PUBLICATIONS OF
THE UNIVERSITY OF EASTERN FINLAND

Dissertations in Health Sciences

ASHIK JAWAHAR DEEN

REGULATION OF HYALURONAN SYNTHESIS
– role of hyaluronan synthase trafficking and UDP-sugars

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Regulation of hyaluronan synthesis – role of hyaluronan synthase trafficking and UDP-sugars

To be presented by permission of the Faculty of Health Sciences, University of Eastern Finland for public examination in lecture hall SN200, Kuopio, on Friday, April 29th 2016, at 12 noon

Publications of the University of Eastern Finland
Dissertations in Health Sciences
Number 341

Department of Biomedicine, School of Medicine, Faculty of Health Sciences,
University of Eastern Finland
Kuopio
2016

Grano Oy
Jyväskylä, 2016

Distributor:
University of Eastern Finland
Kuopio Campus Library
P.O.Box 1627
FI-70211 Kuopio, Finland
http://www.uef.fi/kirjasto

ISBN (print): 978-952-61-2071-3
ISBN (pdf): 978-952-61-2072-0
ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

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Jawahar Deen, Ashik
Regulation of hyaluronan synthesis – role of hyaluronan synthase trafficking and UDP-sugars
University of Eastern Finland, Faculty of Health Sciences
Publications of the University of Eastern Finland. Dissertations in Health Sciences Number 341. 2016. 78 p.

ISBN (print): 978-952-61-2071-3
ISBN (pdf): 978-952-61-2072-0
ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

ABSTRACT

Hyaluronan is a ubiquitous non-sulfated glycosaminoglycan synthesized by the hyaluronan synthase family enzymes (HAS1–3) in the plasma membrane. Hyaluronan is made up of repeating units of disaccharides containing glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) and is present in the pericellular and extracellular matrix of cells. Synthesis and degradation of hyaluronan is important in inflammation, cancer and in normal physiological processes like development and epithelial-to-mesenchymal transition.

This study showed that HAS3 traffic is an indispensable factor in the initiation and maintenance of hyaluronan synthesis and that the plasma membrane level of the enzyme is largely controlled by its endocytosis. Rab10 was identified as the first known protein to enhance HAS3 endocytosis, in a clathrin-dependent pathway. Declining levels of UDP-GlcUA and UDP-GlcNAc provoked HAS3 endocytosis, whereas excess UDP-GlcNAc and sustained O-GlcNAc modification of HAS3 severely inhibited endocytosis and lysosomal degradation of HAS3. HAS3 was continuously recycled between endosomes and the plasma membrane, and the proportion of HAS3 residing in the plasma membrane associated to the availability of UDP-sugars and the extent to which HAS3 is O-GlcNAc modified. Excess UDP-sugars and sustained O-GlcNAcylation of HAS3 also increased the release of HAS3 in extracellular vesicles, while the release was subdued with depletion of UDP-sugars and O-GlcNAcylation. GFAT and GNPDA enzymes functioned in different directions in the maintenance of UDP-GlcNAc content in basic keratinocyte culture conditions, in which GNPDAs returned excess hexosamines back to fructose. In contrast, depletion of UDP-GlcNAc turned the net catalysis by GNPDAs towards more hexosamines and UDP-GlcNAc. Changes in UDP-sugars and hyaluronan synthesis affected basic cellular functions such as proliferation, migration and adhesion to type I collagen. GFAT1 expression was increased in early *in situ* melanoma and declined in deep melanoma tissues, correlating with hyaluronan content.

This thesis work delivers novel information about the traffic of HAS3, its secretion in extracellular vesicles and molecular mechanisms in hyaluronan synthesis regulation. Furthermore, the results suggest that changes in UDP-sugars and hyaluronan synthesis are indicators for progression of malignancy and can perhaps be utilized as prognostic and therapeutic targets.

National Library of Medicine Classification: QU 57, QU 83, QU 141
Medical Subject Headings: Hyaluronic Acid/biosynthesis; Glucuronosyltransferase; Protein Transport; Uridine Diphosphate Sugars; Rab GTP-Binding Proteins; Neoplasms; Melanoma; Extracellular Vesicles

Jawahar Deen, Ashik
Regulation of hyaluronan synthesis – role of hyaluronan synthase trafficking and UDP-sugars
Itä-Suomen yliopisto, terveystieteiden tiedekunta
Publications of the University of Eastern Finland. Dissertations in Health Sciences Numero 341. 2016. 78 s.

ISBN (print): 978-952-61-2071-3
ISBN (pdf): 978-952-61-2072-0
ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

TIIVISTELMÄ

Hyaluronaani on yleisenä esiintyvä sulfatoitumaton glykosaminoglykaani jota tuottavat hyaluronaanisyntaasientsyymit (HAS1–3) solun ulkokalvolla. Hyaluronaani muodostuu toistuvista disakkaridiyksiköistä jotka sisältävät glukuronihapon (GlcUA) ja N-asetyyli-glukosamiinin (GlcNAc). Hyaluronaani sijoittuu solun ulkopinnalle ja soluväliaineeseen ja vaikuttaa vahvasti tulehduksessa, syövässä ja normaaleissa fysiologisissa tapahtumissa kuten sikiön kehityksessä ja epiteeli-mesenkyymitransitiossa.

Tämä tutkimus osoittaa että HAS3:n kuljetus on tärkeä tekijä hyaluronaanisynteesissä ja että tämän entsyymin määrää solukalvolla säätelee suureksi osaksi sen endosytoosi. Rab10 lisäsi HAS3:n klatriinivälitteistä endosytoosia on ensimmäinen proteiini jonka tiedetään vaikuttavan HAS3:n kuljetukseen. HAS3:n endosytoosi väheni UDP-GlcNAc:in ja UDP-GlcUA:n puutteessa, kun UDP-GlcNAc:in ylimäärä ja HAS3:n O-GlcNAcylaatio puolestaan estivät HAS3:n endosytoosia ja lysosomaalista hajotusta. HAS3:a kierrätettiin jatkuvasti solukalvon ja endosomirakkuloiden välillä. UDP-sokerien saatavuus ja HAS3:n O-GlcNAcylaatiotaso määräsivät solukalvolla olevan HAS3:n pitoisuuden ja erittymisen solunulkoisiin vesikkeleihin.

Keratinosyyteissä GFAT ja GNPDA entsyymit katalysoivat samaa reaktiota vastakkaisiin suuntiin; GNPDA palautti ylimäärän heksosamiinia takaisin fruktoosiksi. UDP-GlcNAc:in vähentyminen kuitenkin käänsi nettokatalyysin päinvastaiseksi, heksosamiinin suuntaan. UDP-sokerien ja hyaluronaanisynteesin muutokset vaikuttivat solun jakautumiseen, liikkuvuuteen ja kiinnittymiseen tyyppi I kollageeniin. GFAT1:n ilmeneminen lisääntyi varhaisessa *in situ* melanoomassa ja väheni syvälle edenneessä melanoomassa korreloiden hyaluronaanin pitoisuuden kanssa.

Tämä väitöskirjatyö antaa uutta tietoa HAS3:n liikkeistä, sen erittymisestä solunulkoisiin rakkuloihin, sekä hyaluronaanisynteesin säätelyn molekulaarisista mekanismeista. Tulokset viittaavat myös siihen että muutokset UDP-sokereissa ja hyaluronaanin synteesissä ja pitoisuudessa toimivat kasvaimissa pahanlaatuisuuden indikaattoreina, ja että niistä voidaan kehittää ennustetekijöitä ja hoidon kohteita.

Luokitus: QU 57, QU 83, QU 141
Yleinen Suomalainen asiasanasto: hyaluronaani; biosynteesi; syöpätaudit; melanooma

Just when the caterpillar thought the world was ending,

it turned into a butterfly!

