantified atRA production. Apo-Crbp points were 0, 0.02, 0.04, 0.1, 0.2, 0.5, 1.0, 2.0 and 4.0 µM that correspond to apo:holo ratio of 0, 1:50, 1:25, 1:10, 1:5, 1:2, 1:1, 2:1 and 4:1. Apo-inhibition assays of Rdh enzyme activity combined the microsomal fraction only (200µg) with 1 µM holo(ROL)-CrbpI and quantified RAL production. Apo-Crbp points were 0, 0.04, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0, 10.0 and 20.0 µM that correspond to apo:holo ratio of 0, 1:25, 1:10, 1:5, 1:2, 1:1, 2:1, 4:1,

10:1 and 20:1. Reactions were set up as specified in enzyme activity assays, except for Rdh cofactor conditions, which had 2 mM NADP⁺ only.

4.2.7 Gene expression

Total RNA was isolated with a Purelink miniRNA plus kit (Invitrogen; Life Technologies, Grand Island, NY, USA). RNA was quantified by a NanoDrop 2000 spectrophotometer (Thermo, Wilmington, DE, USA). RNA was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems; Life Technologies). Quantitative polymerase chain reaction (qPCR) was done with pre-designed and optimized primers (Applied Biosystems). mRNA gene expression was measured with a Step One Plus real-time PCR (Applied Biosystems). mRNA gene expression was calculated by the comparative Ct method with 18s as the reference gene.

4.2.8 NAD⁺/NADH Determination

Lung from female, 2-mo-old C57BL/6 or *Rbp1*^{-/-} mice were used for analysis. Tissue was processed and the NAD⁺/NADH ratio was determined using an EnzyChrom NAD⁺/NADH assay kit (BioAssay Systems, Haywood, CA, USA) according to manufacturer's instructions. Changes in absorbance were measured at 565 nm using a Bio-Tek Eon plate reader (Bio-Tek, Winooski, VT, USA). NAD⁺ and NADH concentrations were quantified using an NAD⁺ standard curve, per kit recommendations, normalized per milligram of tissue, and the NAD⁺/NADH ratio was calculated from those values.

4.2.9 Histology

Lung from 4 month old C57BL/6 or *Rbp1*^{-/-} mice was preserved in formalin on collection until tissue was embedded in paraffin, sliced, and stained by the University of Maryland CVID Core Facility for Histology. Hematoxylin and eosin and Sirius red images were captured using an EVOS XL digital microscope (AMG, Bothell, WA, USA).

4.3 Results

4.3.1 *atRA* is deficient in the absence of *CrpbI* expression

Crpb status was first determined through relative gene expression. *Rbp1*, *Rbp2* and *Rbp7* genes code for murine *CrpbI*, *CrpbII* and *CrpbIII* respectively. Relative expression showed that compared to C57BL/6J wild type (WT) lung, *Rbp1* was not detectable, *Rbp2* was significantly reduced and *Rbp7* was significantly elevated (**Figure 4.1A**). *Rbp2* and *Rbp7* are both 50 fold less abundant than *Rbp1* in WT lung, and in *Rbp1*^{-/-} lung, *Rbp7* is 2 fold more abundant than *Rbp2* (Data not shown). In the absence of *CrpbI*, *CrpbIII* is the predominant vitamin A chaperone. Retinoid levels were measured by Fast LC-MRM³ (MRM³) for RA quantification and UPLC-UV detection for ROL and RE. *atRA* was determined to be significantly reduced by 45% and ROL and RE were significantly elevated by 52% and 49% respectively in *Rbp1*^{-/-} compared to WT lung (**Figure 4.1B-D**). **Figure 4.1E** depicts a simplified schematic of the RA biosynthesis pathway and interactions between *Crpb* and retinoid ligands.

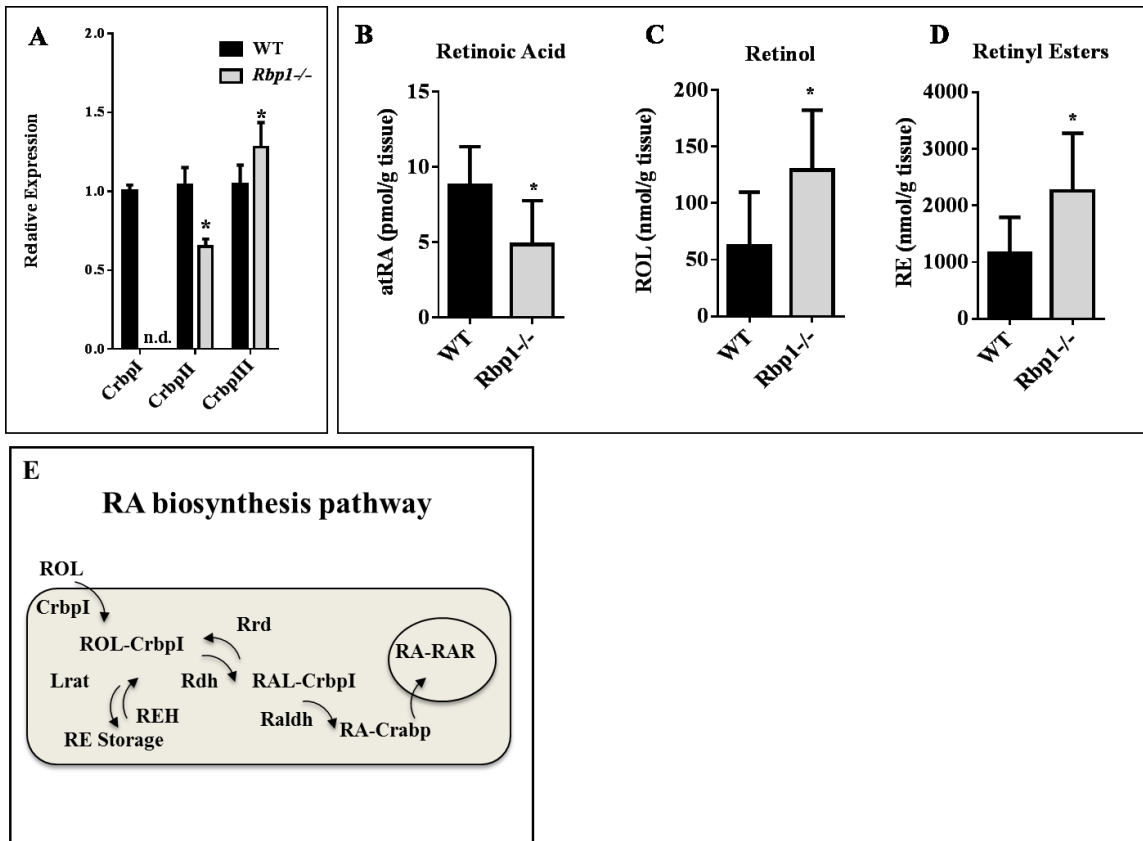


Figure 4.1: *atRA* is deficient in the absence of *CrbpI* expression. **A**, *CrbpI* relative expression is not detectable (n.d.), while *CrbpII* is significantly reduced and *CrbpIII* is significantly increased in the *Rbp1*^{-/-} mouse lung. n=8 for both genotypes, p<0.0072 **B**, *atRA* is reduced 45 % in *Rbp1*^{-/-} mouse lung, while *ROL* **C**, and *RE* **D**, are increased by 52 and 49% respectively, n=24 ± SEM and p<0.0001. **E**, RA biosynthesis pathway.

4.3.2 Retinol dehydrogenase (Rdh) activity is altered in Rbp1^{-/-} lung

Enzyme activity of the atRA biosynthesis enzymes were measured to determine if the absence of CrbpI would have an impact on activity because the ratio of apo:holo CrbpI has been shown to impact regulation of liver enzyme activities.^{3,51-53} **Figure 4.2** depicts the workflow of isolating atRA biosynthesis enzymes from mouse lung to measure their activity in an *in vitro* assay.

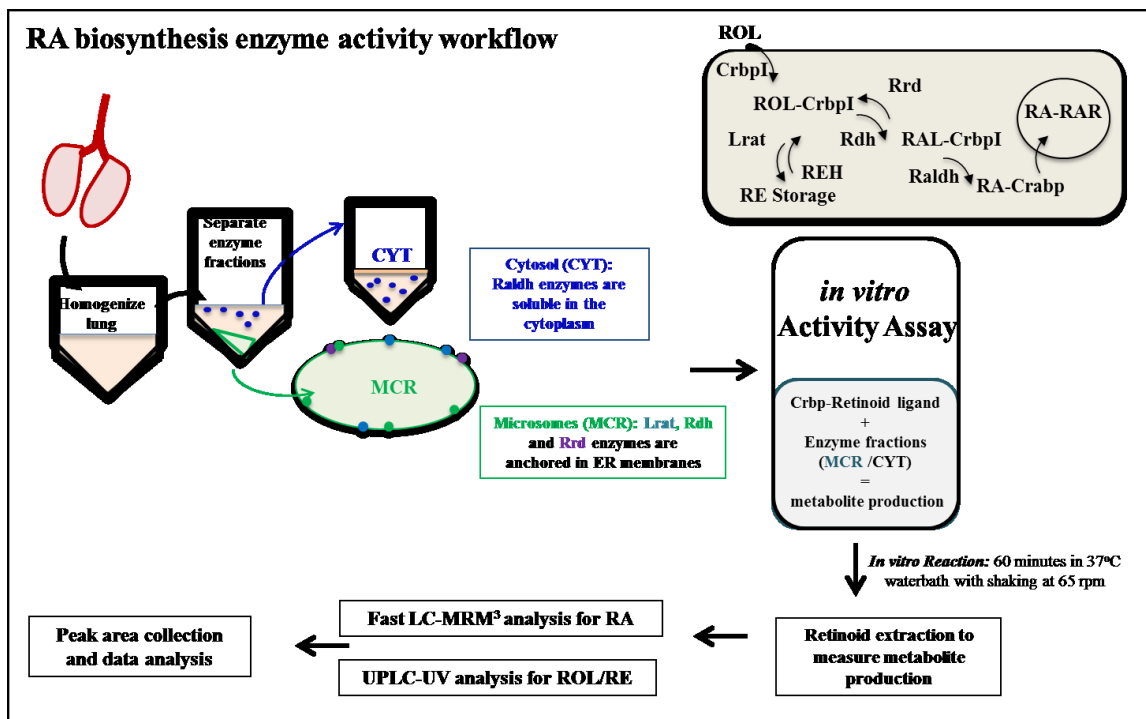


Figure 4.2: RA biosynthesis enzyme activity workflow. Subcellular fractions that contain RA biosynthesis enzymes are isolated from lung tissue and activity is measured *in vitro*. Lung tissue is collected, pooled and homogenized. Homogenate is then centrifuged to separate organelle fractions. Membranes from endoplasmic reticulum (ER), in the microsome (MCR) fraction, and anchored in the ER membrane are Lrat, Reh, Rdh and Rrd enzymes. Cytosol (CYT) contains the soluble Raldh enzymes. The activity of the enzymes is measured *in vitro* by adding Crbp-retinoid substrate and cofactor to react for 60 minutes at 37°C. Metabolite product was then measured by fast LC-MRM³ for RA or HPLC-UV for ROL and RE. Peak areas were collected and data was analyzed.

Figure 4.3A depicts the Rdh enzyme activity. Rdh enzymes are isolated in the microsomal fraction (**Figure 4.2**) and oxidation of ROL to RAL by Rdh enzymes is the rate-limiting step in atRA production.^{3, 51, 54} Raldh enzymes are isolated in the cytosolic fraction (**Figure 4.2**) and conversion of RAL to atRA by the Raldh enzymes is 200 fold more efficient than RAL production by Rdh enzymes.^{3, 51, 54} CrbpI-ROL and CrbpIII-ROL substrate were reacted with WT and *Rbp1*^{-/-} lung enzyme fraction, which a combination of microsomes and cytosol were used to enable quantification of atRA production, which has much greater sensitivity than RAL detection.⁴² Significantly more atRA was produced by *Rbp1*^{-/-} than WT Rdh enzymes. For WT and *Rbp1*^{-/-} Rdh enzymes, CrbpIII-ROL substrate yields significantly more atRA production. The *Rbp1*^{-/-} lung condition (*Rbp1*^{-/-} enzyme fractions with CrbpIII substrate) also showed significantly increased atRA production compared to the WT lung condition (WT enzyme fractions with CrbpI substrate). RE was measured simultaneously to measure Lrat esterification activity as well. *Rbp1*^{-/-} Lrat enzymes produced significantly more RE than WT enzymes (**Supplemental Figure 4.1A-B**). Because atRA quantification was contradictory to the tissue atRA measurements (**Figure 4.1B**), we measured relative gene expression for the Rdh enzymes *Dhrs9*, *Rdh10* and *Rdh12* (**Figure 4.3C**). Relative expression showed significantly increased *Dhrs9*, while *Rdh10* was reduced and *Rdh12* was unchanged. *Rdh10* is more abundant than *Dhrs9*, but not *Rdh12*. (Data not shown) As the ratio of apo:holo-CrbpI has been previously investigated in liver enzyme activity, but not lung enzyme activity, we measured the impact of increasing apo-Crbp concentrations with 1 uM holo(ROL)-Crbp (**Figure 4.3D-E**). The control condition to mimic WT lung measured apo-CrbpI with holo-CrbpI using WT enzyme fractions (black

filled circle). A CrbpI control with *Rbp1*^{-/-} enzyme fractions measured apo-CrbpI with holo-CrbpI (black open circle). The *Rbp1*^{-/-} lung condition measured apo-CrbpIII with holo-CrbpIII using *Rbp1*^{-/-} enzyme fractions (red filled square). CrbpIII was evaluated by combining apo-CrbpI with holo-CrbpIII (blue triangle) or apo-III with holo-CrbpI (green open square) using *Rbp1*^{-/-} enzymes to determine if the holo-Crbp identity has an influence on inhibition or if activity regulation is apo-Crbp specific. Relative activity of Rdh inhibition (**Figure 4.3E**) shows that apo-CrbpI but not apo-CrbpIII inhibits RAL production from ROL, independent of lung microsome genotype or holo-Crbp identity. In addition to Rdh activity inhibition, induced Reh activity was also observed with increasing concentrations of apo-CrbpI but not apo-CrbpIII (**Figure 4.3D**). This is consistent with previous studies showing apo-CrbpI induces Reh activity.³ ROL production increased 5-10 fold from the condition with no apo-CrbpI present to 20 uM apo-CrbpI with $p < 0.0001$. (Data not shown) The corresponding absolute value, rate curves (pmol/min/g) with error bars are provided in **Supplemental Figure 4.1C-D**.