(English proverb)

Acknowledgements

This thesis work was carried out in the Institute of Biomedicine/Anatomy, School of Medicine, at the University of Eastern Finland during the period 2010-2016. I sincerely thank the administrative personnel in the institute for providing the essential facilities to carry out my research work. Special thanks to Eija Vartiainen (Personnel secretary, Institute of Biomedicine) and Karoliina Tenkanen (Financial secretary, Institute of Biomedicine), for your innumerous help, whenever I have bothered you. And it is my utmost responsibility to convey my appreciativeness to Arja Afflekt (Amanuenssi, Faculty of Health Sciences) for the incredible support during the preparation of my thesis defense.

First and foremost, I need to express my sincere gratitude and endless affection to my thesis supervisor, Professor Markku Tammi. He is the best supervisor one could dream of and he surprises me everytime with his vast knowledge in hyaluronan biology, meticulous planning, trouble-shooting skills and most importantly his patience towards me. His valuable suggestions during the difficult times of my research work got me through this thesis and I am where I am only because of his effort and trust in me. I wish to learn so much from you in doing science and thank you for teaching me how to ski and also for encouraging me to do numerous other extra-curricular activities.

My warmest thanks also go to my second supervisors, Dr. Sanna Oikari, Docent Kirsi Rilla and Dr. Katri Makkonen. I am indebted to your valuable suggestions, countless discussions and problem solving skills. I feel so lucky to have your expert guidance in proteomics, microscopy imaging and genomics that helped me steer through my experiments to finish the research works in a reasonable time frame. Without your dedication and support, completion of this thesis would not have been possible. More than supervisors, you have been so friendly and supportive, and made me feel comfortable. I will never miss a chance to collaborate with you in the future.

I also want to thank Professor Raija Tammi for her immense support, constructive criticism and careful evaluation of the research results. Your extensive reading of my manuscripts and advice on many technical issues in experiments helped me to grow as a researcher.

I like to extend my gratitude to my thesis committee members, Docent Sakari Kellokumpu and Adjunct Professor Daniel Abankwa for their critical evaluation of the project goals and research results. Your ideas and useful suggestions are treasured, and thank you for spending your time to improve my thesis.

I want to express my gratitude to my thesis pre-examiners, Professor Risto Renkonen and Professor Jukka Finne for your extensive review and valuable comments to improve my thesis. I am also grateful to Dr. Gina Galli for her valuable and careful revision of the

language in the thesis. I deeply enjoyed our interactive and friendly discussions! Thank you very much for your time, appreciation and most of all your friendship.

My deepest gratefulness goes to my co-authors: Uma Thanigai Arasu, M.Sc., Riikka Kärnä, M.Sc., Docent Sanna Pasonen-Seppänen, Dr. Genevieve Bart, Dr. Antti Hassinen, Piia Takabe, M.Sc., Sara Wojciechowski, M.Sc., Dr. Jukka Häyrinen, Dr. Antti Ropponen, Dr. Ville Koistinen, Kari Törrönen, M.Sc., Ilari Tyni, M.Sc., and Avinash Bathina, M.Sc., for their contribution and sincere efforts with the publications.

I want to thank all the laboratory personnel in Institute of Biomedicine for creating a lovable atmosphere to work. Special thanks to Riikka Kärnä for your immeasurable help in experiments and patience. I also acknowledge the contributions of Kari Kotikumpu, Tuula Venäläinen and Eija Rahunen to this work. I like to extend my thanks to the present and former members of the hyaluronan research group. Especially, my warmest gratitude to Dr. Raquel Melero, Lasse Hämäläinen, M.Sc., Dr. Hanna Siiskonen, Leena Rauhala, M.Sc., Dr. Irina Ermakova, Dr. Hertta Pulkkinen, Dr. Anne Kultti, Dr. Virpi Tiitu, and Dr. Tiina Jokela for your cherished friendship and nice company in conference trips. Lasse Hämäläinen, thank you for your friendship, and enjoyable sailing and fishing adventures.

Piia Takabe, I will always cherish your valuable friendship, limitless discussions and fun-filled outdoor trips. It is fun talking to you, both as a colleague and a friend, and I will always be indebted to your moral support. The most important thing that I admire about you is that, whenever I have a silly or big (real) problem I call you right away and you have had ready-made solutions! I want to thank Leena Rauhala for the “out-of-the-work” talks and enjoyable “Indian restaurant” lunch plans. You have never shied away whenever I knocked your door for help and most of all you are such a sweet person! I want to extend my gratitude to Dr. Irina Ermakova for your friendship and support during your stay in Kuopio. Cooking Indian food with you is always a fun thing to remember! Raquel Melero, you are so much fun to talk to and I will always remember you as the one person to discuss my problems and frustration, and that is because I can relate myself to you in so many levels. Thank you for everything! I like to extend my thanks to Dr. Inga Kretschmer, for your friendship, support and valuable discussions – both in science and life in general. I always admire your enthusiasm and motivation to excel in life.

My wholehearted gratitude to the “volleyball and ping pong clubs” with the “Southern Empire and Tamil Pasanga” gang (Suresh, Rolls John, Rajasekaran, Bala, Jagadish, Shalem, Appu, Ashok and Amzad), as life outside work would be so boring without the company of you guys. It was so much fun with all the “high-profile” games, silly arguments and match-winning performances. Suresh, you have always been a great friend to me and I will cherish our friendship forever. You have so much energy that it is very apt to call you, “the wild-type”. Thank you machan for being there for me in many critical situations and also for everything else! And, Krishnaveni, I wish to extend my deepest gratitude for your

friendship, support and fun-filled discussions in our "Southern Empire" gatherings. Especially, I thank you for your help in many important situations. Niyaz, you were my first and foremost friend in Kuopio and I will always be thankful for your friendship and care on me. It is my responsibility to remember that in my life. I will extend my gratitude to my other good friends in the Indian gang – Lakshmi, Bhima, Sudipta, Kajal, Yuvraj, Varsha and Narasinha Shurpali.

I want to thank my BDU gang for all the fun times and a decade-long friendship! Yashwanth, if not for you I wouldn't be here, as a PhD and I thank you for that. You will always be close to my heart and I will cherish our late night talks, surreal events, fun-filled adventures and many more – to say the least. Arafath, where do I even begin? Our friendship was a roller coaster ride in the past 15+ years and it wouldn't be an overstatement to say that we have grown together. We have had our share of ups and downs but the fact that we are still friends is in itself a great achievement :) You were by my side during my most difficult times, and I knew that it was not an easy task! We fought together, laughed together, had "the talk" a zillion times, and the most special thing about you is that you always make me laugh out loud! Thank you for being one of the best and closest friends I have ever gotten and with you it is always an adventure to begin with. Ponnu (alias Ponnuswamy), I always admire your calm and composed nature, and I mean, how do you maintain that? You are and will be one of my best friends ever and I thank you for being there for me! Vinodh, though many people would say that you are tough to deal with, I always knew your soft side. You are one of the sweetest persons I have ever seen, and I value your friendship more than anything else. I only wish that we could stay as friends forever! Muniesh, you always surprise me with your unconditional affection and faithful friendship. You are so unique and I cherish your friendship with my whole heart. Again, I only wish that we could have spent more time together and may be in the future we will get an opportunity like that. Veena, thank you for always being a great friend to me!

I want to thank my other BDU friends – Asha, Madhi, Kartiga, Vanitha, Prem, Nagendran, Mani, Deepauc, Kannan, Saminathan, Lenin, Madhu Mohan, Tamil Nambi, Venkat, Anusha, Deepan, Anand, Yadu, Sathish, Prasanth Varma, Buvani, Minu and Sankari.