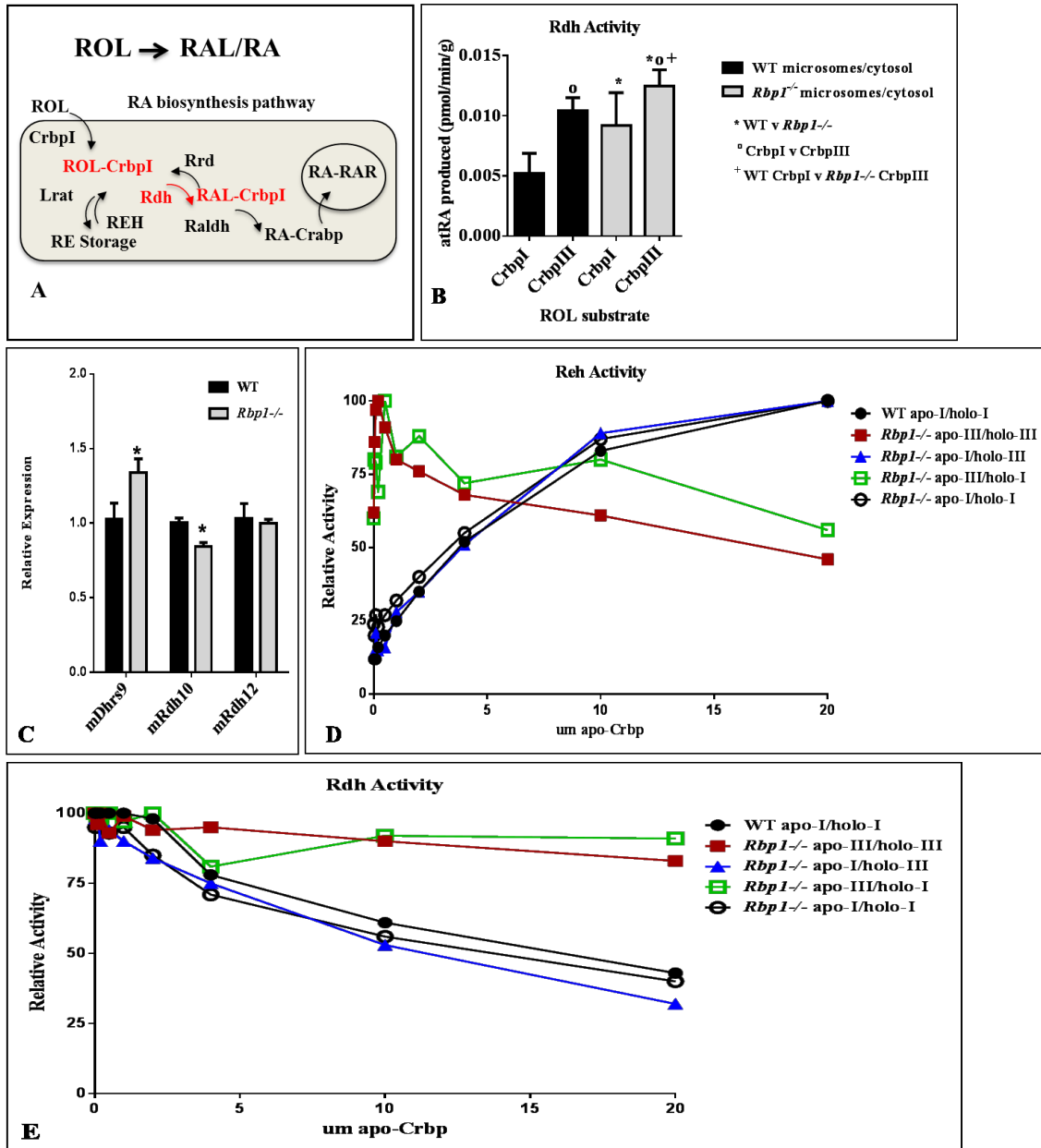


Figure 4.3: *Rdh* activity is altered in *Rbp1*^{-/-} lung. **A**, Scheme of *Rdh* oxidation of ROL to RAL. **B**, atRA production is increased with *Rbp1*^{-/-} lung enzyme fractions due to increased *Rdh* activity as well as with CrbpIII as substrate protein for WT and *Rbp1*^{-/-} enzyme fractions. The *Rdh* activity in the *Rbp1*^{-/-} lung condition is also significantly increased compared to the WT control condition. $n=6 \pm \text{SEM}$ and $p < 0.0257$. **C**, Relative gene expression of *Rdh* enzymes show elevated *Dhrs9* and reduced *Rdh10*. $n=8 \pm \text{SEM}$ for each genotype and relative expression was determined by the $\Delta\Delta\text{CT}$ method, $p < 0.0423$. **D**, Relative activity of induced *Reh* shows *Reh* enzymes producing ROL from RE storage with induction by apo-CrbpI but not CrbpIII, regardless of mouse genotype or holo-Crbp identity. $n=3$ for each condition. $K_m = 1.3 \mu\text{M}$ (WT-apoI/holoI), $1.7 \mu\text{M}$ (*Rbp1*^{-/-} apoI/holoIII) and $1.3 \mu\text{M}$ (*Rbp1*^{-/-} apoI/holoI) **E**, Relative activity of *Rdh* enzyme inhibition. RAL production is inhibited with increasing apo-CrbpI but not apo-CrbpIII regardless of mouse genotype or holo-Crbp identity. $n=3$ for each condition. $K_i = 3.2 \mu\text{M}$ (WT-apoI/holoI), $3.0 \mu\text{M}$ (*Rbp1*^{-/-} apoI/holoIII) and $2.7 \mu\text{M}$ (*Rbp1*^{-/-} apoI/holoI).

4.3.3 Retinal reductase (Rrd) activity is not altered in *Rbp1*^{-/-} lung

We next measured retinal reductase (Rrd) enzyme activity, which reduces RAL to ROL, to determine if the reductase activity is altered and contributing to the reduced atRA biosynthesis (**Figure 4.4A**). 1 uM CrbpI-RAL or 1 uM CrbpIII-RAL substrate was reacted with WT and *Rbp1*^{-/-} lung enzyme fractions to quantify ROL production (**Figure 4.4B**). *Rbp1*^{-/-} Rrd activity showed a significant increase in ROL production compared to WT Rrd activity, as indicated by (*). However, WT and *Rbp1*^{-/-} Rrd activity has significantly reduced ROL production with CrbpIII-RAL substrate compared to CrbpI-RAL substrate, indicated by (o). Comparing the *in vivo* WT and *Rbp1*^{-/-} condition, there is no net change in activity. Dhhrs3 is a retinal reductase enzyme and *Dhhrs3* relative gene expression is significantly elevated in *Rbp1*^{-/-} lung (**Figure 4.4C**). *Dhhrs3* is more abundant than *Rdh10* (13 and 19 fold), *Dhhrs9* (180 and 170 fold) and *Rdh12* (191 and 239 fold) for WT and *Rbp1*^{-/-} lung respectively. *Rbp1*^{-/-} Rrd activity is significantly increased, likely due to the increase in *Dhhrs3* expression. Despite this, net Rrd activity is not altered and likely does not have an impact on reduced atRA biosynthesis. We also measured the NAD⁺/NADH ratio, an indication of the oxidative environment, in WT and *Rbp1*^{-/-} lung tissue to determine if the oxidative environment is impacting the oxidation/reduction flux of ROL/RAL (**Figure 4.4D**). There is not a change in the NAD⁺/NADH ratio, indicating no overall significant change in the oxidative environment.

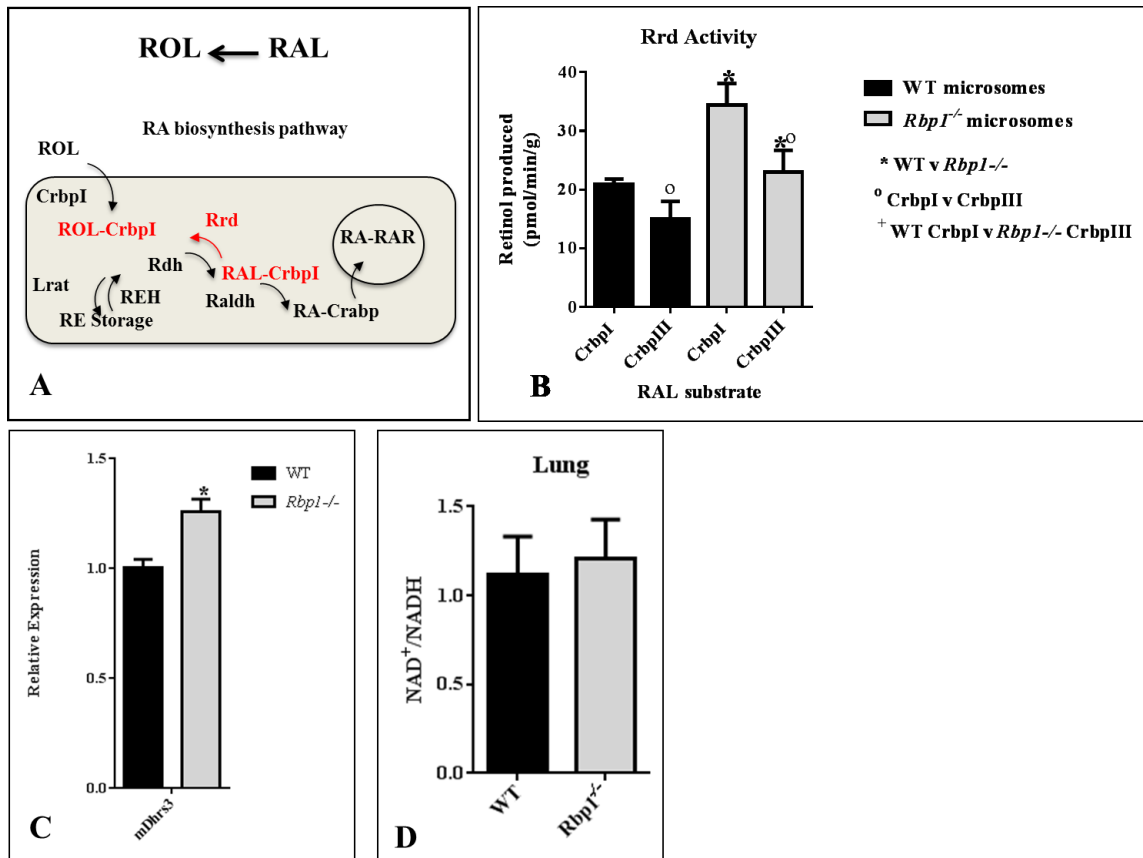


Figure 4.4: *Rrd* activity is not altered in *Rbp1*^{-/-} lung. **A**, *Rrd* metabolism of RAL to ROL. **B**, ROL production by *Rrd* activity is increased in *Rbp1*^{-/-} lung enzyme fractions as compared to WT, but is decreased with CrbpIII as substrate protein for both WT and *Rbp1*^{-/-} *Rrd* enzyme fractions. There is therefore no significant change between *Rrd* activity in the *Rbp1*^{-/-} lung condition compared to the WT control condition. $n=6 \pm \text{SEM}$, $p<0.0022$. **C**, Relative gene expression of *Rrd* enzymes show elevated *Dhrs3*. $n=8 \pm \text{SEM}$ for each genotype and relative expression was determined by the $\Delta\Delta C_T$ method, $p=0.0027$. **D**, Lung tissue measurements of the NAD^+/NADH ratio showed no significant change, $n=6 \pm \text{SEM}$.

4.3.4 Retinal dehydrogenase (*Raldh*) activity is altered in *Rbp1*^{-/-} lung

Because the Rdh enzymes have increased activity and the Rrd enzyme activity is unchanged in the *Rbp1*^{-/-} lung, Raldh activity was assessed to identify a potential mechanism for reduced endogenous atRA. To measure Raldh activity, CrbpI-RAL and CrbpIII-RAL substrate was reacted with WT and *Rbp1*^{-/-} lung enzyme fractions to quantify atRA production (**Figure 4.5A**). With CrbpI-RAL as substrate, *Rbp1*^{-/-} Raldh enzymes had reduced activity compared to WT Raldh enzymes, but not with CrbpIII-RAL substrate, as indicated by (*) (**Figure 4.5B**). There was no significant difference in atRA production, however, between CrbpI-RAL and CrbpIII-RAL substrate for WT and *Rbp1*^{-/-} Raldh enzyme fractions. Comparison of WT and *Rbp1*^{-/-} lung *in vivo* conditions show significantly reduced atRA production in the *Rbp1*^{-/-} condition, as indicated by (+). Relative gene expression (**Figure 4.3C**) shows that *Raldh1* is unchanged, *Raldh2* is significantly increased and *Raldh3* is significantly decreased. *Raldh1* is the most abundant, followed by *Raldh2*, 52 and 31 fold less than *Raldh1* for WT and *Rbp1*^{-/-}, and then *Raldh3*, 22 and 47 fold less than *Raldh2* for WT and *Rbp1*^{-/-} (Data not shown). Because Raldh inhibition by apo-CrbpI was also previously reported in liver cytosol, we evaluated lung cytosol for inhibitory effects of increasing apo-Crbp concentrations with 1 uM holo(RAL)-Crbp. (**Figure 4.5D**) The control condition to mimic WT lung measured apo-CrbpI with holo-CrbpI using WT enzymes (black filled circle) and the *Rbp1*^{-/-} lung condition measured apo-CrbpIII with holo-CrbpIII using *Rbp1*^{-/-} enzymes (red filled square). We evaluated CrbpIII by combining apo-CrbpI with holo-CrbpIII (blue triangle) and apo-III with holo-CrbpI (green open square) using *Rbp1*^{-/-} enzymes to determine if the holo-Crbp identity has an influence on inhibition or if activity regulation is apo-Crbp

specific. Relative activity of Raldh enzyme inhibition showed that atRA production is inhibited with increasing apo-CrbpI but not apo-CrbpIII regardless of enzyme fraction genotype or holo-Crbp identity. The corresponding absolute value, rate curves (pmol/min/g) with error bars are provided in **Supplemental Figure 4.1E**.

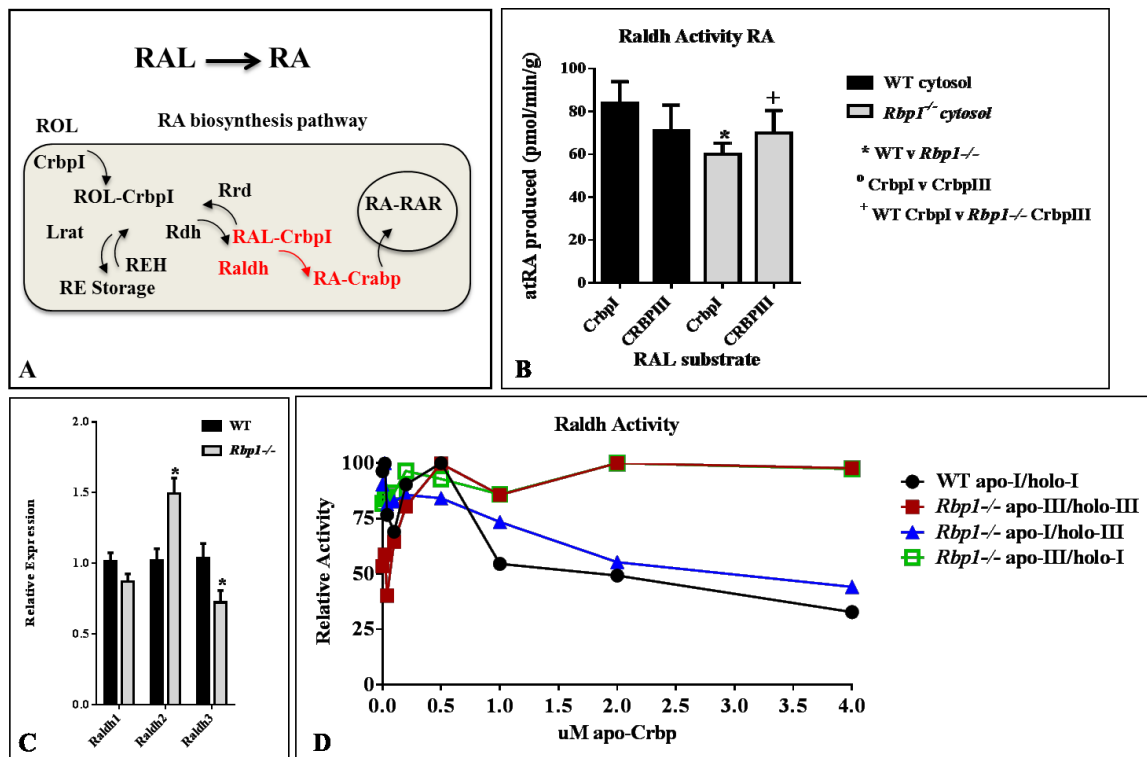


Figure 4.5: Raldh activity is altered in *Rbp1*^{-/-} lung. **A**, Raldh metabolism of RAL is measured by adding Crbp-RAL substrate to the CYT fraction to quantify atRA production. **B**, atRA production by Raldh is decreased in *Rbp1*^{-/-} lung with CrbpI as substrate protein. The Raldh activity in the *Rbp1*^{-/-} lung condition is also significantly decreased compared to the WT control condition. $n=6 \pm \text{SEM}$, $p<0.0429$ **C**, Relative gene expression of Raldh enzymes show significantly elevated *Raldh2* and reduced *Raldh3*. $n=8 \pm \text{SEM}$ for each genotype and relative expression was determined by the $\Delta\Delta C_T$ method, $p<0.0328$. **D**, Relative activity of Raldh enzyme inhibition. atRA production is inhibited with increasing apo-CrbpI but not apo-CrbpIII regardless of mouse genotype or holo-Crbp identity. $n=6$ for each condition. $K_i = 0.45 \text{ uM}$ (WT-apoI/II/III) and 0.99 uM (*Rbp1*^{-/-} apoI/II/III).

4.3.5 *Rbp1*^{-/-} lung microenvironment is characteristic of VAD metaplasia and hyperplasia

As altered vitamin A metabolism and atRA biosynthesis were characterized in *Rbp1*^{-/-} lung as compared to WT lung, the impact on the lung microenvironment was evaluated by histology. VAD causes morphological changes including metaplasia and hyperplasia.⁵⁵⁻⁵⁸ Histological evaluation of WT and *Rbp1*^{-/-} lung was performed for tissue slices stained with hematoxylin and eosin (HE) for morphology and sirius red (SR) for collagen and extracellular matrix (ECM). Images taken at 40x (**Figure 4.6A-B, E-F**) show representative bronchioles for WT (**Figure 4.6A,E**) and *Rbp1*^{-/-} (**Figure 4.6B,F**). The overall structure and organization of the *Rbp1*^{-/-} bronchiole is lost with no definitive basement membrane or cell organization as indicated by the large arrowheads. More specifically, in the 100x images (**Figure 4.6C-D, G-H**) the detail of the cell organization of the WT (**Figure 4.6C, G**) is clearly lost in the *Rbp1*^{-/-} bronchiole (**Figure 4.6D, H**). Basal and goblet cell structures and organization have undergone metaplasia to become undefined squamous like cells, a loss of ciliated epithelium and thickening of some areas of the basement membrane in the *Rbp1*^{-/-} bronchiole are indicated with small arrows. Hyperplasia is observed with accumulation of the epithelial cells, elongation of fibroblasts in the basement membrane and accumulation of collagen/ECM as indicated with the diamondhead arrows. To verify the ECM changes related to atRA loss we measured gene expression of two matrixmetalloproteinase (MMP) enzymes, which are directly modulated by atRA activity. *Mmp2* and *Mmp9* relative expression were both significantly increased (**Figure 4.6I**) with *Mmp2* about 14 fold more abundant than *Mmp9* for WT and *Rbp1*^{-/-} lung. (Data not shown)