Pasi, you are an amazing person and I always get surprised with your unconditional affection towards me. You have put up with me so much that I have started to think that you are a saint! Thank you for your friendship and I wish I can be as good and pure as you are :)

Uma, I have no words to explain my gratitude towards you! I'm sorry that I did not make a good impression on you in our first meeting, but hey, I did not fail the second time – or did I? :) You are so much fun to talk to and the most important thing that I admire about you is that you immensely care for everyone around you and to do that you should have an

amazing personality, which I respect from the bottom of my heart! I admire your presence of mind, problem solving skills and most importantly your near-perfect planning. And I am sorry that I always mess up your plans :) You always make me smile and laugh and I have deeply enjoyed our each and every "out-of-the box" discussions. More than a colleague, you have supported me all the time and, oh boy, you are such a prankster! :) More than anything else, I can relate to you in so many levels and thank you for sharing my burden in every difficult situation. You have given a different flavor to my life and I wish we can be supportive of each other, forever and ever! :) Thank you for everything, and, I am out of words!

Mom and Dad, how can I ever repay you for your affection and all the difficulties you have gone through to push me forward in my career. Life is too short to make sacrifices but you both have proved it wrong for me and for that I am forever indebted to you. If not for your love and hard work, I wouldn't be here in this place in my life. Thank you a million times and more for everything, which I cannot put down in words, and I love you so very much! Jessy, my sweet little sister, your cute memories are still fresh in my mind – the day you were born and how I hit you (accidently) for the first time, how your first word that you said was my name, and the day I felt that you have grown up so much when you cheered mom and me at the airport. I have always felt bad that I couldn't spend much time with you and I have missed so many events in your life. Sorry for being a big brother with boring advices and sorry that I was not around when you were a little girl :) I know how much you love me and you know that I will always be there for you! Life is nothing without you and you will be deep-rooted in my heart, forever and ever! Thank you for all the love and care you have shown towards me. This thesis work is dedicated to you, mom and dad :)

I am grateful for the financial support from the Finnish Cultural Foundation, North-Savo Cultural Foundation, North-Savo Cancer Foundation, K. Albin Johanssons Stiftelse Foundation, Doctoral Program in Molecular Medicine (DPMM), University of Eastern Finland, Finnish Glycoscience Graduate School, Paavo Koistisen Foundation and CIMO Foundation.

love,

Ashik Jawahar Deen

Kuopio, March 2016

List of the original publications

This dissertation is based on the following original publications:

I **Deen AJ**, Rilla K, Oikari S, Kärnä R, Bart G, Häyrinen J, Bathina AR, Ropponen A, Makkonen K, Tammi RH, Tammi MI.
Rab10-mediated endocytosis of the hyaluronan synthase HAS3 regulates hyaluronan synthesis and cell adhesion to collagen.
J. Biol. Chem. 289: 8375-89, (2014).

II **Deen AJ**, Arasu UT, Pasonen-Seppänen S, Hassinen A, Takabe P, Wojciechowski S, Kärnä R, Rilla K, Kellokumpu S, Tammi R, Tammi M, Oikari S.
UDP-sugar substrates of HAS3 regulate its O-GlcNAcylation, intracellular traffic, extracellular shedding and correlate with melanoma progression.
Cell. Mol. Life. Sci. (2016). DOI: 10.1007/s00018-016-2158-5.

III Rilla K, Pasonen-Seppänen S, **Deen AJ**, Koistinen VV, Wojciechowski S, Oikari S, Kärnä R, Bart G, Törrönen K, Tammi RH, Tammi MI.
Hyaluronan production enhances shedding of plasma membrane-derived microvesicles.
Exp. Cell. Res. 319: 2006-18, (2013).

IV Oikari S, Makkonen K, **Deen AJ**, Tyni I, Kärnä R, Tammi RH, Tammi MI.
Hexosamine biosynthesis in keratinocytes – roles of GFAT and GNPDA enzymes in the maintenance of UDP-GlcNAc content and hyaluronan synthesis.
Glycobiology. (2016). DOI: 10.1093/glycob/cww019.

Contents

Abbreviations

4MU	4-methylumbelliferone
ABC	adenosine triphosphate-binding cassette
AC	adenylyl cyclase
ADP	adenosine diphosphate
Arf	ADP ribosylation factor
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
EEA1	early endosomal antigen 1
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
emmprin	extracellular matrix metalloproteinase inducer
EMT	epithelial to mesenchymal transition
ERK	extracellular signal-regulated kinase
ESCs	embryonic stem cells
FAK	focal adhesion kinase
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GFAT	glutamine fructose-6-phosphate amidotransferase
GFP	green fluorescent protein
GF-R	growth factor receptor
GlcNAc	N-acetylglucosamine
GlcUA	glucuronic acid
GNPDA	glucosamine-6-phosphate deaminase
GPCR	G-protein coupled receptor
GPI	glycosylphosphatidylinositol
GTPase	guanosine triphosphate hydrolase
HA	hyaluronan
HABC	hyaluronan binding complex of the cartilage aggrecan G1 domain and link protein
CD44	cluster of differentiation 44/hyaluronan receptor
CREB	cAMP response element binding protein
Dendra2	photoswitchable monomeric fluorescent protein derived from octocoral *Dendronephthya sp.*
DIC	differential interference contrast
HABP	hyaluronan binding protein
HARE	hyaluronan receptor for endocytosis
HAS	hyaluronan synthase
HBP	hexosamine biosynthetic pathway
HEMa	human epidermal melanocytes from adult origin
HSCs	hematopoietic stem cells
HYAL	hyaluronidase
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
IKK	inhibitor of nuclear factor κB kinase
IL	interleukin
IαI	inter-alpha-inhibitor
JAK	Janus kinase
KGF	keratinocyte growth factor
LYVE-1	lymph vessel endothelial hyaluronan receptor 1
MAPK	mitogen activated protein kinase
MDR1	multidrug resistance 1, P-glycoprotein
MMP	matrix metalloproteinase
MRP	multidrug resistance protein
MSCs	mesenchymal stem cells
NF-κB	nuclear factor κB
O-GlcNAc	O-linked-N-acetylglucosamine

OGT	O-GlcNAc transferase
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PLC	phospholipase C
Rab	Ras related in brain
Ras	a small guanosine triphosphatase
RHAMM	receptor for hyaluronan mediated motility
RTK	receptor tyrosine kinase
*Se*HAS	hyaluronan synthase from *Streptococcus equisimilis*
SEM	scanning electron microscope
Shh	sonic hedgehog protein
siRNA	short interfering RNA
SP1/3	specificity protein 1/3
Src kinase	sarcoma kinase
STAT	signal transducer and activation of transcription
TEM	transmission electron microscope
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TSG	tumor necrosis factor alpha stimulated gene
UDP	uridine diphosphate
UGDH	UDP-glucose dehydrogenase
YY1	ying-yang 1

1 Introduction

Hyaluronan (or hyaluronic acid or hyaluronate or "HA") is a unique glycosaminoglycan in the extracellular matrix and pericellular space, present in vertebrate tissues and in some bacteria (Tammi et al, 2011). Hyaluronan is negatively charged and possesses a very simple chemical structure with repeating disaccharide units of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc). Hyaluronan is not known to be associated with a core protein or lipid (Hubbard et al, 2012). Due to its anionic nature hyaluronan acts as a space-filler in tissues and it binds to cell surface receptors like CD44, ICAM-1, HARE and LYVE-1. Additionally, hyaluronan interacts with RHAMM, which is a soluble protein without a membrane spanning domain but can be transported to the cell surface by stimuli. Via these interactions, hyaluronan influences numerous cellular functions like proliferation, migration, differentiation and invasion (DeAngelis, 2012, Jiang et al, 2011a, Tammi et al, 2011, Toole, 2004a, Weigel & DeAngelis, 2007a, Zhou et al, 2003). Although the effects of hyaluronan through multiple cell surface receptors have been extensively studied, contributions of hyaluronan synthases and associated molecules in the signaling network are not so well known (Toole, 2004).