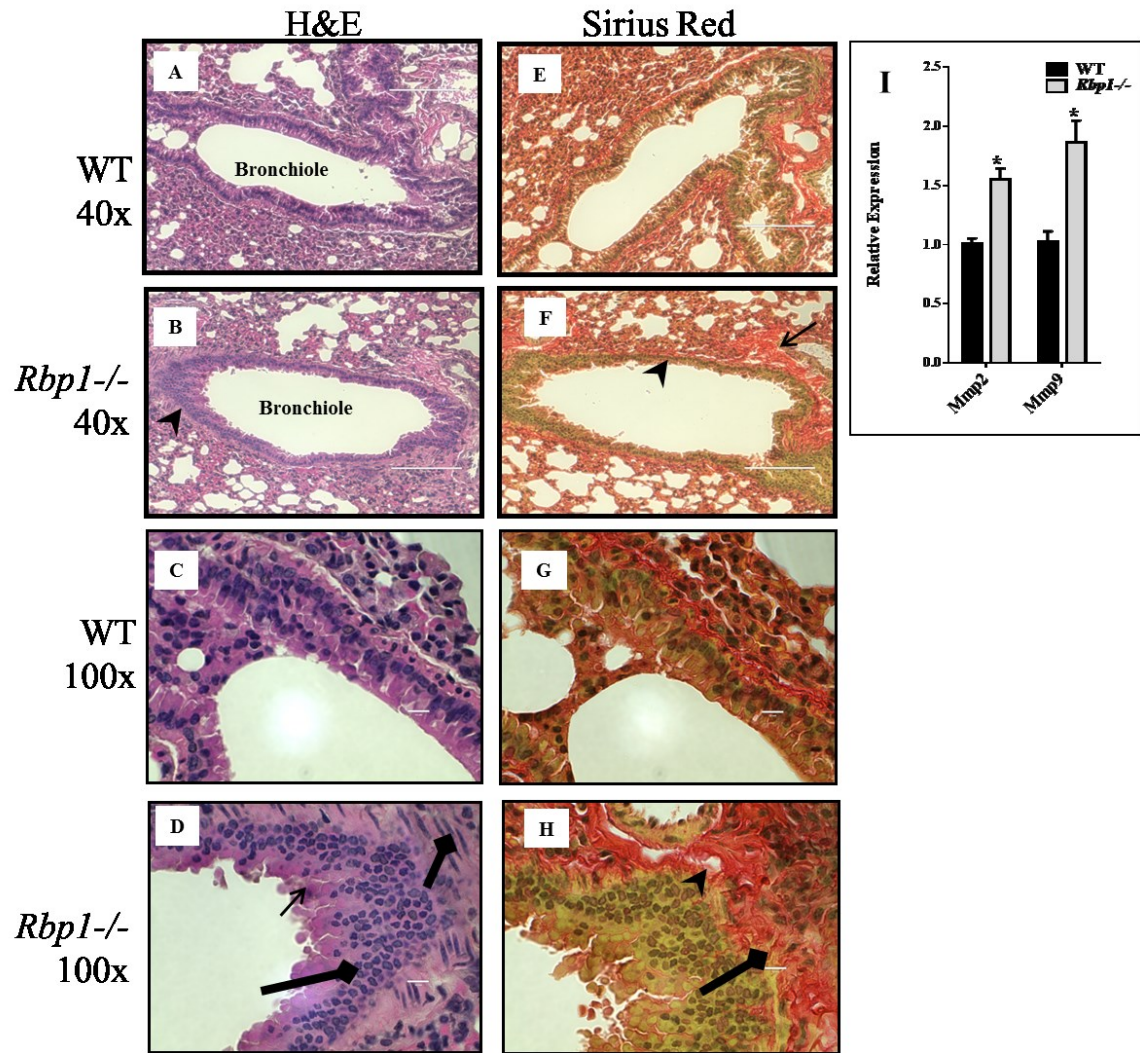


Figure 4.6: *Rbp1*^{-/-} lung is characteristic of VAD lung metaplasia and hyperplasia. Tissues were formalin fixed at collection and were stained by hematoxylin and eosin (H&E) for morphology (A-D) and sirius red (SR) for extracellular matrix (E-H) visualization. Images were taken at 40x (A-B, E-F) and 100x (C-D, G-H). WT tissues (A, C, E, G) show normal bronchiole morphology. *Rbp1*^{-/-} tissues (B, D, F, H) showed 1) a loss of basal and goblet cell structure and organization characteristic of VAD metaplasia (small arrow); 2) accumulation of epithelial cells, accumulation of ECM and elongation of fibroblasts characteristic of early hyperplasia (diamond arrow); and 3) a loss of bronchiole structure and organization including thickening of the basement membrane/bronchiole wall characteristic of VAD morphology (large arrowhead). I, Relative gene expression shows elevated ECM matrix metalloproteinases, *Mmp2* and *Mmp9*. n=8 ± SEM for each genotype and relative expression was determined by the $\Delta\Delta C_T$ method, p<0.001.

4.4 Discussion

4.4.1 *Rbp1*^{-/-} lung has altered vitamin A metabolism, atRA biosynthesis and homeostasis in the absence of *CrbpI*

Altered vitamin A metabolism and atRA biosynthesis observed in the *Rbp1*^{-/-} lung is described in **Figure 4.7**. Because *CrbpI* loss and VAD have been associated with several human lung diseases, we used an *Rbp1*^{-/-} mouse model to probe the atRA biosynthesis pathway in the lung.^{2, 22, 40, 55, 58-59} In the absence of *CrbpI*, atRA is reduced 45%, with *CrbpIII* as the predominant ROL chaperone (**Figure 4.1**). ROL uptake, which is increased in the *Rbp1*^{-/-} lung (**Figure 4.1C**), may be due to a two-fold increase in *Stra6* membrane uptake receptor gene expression. (**Supplemental Figure 4.2A**). With predominantly holo-*CrbpIII* present in *Rbp1*^{-/-} lung, RE quantification was increased (**Figure 4.1D**) and Lrat esterification of ROL for RE storage formation was confirmed to be significantly elevated (**Supplemental Figure 4.1B**). Since apo-*CrbpIII* is unable to induce Reh activity, mobilization of ROL from RE stores is impaired (**Figure 4.3D**) With ROL metabolism being directed towards RE storage formation without regulation for RE hydrolysis flux for ROL production, ROL delivery to *Rdh* enzymes for atRA biosynthesis may be contributing to atRA tissue deficiency. Future studies to determine *CrbpIII* binding affinities and physical interactions, or lack thereof, for ROL metabolizing enzymes would be beneficial to better understand the functional role of *CrbpIII* in vitamin A metabolism, and atRA biosynthesis.

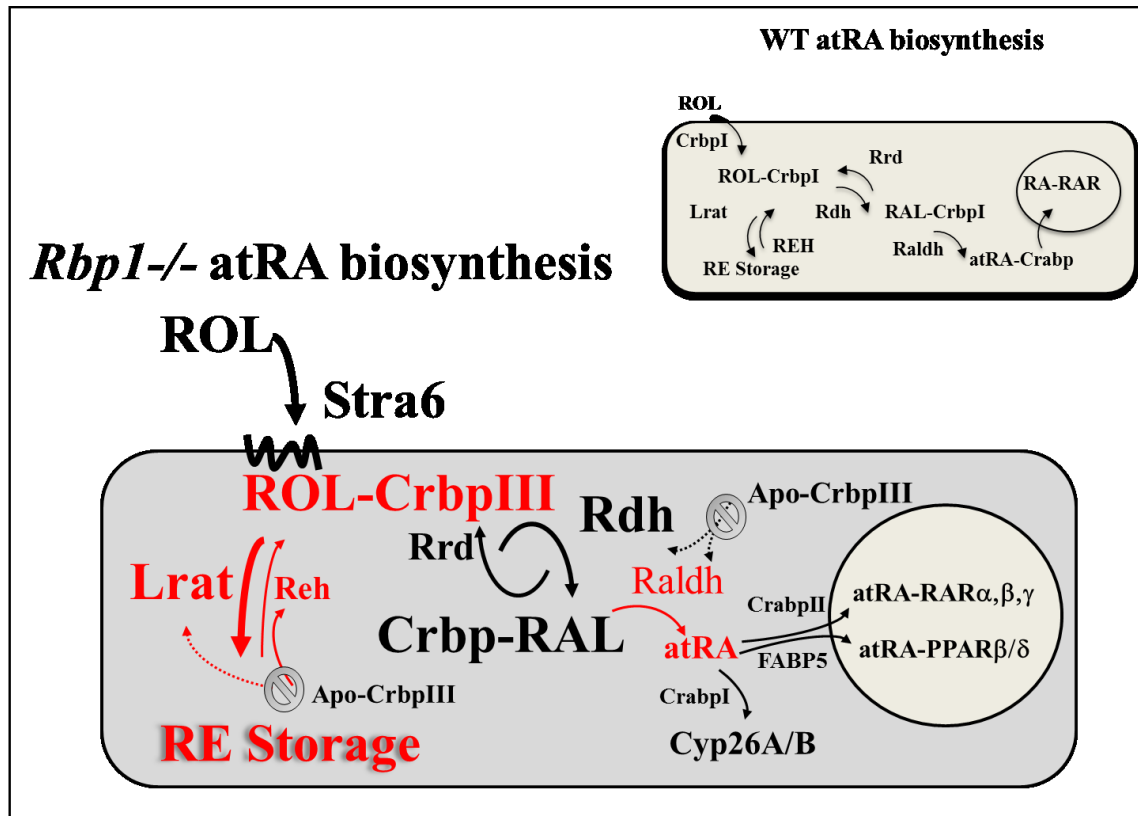


Figure 4.7: Altered retinoid homeostasis in *Rbp1*^{-/-} lung. Stra6, the ROL membrane uptake receptor, expression is elevated. Lrat, the metabolizing enzyme for RE storage formation, has increased activity, while Reh is less efficient at hydrolyzing RE to ROL in the absence of CrbpI. ROL and RE levels are increased, reflecting the increase in Stra6 expression and Lrat activity in the absence of CrbpI. Rdh enzyme expression and activity is increased, while Raldh activity is reduced. Apo-CrbpIII does not inhibit Rdh or Raldh activity as apo-CrbpI does. Cyp26A and Cyp26B expression is elevated about 4 fold. Endogenous atRA is reduced by 45%. RAR α , β and γ receptor are unchanged.

We used *in vitro* activity assays to evaluate the individual atRA biosynthesis reaction steps in an effort to determine the mechanism(s) by which atRA is reduced with CrbpI loss in *Rbp1*^{-/-} lung (**Figure 4.2**). Enzyme activity of the first (rate-limiting) step of ROL oxidation to RAL by Rdh activity revealed that the *Rbp1*^{-/-} activity condition is more active than the WT activity condition (**Figure 4.3B**). This reveals that Rdh function is not the source of reduced atRA in the *Rbp1*^{-/-} lung as Rdh function was previously observed to be reduced in the *Rbp1*^{-/-} mammary.⁴⁷ Because there is a tissue specific activity profile, Rdh enzyme gene expression was evaluated and revealed that *Dhrs9* gene expression is significantly elevated in the *Rbp1*^{-/-} lung, but was not significantly elevated in the *Rbp1*^{-/-} mammary.⁴⁷ The increase in *Dhrs9* expression is contributing to the increase in Rdh activity in the *Rbp1*^{-/-} lung specifically. As the apo:holo CrbpI ratio has been shown to have regulatory effects on atRA biosynthesis enzyme activities in other models, we compared regulatory effects of apo-CrbpI and apo-CrbpIII on lung Rdh activity.^{51,53} Apo-CrbpI inhibited Rdh activity regardless of the enzyme fraction genotype or holo-Crbp identity, while apo-CrbpIII did not inhibit Rdh activity in any condition (**Figure 4.3E**).

Rrd activity was measured to determine if RAL reduction to ROL is contributing to atRA deficiency. ROL production from holo(RAL)-CrbpIII was reduced compared to holo(RAL)-CrbpI with WT and *Rbp1*^{-/-} enzyme fractions, while *Rbp1*^{-/-} enzyme fractions had increased activity compared to WT enzyme fractions. The activity of the WT and *Rbp1*^{-/-} conditions however showed not net change, hence the decrease in endogenous atRA and increased in ROL levels are not due to an increase in RAL reduction to RAL. Relative gene expression of the Rrd enzyme, *Dhrs3*, showed that

Dhrs3 is elevated in *Rbp1*^{-/-} lung which accounts for the increase in enzyme activity seen in the *Rbp1*^{-/-} fractions compared to WT fractions (*), but holo-CrbpIII does not produce ROL as efficiently as holo-CrbpI to cancel the effects of increase *Dhrs3* expression for no net change in Rrd activity in the *in vivo* *Rbp1*^{-/-} condition.

The second biosynthesis step is an irreversible oxidation reaction of RAL oxidation to atRA by Raldh enzymes, which are ~200 fold more efficient than Rdh enzymes.³ *Rbp1*^{-/-} enzymes are 28% less active than WT enzymes with CrbpI as substrate. Raldh activity is reduced, consistent with less atRA production. As apo-CrbpI has inhibitory effects on Raldh enzymes from liver, we investigated the inhibitory effects of apo-CrbpI and apo-CrbpIII on Raldh enzymes isolated in lung cytosol.⁵¹ Similar to Rdh activity, apo-CrbpI, but not apo-CrbpIII, exhibited increasing inhibitory effects on Raldh activity as apo-CrbpI concentrations increase, regardless of mouse genotype or holo-Crbp identity.

As Cyp26 activity functions to catabolize atRA for excretion, we measured relative gene expression of *Cyp26A1*, *Cyp26B1* and *Cyp26C1* in WT and *Rbp1*^{-/-} lung to determine if Cyp26 may be contributing to endogenous atRA loss (**Supplemental Figure 4.2B**).⁶⁰⁻⁶¹ *Cyp26C1* was not detectable in WT or *Rbp1*^{-/-} lung. *Cyp26A1* and *Cyp26B1* expression was significantly elevated in *Rbp1*^{-/-} lung compared to WT lung with *Cyp26B1* in greater abundance. Increased gene expression of *Cyp26A1* and *Cyp26B1* may be contributing to reduced endogenous atRA levels in *Rbp1*^{-/-} due to an increase of atRA catabolism. To confirm the extent of atRA catabolism, Cyp26 activity would need to be evaluated.

Cyp26 enzymes are isolated in mitochondria and microsome fractions and additional atRA catabolism studies would be beneficial in future works to understand atRA homeostasis in the *Rbp1*^{-/-} lung.⁶⁰

Additional evaluation of RAR gene expression shows that RAR α , β and γ are not changed in the *Rbp1*^{-/-} lung and, as such, altered gene transcription effects in *Rbp1*^{-/-} lung are likely due to the lack of available atRA ligand as a result of CrbpI loss (**Supplemental Figure 4.2C**). Reduced atRA ligand availability is due to a combination of alteration in atRA homeostasis including flux of ROL towards storage ester formation, reduced Raldh activity and even possibly increased catabolism by Cyp26A and Cyp26B activity.

4.4.2 Dysfunctional atRA biosynthesis results in an altered microenvironment that resembles VAD metaplasia and hyperplasia

VAD lung morphological changes have been well described in developing and mature lung that result in functional complications associated with premalignant and preinvasive lung disease. These morphological changes include basal and goblet cell metaplasia, loss of ciliated epithelium and thickening of the basement membrane of the bronchioles.^{9, 21-25, 55-59} *Rbp1*^{-/-} lung morphology is consistent with the VAD bronchiole morphological defects described. CrbpI loss and atRA biosynthesis dysfunction have been identified as early changes in other nonmalignant conditions like endometriosis as well as premalignant conditions which may progress to malignancies.^{39-41, 48} In lung tissue, metaplasia and hyperplasia precedes fibrosis. Fibrosis is defined as excessive accumulation of ECM and remodeling of lung architecture and is characteristic of chronic lung disease, which if not treated, will cause irreversible damage, limiting lung function and in severe cases, death.² Fibrosis develops with altered regulation of inflammation, oxidative environment, and coagulation that causes fibroblast activation and proliferation, epithelial injury and epithelial-mesenchymal transition (EMT) and excessive ECM

accumulation.² Because *Crbp1* loss results in altered atRA and lung tissue homeostasis and the defects observed are early occurrences which precede irreversible lung damage, future works to modulate endogenous *Crbp1* and atRA levels may be a novel approach in therapy for lung disease.

4.5 Acknowledgements

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Chapter 5: Discussion

5.1 Retinoic acid quantification

Interest in vitamin A research has been going on since the early 1900's where additional nutrients other than proteins, fats, salts and starches, were identified as necessary for growth, development, immunity and survival.¹⁻⁶ Further work included identification of VAD symptoms which led to vitamin A to be crystallized in 1937 by Holmes and Corbet.^{1,7} As investigation into vitamin A metabolism progressed, analytical methods for quantification were developed.

RA quantification has been difficult due to low endogenous abundance.⁸ Indirect methods for RA include an *in vitro* reporter assay and transgenic RA reporter mouse strains.⁹⁻¹²

RAR receptor quantification has also been used as a RA indicator. In this case, RA levels were assumed to mimic the RAR levels.¹² HPLC-UV has been used for direct quantification, but sensitivity is often not sufficient for the low endogenous abundance of RA.¹³⁻¹⁴ Specificity for RA isomers is also desirable, as the isomers have different affinity for nuclear receptors and subsequently activate nuclear receptors for different biological functions.^{9, 15-18} *atRA* is the predominant isomer for RAR α,β,γ and PPAR β/δ activation, so a method sensitive enough for endogenous quantification with specificity to distinguish RA isomers is necessary to understand their role in biological activity.^{8-9, 19-21}

An LC-MS/MS method using a triple quadrupole mass spectrometer was developed, capable of a limit of detection to 10 fmol with specificity to separate RA isomers using selective reaction monitoring for mass identification.⁸ The selectivity and specificity were great improvements over previously established RA detection methods, both indirect and

direct. As retinoids are lipid soluble, interferences due to the presence of lipids, along with the complexity of biological matrices has still been problematic.

Most recently, fast LC multiplexing to MRM³ (multiple reaction monitoring cubed), which combines fast HPLC, for RA isomer separation, with MRM³ mass spectrometry detection of *m/z* transitions of ion fragments specific for RA.²² Previously, with the LC-MRM assay, only a single *m/z* transition (301.2 to 205.1) was used for detection; however, with MRM³, a second generation *m/z* transition (301.2 to 205.1 to 159.1) is used for detection.²² The addition of the second generation *m/z* transition has enhanced selectivity for RA, which has reduced interferences of complex biological matrices. Using these techniques, we have been able to characterize endogenous atRA levels in mouse tissues not previously reported, as well as, atRA status in several human conditions.²³⁻²⁴ These direct atRA quantification measurements have been a missing piece of information in the retinoid field and in the understanding of atRA activity on its targets. Direct atRA quantification in this body of work has enabled significant progress in understanding the role of CrbpI in vitamin A metabolism, atRA biosynthesis and retinoid pathway homeostasis.