Vertebrate hyaluronan is synthesized by a family of hyaluronan synthases (HAS1–3) and the synthesis takes place when the HAS enzyme is present in the plasma membrane (Rilla et al, 2005) and extrudes the growing chain of the hyaluronan molecule directly into the extracellular space (DeAngelis, 2012, Toole, 2004). Regulation of hyaluronan synthesis takes place at different levels, starting from transcriptional regulation of *HAS* genes, post-translational regulation of HAS proteins and their modifications, and, as shown recently, by changes in the cytosolic levels of the precursor sugars, i.e. UDP-GlcUA and UDP-GlcNAc (Jokela et al, 2008a, Jokela, 2011, Tammi et al, 2011). Several growth factors and cytokines like TGF-β, EGF, PDGF, IL-1β, IFN-γ, transcription factors like SP1, SP3, YY1, and other signaling molecules like the cAMP activator forskolin and retinoic acid, are known to affect and modify *HAS* gene expression and hyaluronan synthesis (Jiang et al, 2011, Jokela et al, 2011, Makkonen et al, 2009, Monslow et al, 2004, Saavalainen et al, 2005, Sironen et al, 2011, Vigetti et al, 2011). However, post-translational regulation of HAS proteins is less studied. The roles of post-translational modifications such as phosphorylation, ubiquitination and O-GlcNAcylation on the activity of HASs, and their impact on hyaluronan synthesis have been reported recently (Karousou et al, 2010, Vigetti et al, 2011, Vigetti et al, 2012). Hyaluronan synthesis is also affected by any change in the cytosolic pools of UDP-GlcUA and UDP-GlcNAc. Several compounds like 4MU, mannose and glucosamine change the cytosolic levels of these UDP-sugars (Jokela et al, 2008, Jokela et al, 2013, Rilla et al, 2004, Tammi et al, 2011) and consequently the synthesis of hyaluronan.

Synthesis and metabolism of hyaluronan have a major impact on different aspects of malignancies, including even their multidrug resistance (Sironen et al, 2011). In most of the cancers of epithelial origin, the amount of hyaluronan in cancer cells differs significantly from normal cells. There is either a direct or inverse correlation of hyaluronan level with the tumor grade and clinical prognosis of cancer patients. Increased hyaluronan levels directly correlate with the tumor grade and poor prognosis in breast, gastric, ovary and colon adenocarcinomas (Sironen et al, 2011, Tammi et al, 2008a). On the other hand, in squamous cell carcinomas of the skin, mouth, larynx and lung, decreased hyaluronan levels correlate with the tumor grade and poor prognosis (Tammi et al, 2008a), thereby showing an inverse correlation. An explanation for this dichotomy may come from the recent paper indicating that degradation of hyaluronan allows carcinogenesis, while signals induced by intact, very high molecular mass hyaluronan shield against cancers (Tian et al, 2013). Indeed, hyaluronan degradation products induce inflammation and growth factor response, which can prime the tissues for cancer and promote its progression. Therefore, chronically enhanced production and degradation of hyaluronan appears to be the malefactor.

The present study aims at revealing the role of intracellular trafficking of HAS in hyaluronan synthesis. It shows that hyaluronan synthase 3 (HAS3) traffic to and from the plasma membrane regulates hyaluronan synthesis and the synthesis, in turn, acts as a stimulus for the shedding of HAS3-containing extracellular vesicles. Rab10 is identified as the first known protein regulator of HAS traffic. The study also shows that cellular levels of UDP-GlcNAc and UDP-GlcUA modulate this traffic and thereby regulate hyaluronan synthesis and shedding of HAS3 in extracellular vesicles. UDP-GlcNAc levels also dictate the O-GlcNAc modification of HAS3, and consequently HAS3 traffic and shedding of HAS3-dependent extracellular vesicles. Maintenance of cellular UDP-GlcNAc level is feedback controlled by GFAT1 and GNPDA enzymes.

2 Review of the Literature

2.1 HYALURONAN AND HYALURONAN SYNTHASES

2.1.1 Hyaluronan – structure and properties

Hyaluronan was discovered in 1934 in the vitreous body of the bovine eye (Meyer & Palmer, 1934) and its chemical formula was described 20 years later (Weissmann et al, 1954. Hyaluronan is a unique non-sulfated glycosaminoglycan (GAG), made up of repeated disaccharide units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA): [-β(1,4)-GlcUA-β(1,3)-GlcNAc-]$_n$. Hyaluronan usually consists of 2,000–25,000 disaccharide units with a relative molecular mass of 10^6–10^7 Da, and extended length of 2–25 μm (Toole, 2004). Under physiological pH, hyaluronan is hydrophilic due to the fact that the carboxyl groups on the GlcUA residues are negatively charged (Scott, 1989). However, twists in the hyaluronan chain form hydrophobic patches, allowing interactions with other hyaluronan chains, proteins, lipids and membranes (Scott et al, 1992). Unlike other glycosaminoglycans, hyaluronan is not covalently linked to core proteins (Fraser et al, 1997). This particular fact, along with the discovery that it is not sulfated, makes hyaluronan a different yet special component of the extracellular matrix. The physicochemical properties of hyaluronan with its ability to bind water molecules, formation of viscous gels and filtering effects on molecular level makes hyaluronan a likely candidate in several medical applications in physiological and pathological conditions (Jiang et al, 2011, Laurent & Fraser, 1992, Sironen et al, 2011, Toole, 2004).

2.1.2 Hyaluronan synthases – discovery and structure

Hyaluronan synthase gene, termed as *HasA* was first discovered and cloned in group A *Streptococcus pyogenes* (DeAngelis et al, 1993). Later *HAS* genes were studied in vertebrates including human cells, bacteria and a virus (Weigel & DeAngelis, 2007). Hyaluronan synthesis takes place in a selected group of bacteria like the Gram-positive streptococci (*S. pyogenes, S. equisimilis, S. uberis and S. zooepidemicus*) and the Gram-negative *Pasteurella multocida* (Tammi et al, 2011). About two decades ago, three highly conserved genes for *HAS* isoforms (*HAS1, HAS2 and HAS3*) were published in several eukaryotes including human (Itano & Kimata, 1996b, Shyjan et al, 1996, Watanabe & Yamaguchi, 1996), mouse (Itano & Kimata, 1996a, Spicer et al, 1996, Spicer et al, 1997) and frog genomes (DeAngelis, 1996, Rosa et al, 1988, Spicer & McDonald, 1998, Vigetti et al, 2003). Human *HAS1*–3 genes are located in chromosomes 19, 8 and 16 respectively (Spicer & McDonald, 1998, Tammi et al, 2011). Splice variants have been reported for individual HASs i.e. *HAS1* variants are reported in diseases such as Waldenström's macroglobulinemia (Adamia et al, 2003),

multiple myeloma (Adamia et al, 2005) and bladder cancer (Golshani et al, 2007), and *HAS3* has three variants (Gene-database, NCBI).

HAS protein isoforms in mammals share an identity of 55–71% at the amino acid level. Among the three different HASs, HAS2 and HAS3 are most closely related (Spicer & McDonald, 1998, Toole, 2004). Streptococcal HAS (spHAS) is the only HAS protein that is biochemically purified and partially characterized with relation to its topology. To date, the three-dimensional structure of mammalian HASs have not been resolved, and only predictions are available that have been derived from biophysical data (Tammi et al, 2011, Weigel et al, 1997, Weigel & DeAngelis, 2007). Mammalian HAS isoforms are integral membrane proteins, predicted to possess 4–6 transmembrane and 1–2 membrane associated domains, in addition to the cytoplasmic "catalytic" domain (Weigel et al, 1997, Weigel & DeAngelis, 2007). The molecular weight of all HAS isoforms is ~63–65 kDa.

2.1.3 Function of hyaluronan synthases and their differences

In Xenopus, differential localization of *HAS* expression is observed during embryonic development. *HAS1* and *HAS2* are homogenously expressed throughout the embryo but *HAS3* is localized to the inner ear and the cement gland (Camenisch et al, 2000, Tammi et al, 2011). Also, in mouse development, *HAS2* expression is observed throughout all the stages of the embryo and *HAS1* disappears after the embryonic day 8.5 (Tien & Spicer, 2005). *HAS3*, on the other hand, is only expressed at the later stages of embryonic development, especially during the formation of sensory organs. *HAS2* is the only highly expressed isoform during heart valve development between the embryonic days 8.5–9.5. In fact, mice with *HAS2* knockout die from severe cardiovascular defects already in embryonic days 9.5–10. However, mice with *HAS1* and *HAS3* knockout are born and continue to live with no obvious structural changes or malfunctions (Bai et al, 2005, Camenisch et al, 2000, Kobayashi et al, 2010, Tien & Spicer, 2005).

HAS isoforms differ in their affinity to the UDP-sugar substrates, with HAS1 showing the lowest and HAS3 the highest affinity (Itano et al, 1999, Rilla et al, 2013a, Tammi et al, 2011). Also, HAS isoforms are reported to differ in the size of the hyaluronan chains produced but this appears to depend on the conditions of synthesis. In plasma membrane preparations of COS1 cells transfected with HAS isoforms, HAS3 produced lower molecular weight hyaluronan (1×10^5–1×10^6 Da) compared to HAS1 and HAS2 (2×10^5–2×10^6 Da) (Itano et al, 1999). However, in CHO cell plasma membrane preparations with transfected HASs, HAS2 produced high molecular weight hyaluronan (3.9×10^6 Da) compared to HAS1 and HAS3 (0.12×10^6 Da and 0.12–1.0×10^6 Da respectively) (Brinck & Heldin, 1999). In intact CHO cells, all isoforms of HAS produced high molecular weight hyaluronan (3.9×10^6 Da) (Brinck & Heldin, 1999, Itano et al, 1999). In case of aortic smooth muscle cells, HAS1 and HAS2 produced high molecular weight hyaluronan (2–10×10^6 Da), while HAS3 produced low molecular weight hyaluronan (2×10^6 Da) (Wilkinson et al, 2006). The ambiguity

between different cell types and sample preparations (i.e. whole cells vs membrane preparations) on HAS's ability to produce high or low molecular weight hyaluronan suggests that additional factors such as cellular environment, post-translational modifications and trafficking of HASs are involved in the duration and extension of hyaluronan synthesis. HAS2 is the most common and universally expressed HAS isoform in mammalian cells while HAS3 is often over-expressed during inflammation and cancer (Tammi et al, 2011). The reason behind this differential expression of HAS is still unclear.

2.2 BIOSYNTHESIS AND REGULATION OF HYALURONAN SYNTHESIS

2.2.1 Mechanism of hyaluronan biosynthesis

Hyaluronan is synthesized by a family of hyaluronan synthases, HAS1–3, in the plasma membrane and simultaneously extruded into the extracellular space (Rilla et al, 2005, Toole, 2004). HASs utilize UDP-sugars i.e. UDP-GlcNAc and UDP-GlcUA as substrates and need Mg^{2+} or Mn^{2+} to synthesize hyaluronan (Weigel & DeAngelis, 2007). The mechanism of hyaluronan synthesis does not require any primer to initiate polymerization (Weigel & DeAngelis, 2007). Human and mouse HASs add the precursor sugars to the reducing end of the growing hyaluronan chain (Prehm, 1983a, Prehm, 1983b, Prehm, 2006), whereas HAS in *Xenopus laevis* utilizes the non-reducing end (Bodevin-Authelet et al, 2005) similar to the activity of HAS in *Pasteurella multocida* (DeAngelis, 1999). HAS activity in cell homogenates was first discovered in 1959 (Markovitz et al, 1959) and it was described only later (in 1984) that unlike other GAGs, hyaluronan synthesis takes place in the inner face of the plasma membrane and not in the Golgi apparatus (Prehm, 1984). Specialized microvillus-like plasma membrane protrusions are particularly active in hyaluronan synthesis while the detailed molecular machineries are still unclear. These hyaluronan-rich, microvillus-like plasma membrane protrusions (Kultti et al, 2006, Rilla et al, 2008) have also been identified *in vivo* in rat peritoneal cells (Koistinen et al, 2015). Hyaluronan may remain attached to HASs while being synthesized. The pericellular hyaluronan coat acts as a scaffold to maintain the plasma membrane protursions (Rilla et al, 2008). The export and translocation of hyaluronan from inside of the cells to the extracellular space has been a debated issue. The adenosine triphosphate-binding cassette (ABC) transporters such as MRP5 are proposed to be involved in the export of hyaluronan in fibroblasts (Schulz et al, 2007). However, in breast cancer cells the translocation of hyaluronan is not dependent on ABC transporters (Thomas & Brown, 2010). In a recently published report, *Streptococcus equisimilis (Se)* HAS reconstituted in proteoliposomes both synthesized and translocated hyaluronan (Hubbard et al, 2012). In addition, purified *Se*HAS in liposomes showed the presence of an intraprotein pore in HAS and translocation of hyaluronan via the activity of HAS itself (Medina et al, 2012). More support for the HAS pore hypothesis comes from reports that show homo- and heteromers among different HASs, since the complexes

would facilitate the formation of a pore for hyaluronan extrusion (Bart et al, 2015, Karousou et al, 2010).

2.2.2 Transcriptional regulation of HAS

Hyaluronan synthesis and its regulation are important aspects of both physiological and pathophysiological conditions including embryonic development, wound healing, inflammation and cancer. Several endogenous factors and artificial compounds influence hyaluronan synthesis. These regulators can act in one of the following levels: 1) transcriptional and translational regulation of *HAS,* 2) post-translational regulation of *HAS* activity and 3) availability of the hyaluronan precursor sugars, UDP-GlcNAc and UDP-GlcUA. Some of these factors influencing hyaluronan synthesis are presented in Table 1 and in Fig. 1.

Table 1. Factors affecting hyaluronan synthesis. Modified from (Kultti, 2009b, Siiskonen, 2013c).

a) Factors increasing hyaluronan synthesis

↓ decreased, ↑ increased, - not changed, NE not expressed, empty not studied

Agent	Cell/tissue	HA	HAS1	HAS2	HAS3	Reference
cyclic phosphatidic acid	fibroblast	↑	-	↑	-	(Maeda-Sano et al, 2014)
lyso phosphatidic acid	fibroblast	↑		↑		(Maeda-Sano et al, 2014)
P2Y14 (UDP-glucose receptor)	keratinocyte	↑		↑		(Jokela et al, 2014)
Sonic hedgehog (Shh)	mouse limb			↑		(Liu et al, 2013)
UVB irradiation	keratinocyte	↑	↑	↑		(Rauhala et al, 2013)
Kaposi sarcoma-associated herpesvirus	endothelium	↑	↑			(Dai et al, 2015)
Glucosamine	kidney epithelium	↑	-	↓		(Rilla et al, 2013b)
full-length adiponectin	fibroblast	↑	NE	↑	-	(Akazawa et al, 2011)
adiponectin	fibroblast	↑		↑		(Yamane et al, 2011)
constitutively active PI3K transfection	mammary carcinoma cell	↑				(Misra et al, 2005)
compound K	keratinocyte	↑	-	↑	-	(Kim et al, 2004)
dehydroepiandrosterone	uterine fibroblast	↑				(Tanaka et al, 1997)