5.2 *Rbp1*^{-/-} mouse model

Previously, the *Rbp1*^{-/-} mouse has been used to understand the role of CrbpI in retinoid homeostasis.²⁵ On a vitamin a sufficient diet, no apparent phenotypes were observed, however, when placed on a vitamin A deficient diet, retinoid stores were depleted faster than WT. Pups from VAD dams had slowed growth and were smaller and weaker than

the WT cohorts. VAD symptoms also became apparent including blindness, testicular degeneration and squamous keratinizing metaplasia.²⁵ The *Rbp1*^{-/-} mouse model has also shown that CrbpI modulates glucose homeostasis in mouse and *Rbp1*^{-/-} mice were resistant to diet induced obesity.²⁶ In both studies, CrbpI was determined to be playing a role in retinoid homeostasis and retinoid target effects.

This body of work used the *Rbp1*^{-/-} model to further investigate the role CrbpI has in vitamin A metabolism, atRA biosynthesis and retinoid homeostasis. As the *Rbp1*^{-/-} model is a global knock-out, we analyzed multiple tissues to determine if CrbpI regulation and effects of loss were consistent amongst the tissues or if there were tissue specific effects. Ultimately, these data will provide future insight on the function of CrbpI.

atRA deficiency was consistent amongst the extrahepatic tissues measured in the *Rbp1*^{-/-} mouse and to similar extents.²³⁻²⁴ Hepatic atRA was unchanged; however, ROL and RE were significantly reduced in the liver, while significantly elevated in the serum and extrahepatic tissues.²³ This is consistent with liver storage is being depleted in order to provide extrahepatic tissues vitamin A substrate to correct for the local atRA deficiency. These studies and others indicate that in the absence of CrbpI, sufficient ROL is being taken up into extrahepatic tissues; but there is a local atRA biosynthesis and retinoid homeostasis dysfunction contributing to reduced endogenous atRA abundance.

Our studies indicate that tissues that have the alternative CrbpI homolog, CrbpIII, are not capable of functional compensation by CrbpIII to replace CrbpI function.²³ CrbpI appears to have multiple functional roles, such as chaperoning ROL and RAL to specific

metabolizing enzymes, ligand protection from nonspecific oxidation, and regulation of metabolizing enzyme activity; and its loss results in local atRA deficiency.²⁷⁻³¹ As CrbpI is a chaperone for ROL and RAL for atRA biosynthesis, comparing CrbpIII as a chaperone, CrbpIII does not bind to ROL as strongly as CrbpI and is “promiscuous” in its ligand delivery. CrbpIII as a substrate has increased atRA production, presumably because ROL is being released in a less controlled manner and is more readily available to metabolizing enzymes. Despite this, atRA production is still significantly reduced.²³

Further investigation of apo-Crbp regulation was done to determine if apo-CrbpIII is able to regulate enzyme activity as apo-CrbpI has been reported to do.^{27-28, 30} Apo-CrbpI has inhibitory effects on Lrat, Rdh and Raldh enzymes and also induces Reh.²⁷ Apo-CrbpIII did not show any of these regulatory effects, so in the *Rbp1*^{-/-} mouse, where there is increased ROL uptake, there is also increased esterification to RE storage and decreased RE hydrolysis. Without CrbpI present, regulation of Lrat/Reh flux is gone. It is unclear with these studies if CrbpIII directs ROL towards esterification preferentially to atRA biosynthesis and remains an important question to follow up these studies. CrbpIII interactions with metabolizing enzymes have yet to be investigated and would provide additional insight as to the flux of ROL delivery to Lrat vs. Rdh enzymes.

RA biosynthesis enzymes' gene expression profiles between mammary and lung tissues varied in the *Rbp1*^{-/-} mouse which means that CrbpI loss and atRA deficiency precede gene expression changes. These enzyme activities are not only dependent on the substrate present, but also the cofactor availability.²⁷⁻³¹ Enzymes activities and oxidative environments differed between mammary and lung tissue, so the local environment may impact the severity of atRA biosynthesis dysfunction under conditions of stress.³²⁻³⁴

The tissue susceptibility in the *Rbp1*^{-/-} mouse is additionally supported by the common microenvironmental changes seen in the histology. For mammary, endometrium and lung hypercellularity and ECM accumulation are observed. These are early microenvironmental changes in proliferative diseases. Though *Rbp1*^{-/-} mouse tissue do not become malignant, additional stress in these tissues may be the trigger to potentiate more severe alterations.³⁵⁻³⁶

The *Rbp1*^{-/-} mouse model has made it possible to make valuable contributions to the retinoid field with direct tissue measurements and to probe the role of CrbpI in vitamin A metabolism, atRA biosynthesis and tissue homeostasis. Additionally, we have been able to comparatively characterize CrbpIII. CrbpIII is an iLBP and homolog of CrbpI, however, is not able to functionally compensate for CrbpI regulation in retinoid homeostasis and subsequently tissue homeostasis. Whether CrbpIII has primary functions other than vitamin A metabolism and atRA biosynthesis or has a specific spatiotemporal action is not yet resolved and additional characterization of CrbpIII remains to be done.

5.3 The role of vitamin A in health maintenance and the future of retinoids in therapeutics

Vitamin A was discovered nearly a century ago and since its discovery, vitamin A has been shown to be essential for life.¹⁻⁶ Early 20th century observations related growth retardation and illness with dietary deficiencies in the poor, which are now understood to be symptoms of VAD. Individuals with diets rich in vitamin A animal sources not only grew better but also were more resistant to sickness.^{1, 37-39} A poor diet commonly resulted

in night blindness and other eye issues which were typically treated with cod liver oil.^{1, 6, 40} Night blindness is a hallmark of VAD, and we now know that liver is the body's main storage for the lipid soluble vitamin A. Treatments and cures for VAD symptoms was simply to replenish vitamin A through diet, which may not have been completely understood at the time but the clinical effect was clear.¹ Currently, VAD is still a problem for populations afflicted with poverty and according to the World Health Organization (WHO), is the leading cause of blindness with 50% mortality within 12 months of loss of sight.

Dietary VAD is understood to predispose persons to illness and disease, but cellular deficiency occurs when vitamin A is not adequately metabolized to atRA for activation of nuclear receptors and subsequent gene regulation. Numerous vitamin A pathway proteins have been identified and characterized to understand how vitamin A is metabolized including, ROL membrane uptake receptors, ROL and RA chaperone proteins, retinoid specific metabolizing enzymes and RA target nuclear receptors.^{15-18, 27, 41} As retinoid research progressed, as discussed in detail in the introduction, *in vitro* and *in vivo* studies demonstrated several mechanisms of action of atRA, both genomic and nongenomic. RA treatment and restoration of silenced CrbpI led to clinically desirable effects in the *in vivo* models.^{35-36, 42- 45} Because of the promising outcomes of these studies for potential in clinical applications, vitamin A and RA has been pursued as a therapeutic for a number of diseases and is used successfully to treat acute promyelocytic leukemia in 80-95% of cases when dosed with arsenic trioxide.⁴⁶ Other cancers that use retinoid therapy are neuroblastoma, cutaneous T cell lymphoma and Kaposi's sarcoma. Retinoids have been FDA approved for topical therapeutic use in several skin conditions as well.⁴⁷ A number

of clinical trials for natural and synthetic RA and retinoids for use in solid tumor therapy have reported, but none so far have been successful enough for FDA approval. The consensus of many of these trials is that more needs to be understood about retinoid therapy and how the beneficial effects of inhibition of tumor cell proliferation and induction of apoptosis reported from *in vivo* studies, may be achieved without side effects associated with vitamin A toxicity. The successful use of atRA in treating APL is indicative of the potential of vitamin A and RA therapy for disorders associated with deficiencies.⁴⁸⁻⁵⁰

5.4 CrbpI as a potential tissue biomarker for atRA status

For research use, direct RA measurements are reliable in complex biological matrices and are sensitive enough to use ~10 mg of tissue and ~100 μ L of serum or plasma.⁹ How does this correlate to clinical use? There are two major drawbacks for clinical use of direct atRA measurements. First, retinoid extractions require their own samples; and second, circulating retinoids are detectable in serum and plasma, however, are not representative of tissue levels.^{9, 51-52} This means that using serum or plasma is not feasible for representative use of atRA levels in say a tumor and that additional biopsies would be needed. In cases that biopsies are difficult to acquire or are needed for other diagnostic techniques, direct atRA measurements will not be possible. How can this information then be useful in a clinical setting? This body of work has shown that when CrbpI is reduced, atRA is also reduced in *Rbp1*^{-/-} mouse tissues, human tissues and in the induced endometriosis mouse lesions. However, other studies, as well as this work, show limited information in terms of CrbpI utility as a biomarker. Formal validation of CrbpI as a

biomarker would need to be performed for clinical application. CrbpI investigations which support validation studies for CrbpI utility as a biomarker include: (1) CrbpI overexpression has also shown elevated atRA levels in cell culture (unpublished data); and (2) CrbpI was compared to a currently used biomarker, CD10, in the endometrium for endometrial diseases. In the latter, CrbpI loss corresponded with nonmalignant endometriosis and CrbpI loss increased with increasing tumor grades, specifically in areas that lacked differentiation.⁵³⁻⁵⁴ A number of biomarkers can be identified from a tissue biopsy and including CrbpI to a profile is a reasonable substitution for atRA quantification when retinoid therapy is being considered. Being able to target the patient population that would have improved prognosis with retinoid therapy would be the goal. If CrbpI is not changed or elevated, retinoid therapy will more likely lead to toxicity associated side effects and potentially be detrimental for patient prognosis. However, patients with reduced CrbpI could benefit from the clinically desirable outcomes that have been demonstrated in the *in vitro* and *in vivo* studies as previously discussed.