Agent	Cell/tissue	HA	HAS1	HAS2	HAS3	Reference
EGF	fibroblast	↑				(Heldin et al, 1989)
EGF	fibroblast	↑	↑	↑	↑	(Yamada et al, 2004)
EGF	keratinocyte	↑	-	↑	↑	(Pasonen-Seppanen et al, 2003)
EGF	keratinocyte	↑		↑		(Saavalainen et al, 2005)
EGF	oral mucosal cell	↑	↑	↑	↑	(Yamada et al, 2004)
EGF	neural crest cell	↑				(Erickson & Turley, 1987)
EGF	mesothelial cell	↑				(Honda et al, 1991)
EGF	cumulus cell	↑				(Tirone et al, 1997)
EGF	lung adenocarcinoma cell	↑	NE	↑	↑	(Chow et al, 2010)
17β-estradiol	uterine fibroblast	↑				(Tanaka et al, 1997)
estrogen	endometrium	↑				(Tellbach et al, 2002)
estrogen	uterine epithelium	↑				(Mani et al, 1992)
bFGF	fibroblast	↑				(Heldin et al, 1989)
FGF2	dental pulp	↑	↑	↑	-	(Shimabukuro et al, 2005b)
FGF2	periodontal ligament	↑	↑	↑	-	(Shimabukuro et al, 2005a)
FGF	fibroblast	↑	↑	↑	↑	(Kuroda et al, 2001a)
forskolin	orbital fibroblast	↑	↑	-	↑	(van Zeijl et al, 2010)
forskolin	human embryonic kidney cell	↑		↑		(Makkonen et al, 2009)
FSH	cumulus cell	↑				(Tirone et al, 1997)
glucose	mesangial cell	↑				(Ren et al, 2009)
HGF	epithelial cell	↑				(Zoltan-Jones et al, 2003)
IFN-γ	keratinocyte	↑	-	NE	↑	(Sayo et al, 2002)
IFN-γ	fibroblast	↑				(Sampson et al, 1992)
IGF	fibroblast	↑	↑	↑	↑	(Kuroda et al, 2001b)

Agent	Cell/tissue	HA	HAS1	HAS2	HAS3	Reference
IGF	mesothelial	↑				(Honda et al, 1991)
IL-1	fibroblast	↑				(Sampson et al, 1992)
IL-1β	fibroblast	↑	↑	↑	↑	(Yamada et al, 2004)
IL-1β	fibroblast	↑	↑	↑	↑	(Kaback & Smith, 1999)
IL-1β	uterine fibroblast	↑	↑	↑	↑	(Uchiyama et al, 2005)
IL-1β	synoviocyte	↑				(Kawakami et al, 1998)
IL-1β	synoviocyte	↑	-	-	↑	(Oguchi & Ishiguro, 2004)
IL-1β	orbital fibroblast	↑	↑	↑	↑	(van Zeijl et al, 2010)
IL-1β	umbilical vein endothelial cell	↑	NE	↑	-	(Vigetti et al, 2010)
IL-1β	lung adenocarcinoma cell	↑	NE	↑	↑	(Chow et al, 2010)
IL-6	fibroblast	↑				(Duncan & Berman, 1991)
KGF	keratinocyte	↑	-	↑	↑	(Karvinen et al, 2003b)
KGF	keratinocyte	↑				(Jameson et al, 2005)
leukemia inhibitory factor	osteoblast	↑	-	↑	NE	(Falconi & Aubin, 2007)
PTH	osteoblast	↑				(Midura et al, 1994)
PDGF	fibroblast	↑				(Heldin et al, 1989)
PDGF	mesothelial	↑				(Heldin et al, 1992)
PDGF	mesothelial	↑	-	↑	-	(Jacobson et al, 2000)
PDGF	vascular endothelial cell	↑		↑		(Suzuki et al, 2003)
PDGF	vascular smooth muscle cell	↑		↑		(Evanko et al, 2001)
PDGF	trabecular meshwork	↑		↑		(Usui et al, 2003)
PDGF	fibroblast	↑	↑	↑	-	(Li et al, 2007a)
PDGF	cardiomyocyte	↑				(Hellman et al, 2010)
PMA	fibroblast	↑				(Suzuki et al, 1995)
poly I:C	smooth muscle cell	↑				(de la Motte et al, 2003)

Agent	Cell/tissue	HA	HAS1	HAS2	HAS3	Reference
progesterone	uterine fibroblast	↑	↓	↓	↑	(Uchiyama et al, 2005)
prostaglandin D2	orbital fibroblast	↑	↑	↑	↑	(Guo et al, 2010)
prostaglandin J2	orbital fibroblast	↑				(Guo et al, 2010)
prostaglandin E2	synoviocyte		↑			(Stuhlmeier, 2007)
retinoic acid	epidermis	↑				(King & Tabiowo, 1981)
retinoic acid	epidermis	↑				(Tammi & Tammi, 1986)
retinoic acid	keratinocyte	↑		↑		(Saavalainen et al, 2005)
retinoic acid	keratinocyte	↑	-	↑	↑	(Pasonen-Seppanen et al, 2008)
retinyl retinoate	epidermis	↑		↑		(Kim et al, 2010)
testosterone	rooster comb	↑				(Jacobson, 1978)
TGF-β	fibroblast	↑				(Heldin et al, 1989)
TGF-β	fibroblast	↑	↑	↑		(Sugiyama et al, 1998)
TGF-β	keratinocyte	↑	↑	-		(Sugiyama et al, 1998)
TGF-β1	vascular endothelial cell		-	↑	-	(Suzuki et al, 2003)
TGF-β1	lung adenocarcinoma cell	-	NE	↑	-	(Chow et al, 2010)
TGF-β	trabecular meshwork	↑	-	↑	-	(Usui et al, 2003)
TGF-β	synoviocyte	↑	↑	-	-	(Oguchi et al, 2004)
TGF-β	synoviocyte	↑	↑	-	↓	(Stuhlmeier et al, 2004a)
TNF-α	synoviocyte	↑	-	-	↑	(Oguchi et al, 2004)
TNF-α	fibroblast	↑				(Sampson et al, 1992)
TNF-α	umbilical vein endothelial cell	↑	NE	↑	-	(Vigetti et al, 2010)
TNF-β	umbilical vein endothelial cell	↑	NE	↑	-	(Vigetti et al, 2010)
tunicamycin	smooth muscle cell	↑				(Majors et al, 2003)
tunicamycin	smooth muscle cell	↑				(Lauer et al, 2009)

b) Factors decreasing hyaluronan synthesis

↓ decreased, ↑ increased, - not changed, NE not expressed, empty not studied

Agent	Cell/Tissue	HA	HAS1	HAS2	HAS3	Reference
Pirfenidone	fibroblast	↓	↓	↓	↓	(Chung et al, 2014)
benzbromarone	fibroblast	↓				(Prehm et al, 2004)
5,7-dihydroxy-4-methylcoumarin	pancreatic cancer	↓				(Morohashi et al, 2006)
6,7-dihydroxy-4-methylcoumarin	pancreatic cancer	↓				(Morohashi et al, 2006)
dipyridamole	fibroblast	↓				(Prehm et al, 2004)
estradiol	vascular smooth muscle cell	↓	↓	-	-	(Freudenberger et al, 2011)
glucocorticoid	epidermis	↓				(Agren et al, 1995)
glucocorticoid	fibroblast			↓		(Zhang et al, 2000)
glucocorticoid	synoviocyte	↓	-	↓	↓	(Stuhlmeier & Pollaschek, 2004b)
hydrocortisone	mesothelial cell	↓	-	↓	-	(Jacobson et al, 2000)
indomethacin	fibroblast	↓				(August et al, 1994)
indomethacin	fibroblast	↓				(Prehm et al, 2004)
mannose	keratinocyte	↓				(Jokela et al, 2008a)
MβCD	smooth muscle cell	↓				(Sakr et al, 2008)
MβCD	breast cancer cell	↓	NE	↓	-	(Kultti et al, 2010)
mefenamic acid	fibroblast	↓				(August et al, 1994)
4-MU	fibroblast	↓				(Nakamura et al, 1995)
4-MU	fibroblast	↓		↓	-	(Kakizaki et al, 2004)
4-MU	uterine fibroblast	↓				(Tanaka et al, 2007)
4-MU	keratinocyte	↓				(Rilla et al, 2004)
4-MU	melanoma cell	↓				(Kudo et al, 2004)
4-MU	melanoma cell	↓				(Yoshihara et al, 2005)
4-MU	pancreatic cancer cell	↓				(Nakazawa et al, 2006)
4-MU	breast cancer cell MCF-7	↓	NE	↓	-	(Kultti et al, 2009b)
4-MU	breast cancer cell MDA-MB-361	↓	NE	NE	↓	(Kultti et al, 2009b)
4-MU	melanoma cell	↓	NE	↓	↓	(Kultti et al, 2009b)
4-MU	ovarian cancer cell	↓	NE	NE	↓	(Kultti et al, 2009b)
4-MU	smooth muscle cell	↓	↓	↓	↓	(Vigetti et al, 2009b)
4-MU	fibroblast	↓				(Edward et al, 2010)
4-MU	breast cancer cell	↓	NE	↓	-	(Urakawa et al, 2012)
progesterone	uterine fibroblast	↓				(Tanaka et al, 1997)
S-decyl-glutathione	fibroblast	↓				(Prehm et al, 2004)

Agent	Cell/Tissue	HA	HAS1	HAS2	HAS3	Reference
TGF-β1	synoviocyte	↓	↓			(Kawakami et al, 1998)
TGF-β	mesothelial cell		↑	↓	-	(Jacobson et al, 2000)
TGF-β	keratinocyte	↓	-	↓	↓	(Pasonen-Seppänen et al, 2003)
TGF-β	keratinocyte	↓	-	NE	↓	(Sayo et al, 2002)
trequinsin	fibroblast	↓				(Prehm et al, 2004)
vesnarinone	myofibroblast	↓				(Ueki et al, 2000)
valspodar	fibroblast	↓				(Prehm et al, 2004)
verapamil	fibroblast	↓				(Prehm et al, 2004)
vitamin D	osteoblast	↓				(Takeuchi et al, 1989)

HAS genes are often regulated simultaneously (Kultti et al, 2009a, Vigetti et al, 2009) and transcriptional regulation of *HAS* genes often correlate with changes in the synthesis of hyaluronan (Jacobson et al, 2000, Pienimaki et al, 2001, Yamada et al, 2004). Growth factors, hormones, cytokines and artificially synthesized chemical compounds are known to alter *HAS* transcriptional activity and thereby hyaluronan synthesis (Jacobson et al, 2000, Karvinen et al, 2003b, Yamada et al, 2004, Zhang et al, 2000). The *HAS* isoforms respond differently to external stimuli, based on the cell type and treatment conditions (Jacobson et al, 2000). Growth factors are among the most studied effectors of hyaluronan synthesis. In keratinocytes, growth factors like epidermal growth factor (EGF) and keratinocyte growth factor (KGF) increase the mRNA levels of *HAS2* and *HAS3* in monolayer and organotypic cultures (Karvinen et al, 2003b, Pasonen-Seppanen et al, 2003, Sayo et al, 2002). Transforming growth factor β has differential effects on *HAS* expression, as it increases *HAS2* mRNA and protein levels in vascular endothelial cells (Suzuki et al, 2003) but suppresses *HAS3* and *HAS2* mRNA expression, respectively, in synoviocytes and keratinocytes (Pasonen-Seppanen et al, 2003, Stuhlmeier & Pollaschek, 2004a). In the case of *HAS1*, growth factors and cytokines like TGF-β and interleukin (IL)-1β act as inducers of *HAS1* mRNA expression (Stuhlmeier & Pollaschek, 2004a, Stuhlmeier & Pollaschek, 2004b). The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediates the IL-1β-induced upregulation of *HAS1* mRNA expression in synoviocytes (Kao, 2006, Stuhlmeier & Pollaschek, 2005). IL-1β, tumor necrosis factor (TNF)-α, and TGF-β induce upregulation of *HAS2* mRNA expression in endothelial cells (Vigetti et al, 2010). *HAS2* expression is also upregulated by TNF-α treatment in keratinocytes (Saavalainen et al, 2007). In fibroblasts, *HAS1* and *HAS2* mRNA levels are increased by TGF-β treatment (Sugiyama et al, 1998). In keratinocytes, interferon (IFN)-γ, IL-13 and IL-4 treatments increase *HAS3* mRNA expression (Ohtani et al, 2009, Sayo et al, 2002).

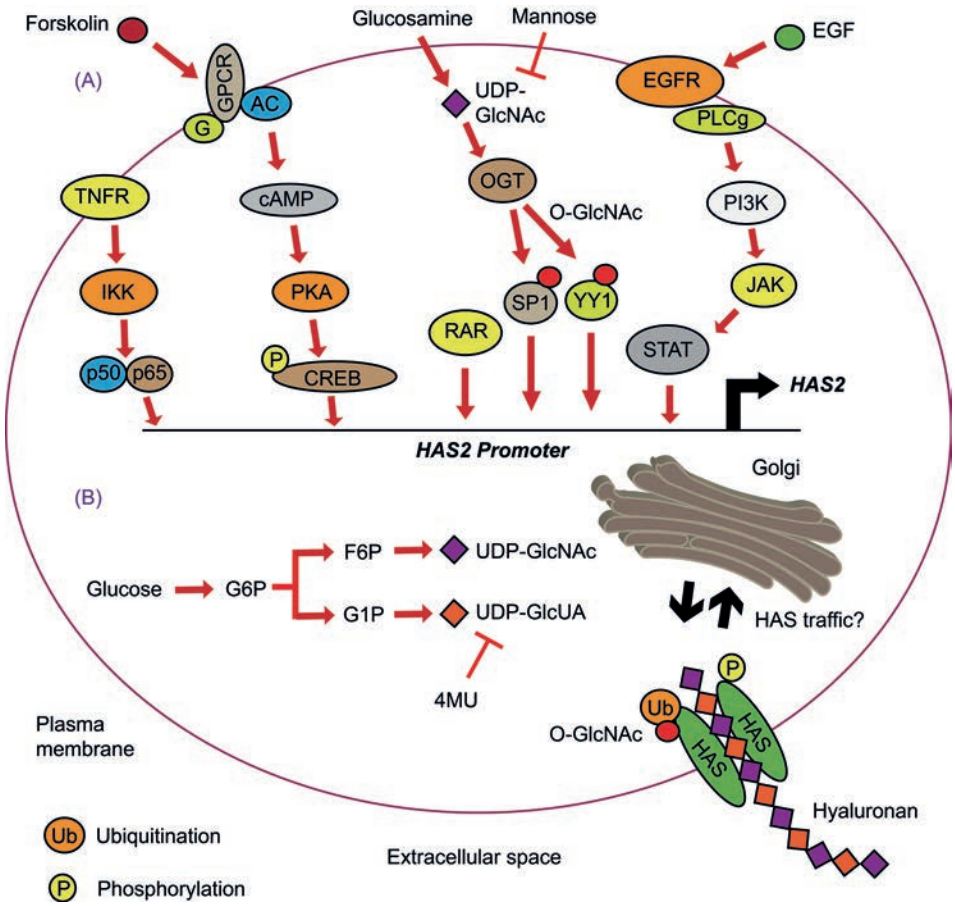

Figure 1. Post-transcriptional and post-translational regulation of hyaluronan synthases. (A) The functional binding sites for transcriptional factors p50, p65, CREB, RAR, SP1, YY1 and STAT are present in the promoter of human HAS2. And the signaling cascade events leading to the binding of transcription factors are presented here. (B) Metabolites from glucose yield UDP-activated precursor sugars for building hyaluronan. HAS resides predominantly in Golgi apparatus but is active in synthesizing hyaluronan only when present in plasma membrane. However, the molecular steps involved in HAS traffic to and from plasma membrane is still unresolved. HAS utilizes UDP-GlcUA and UDP-GlcNAc precursor sugars to synthesize hyaluronan and extrude the growing chain into the extracellular space. HASs can be post-translationally modified with phosphorylation, ubiquitination and O-GlcNAcylation and the significance of these modifications in regulating HAS activity and traffic is still not clearly understood (modified from Tammi et al, 2011). Abbreviations are explained in page numbers 11–12.