Combining epigenetic therapeutic approaches (demethylation agents and HDAC inhibitors) with traditional chemotherapy is becoming more common.⁵⁵⁻⁶⁴ As these endogenous “genetic reset” approaches have been shown to restore CrbpI levels *in vitro* and *in vivo*, CrbpI monitoring may also be of prognostic use. Additionally, CrbpI and atRA loss are early changes, and CrbpI has the potential to be a marker for a chemopreventative therapeutic approach. The need for biomarkers for use in retinoid therapy is there, and preliminary investigations for CrbpI to be a biomarker have been promising.

5.5 Future CrbpI studies

All of our *Rbp1*^{-/-} mouse retinoid studies were performed in whole tissue homogenates. atRA biosynthesis can be cell specific and a whole tissue homogenate is likely causing a dilution effect, which may be masking a more profound atRA loss in a specific cell type. Being able to quantify atRA and vitamin A pathway proteins in a cell specific manner would be greatly beneficial. Primary cell isolation would be possible for some tissues and cell types and would be one means for these studies. More desirable would be to relate spatial resolution of atRA and vitamin A pathway proteins with histological comparisons. Mass spectrometry imaging (MSI) is currently being used to evaluate spatial resolution of targets from tissue slices for small molecules, peptides and lipids.⁶⁵⁻⁶⁸ The low abundance of the desired retinoid targets may be problematic for detection, but as this technology improves and methodologies are developed, there is great potential for these studies to be undertaken. This approach to CrbpI characterization would provide additional insight to CrbpI function in atRA and tissue homeostasis.

5.6 References

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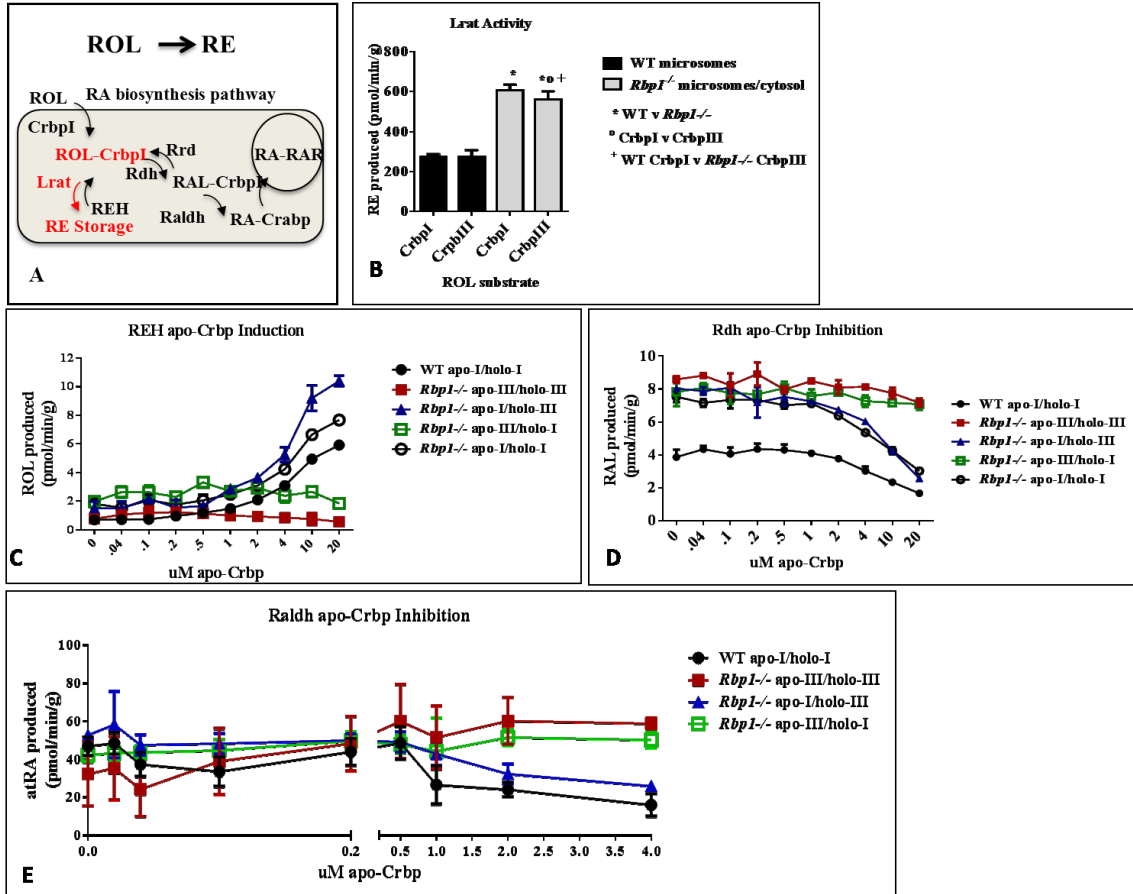
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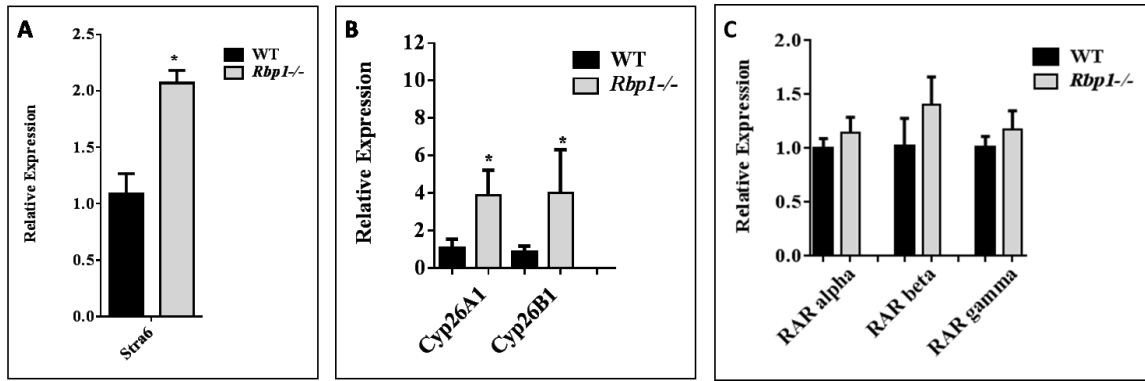
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Appendix A: Chapter 4 Supplemental Figures



Supplemental Figure 4.1: Additional enzyme activities. **A**, Lrat activity determined by measuring RE produced from Crbp-ROL substrate. Lrat is present in the microsome fraction, however RE was measured simultaneously from the Rdh activity assay which combined microsome and cytosol fractions to determine Lrat activity. **B**, RE production by Lrat is increased in *Rbp1*^{-/-} lung, however is decreased with CrbpIII as substrate protein by the *Rbp1*^{-/-} Lrat. The Lrat activity in the *Rbp1*^{-/-} lung condition is significantly increased compared to the WT control condition. $n=6 \pm \text{SEM}$, $p < 0.0477$ **C**, Rate curves (pmol/min/g) of ROL production from RE by REH induction with increasing apo-CrbpI but not apo-CrbpIII. $n=3$ per condition. **D**, Rate curves (pmol/min/g) of Rdh inhibition for RAL production by apo-CrbpI but not apo-CrbpIII. $n=3$ per condition. **E**, Rate curves (pmol/min/g) of Ralsh inhibition for RA production by apo-CrbpI but not apo-CrbpIII. $n=6 \pm \text{SEM}$ per condition.



Supplemental Figure 4.2: Relative gene expression for *Stra6*, *Cyp26*, and *RAR*. (A) *Stra6*, the cell membrane vitamin A uptake receptor, is elevated in *Rbp1*^{-/-}, $n=6 \pm \text{SEM}$, $p<0.0004$. (B) *Cyp26A* and *Cyp26B* are elevated in *Rbp1*^{-/-} lung. (C) RAR α , β and γ are not changed. $n=8 \pm \text{SEM}$ for each genotype and relative expression was determined by the $\Delta\Delta C_T$ method, $p<0.0035$.

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