HAS2 transcription is also regulated by a variety of transcription factors such as specificity protein (SP) 1 and 3, signal transducer and activator of transcription 3 (STAT3) and cyclic adenosine monophosphate (cAMP) response element binding protein 1 (CREB1) (Makkonen et al, 2009, Monslow et al, 2004, Saavalainen et al, 2005). *HAS2* transcription is also regulated by EGF and retinoic acid (RA) (Saavalainen et al, 2005) and platelet derived growth factor-BB (PDGF-BB) (Jacobson et al, 2000). Hormones such as hydrocortisone and other glucocorticoids have been shown to downregulate *HAS2* mRNA expression and its stability in dermal fibroblasts and osteoblasts (Jacobson et al, 2000, Zhang et al, 2000). *HAS2*

transcription is induced by adiponectin through an adenosine monophosphate kinase pathway (Yamane et al, 2011). A natural RNA interfering anti-sense *HAS2* (AS-*HAS2*) transcript has been described, and shown to stabilize and/or reinforce *HAS2* mRNA expression depending on the cell type. For example, AS-*HAS2* RNA is shown to inhibit *HAS2* mRNA expression in osteosarcoma cells while enhancing it in kidney epithelial and aortic smooth muscle cells (Chao & Spicer, 2005, Michael et al, 2011, Vigetti et al, 2014). A detailed summary of regulation of the hyaluronan synthesis by several factors is presented in Fig. 1 and Table 1.

2.2.3 Regulation of HAS activity by trafficking and post-translational modifications

The enzymatic activity of HAS is associated with its plasma membrane localization, which involves traffic from the Golgi to the plasma membrane (Rilla et al, 2005). Additionally, several post-translational modifications of HAS are reported to influence its activity. There are multiple potential phosphorylation sites in the cytoplasmic tail and intracellular domains of HAS (example of sites: HAS2 – T110, S323, Y326, T412 and Y546; HAS3 – T6, Y329, Y333 and Y347) (www.phosphosite.org). Phosphorylation of HAS is expected to regulate its enzymatic activity and hyaluronan synthesis (Anggiansah et al, 2003, Bourguignon et al, 2007, Goentzel et al, 2006, Ohno et al, 2001, Vigetti et al, 2011). Recently, other modifications of HAS such as ubiquitination and O-GlcNAcylation have emerged. Ubiquitination exists in lysine 190 of HAS2 and a point mutation in this amino acid inactivates HAS2. In the same study it has been shown that HAS2 can form homodimers and also heterodimers with HAS3, and a failure to ubiquitinate the lysine 190 residue results in a dominant negative effect on the activity of HAS2/HAS2 homomers (Karousou et al, 2010). Serine 221 of HAS2 has been shown to be O-GlcNAc modified, and the modification increases HAS2 half-life and enzyme activity by suppressing proteosomal degradation (Vigetti et al, 2012). Thus, these studies suggest that post-translational modifications of HAS enzymes may play significant roles in regulating the enzymatic activity of HASs. A recent study on HAS oligomerization revealed that all HAS isoforms have the tendency to form homo- and heteromers, and the interaction is suggested to be present mainly in the N-terminal region, although the C-terminus also offers interaction potential. When presented together, HAS1 tends to inhibit the synthesis of hyaluronan by HAS2 and HAS3, which demonstrates the functional cooperation between the HASs as oligomers (Bart et al, 2015). Since there are very few published reports on this issue, studying post-translational modifications and trafficking of HAS, and their functional effects on the enzymes is of utmost significance.

2.2.4 Biosynthesis of UDP-sugars

Glucose metabolism is involved in the production of precursors for all glycoconjugates. Glycosaminoglycans (GAGs) like hyaluronan, containing an amino sugar (N-acetylglucosamine or N-acetylgalactosamine) and uronic acids (glucuronic acid or iduronic

acid) are particularly heavy consumers of the glucose-derived precursors (Afratis et al, 2012, Esko et al, 2009, Gandhi & Mancera, 2008) (Fig. 1) (II, Fig. 1). The synthesis of GAGs involves two different pathways of glucose metabolism, i.e. hexosamine and glucuronic acid biosynthesis. Hexosamine biosynthetic pathway (HBP) combines the metabolism of glucose, amino acids, fatty acids and nucleotides to synthesize uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) that exists in equilibrium with N-acetylgalactosamine (UDP-GalNAc) (Hanover et al, 2012). Glucuronic acid for glycoconjugate synthesis is derived from glucose and UDP to make uridine diphosphate glucose (UDP-Glc), which is then converted to UDP-GlcUA. In some cases, GlcUA is isomerized into iduronic acid (IdoA) after incorporation into the polymer (Li, 2010). HBP accounts for 2-5% of total glucose metabolism and is associated with the synthesis of glycolipids, proteoglycans, glycosylphosphatidylinositol (GPI) anchors, extracellular proteins with N-and O-linked oligosaccharides, and intracellular proteins with a single N-acetylglucosamine (O-GlcNAcylation) (Fantus et al, 2006). UDP-GlcUA biosynthesis begins with the conversion of glucose-6-P to glucose-1-P by phosphoglucomutase, and is followed by UDP-glucose formation from glucose-1-P by UDP-glucose pyrophosphorylase. The last step, from UDP-Glc to UDP-GlcUA, is considered the rate-limiting step in this pathway, catalyzed by UDP-glucose dehydrogenase (UGDH) (Freeze & Elbein, 2008). In HBP, glutamine fructose-6-phosphate amido transferase 1 and 2 (shortly, GFAT) and glucosamine-6-phosphate deaminase 1 and 2 (shortly, GNPDA) are the enzymes involved in the formation of glucosamine-6-P and this is considered the rate-limiting step of HBP (Marshall et al, 1991, Varki et al, 2008). Glucosamine-6-P is next N-acetylated, followed by conversion to GlcNAc-1-P and eventually UDP-GlcNAc by UDP-N-acetylglucosamine pyrophosphorylase. (Varki et al, 2008). It has been postulated that the function of GNPDA works in the reverse direction, from glucosamine-6-P to fructose-6-P. Degradation of glycoconjugates in the lysosomes also provides GlcUA and GlcNAc for reuse by the cells. Liver lysosomes for example can contribute up to 80% of GlcNAc from degradation of glycoconjugated proteins (Aronson & Docherty, 1983, Varki et al, 2008).

2.2.5 Regulation of UDP-sugar pools

Since the synthesis of hyaluronan depends on its precursor sugars i.e. UDP- glucuronic acid (UDP-GlcUA) and UDP-N-acetylglucosamine (UDP-GlcNAc), any change in the cytosolic level of these substrates regulates hyaluronan synthesis. 4-methylumbelliferone (4MU), a coumarin derivative, is a well-known suppressor of UDP-GlcUA content and thereby hyaluronan synthesis, as reported in a variety of cell lines such as skin fibroblasts (Nakamura et al, 1997, Nakamura et al, 1995), keratinocytes (Rilla et al, 2004), mesothelial cells (Rilla et al, 2008) and melanoma cells (Kudo et al, 2004). The facile glucuronidation of 4MU leads to a rapid depletion of UDP-GlcUA sugar pool in the cells. 4MU also influences the transcription of *HAS2* and *HAS3* genes (Kakizaki et al, 2004, Kultti et al, 2009b), perhaps through ceramide signaling (Qin et al, 2016). Mannose, a C-2 epimer of glucose, reduces the

cellular UDP-GlcNAc levels and thereby affects hyaluronan synthesis, as shown in cultured keratinocytes (Jokela et al, 2008). Although our understanding of the mechanism of action of mannose is incomplete, it has been suggested that mannose-6-phosphate targets the HBP pathway (Jokela et al, 2008) but not by transcriptional regulation of GFAT1 and 2 (Jokela et al, 2008). Instead, mannose is suggested to affect the enzymatic activity of glucosamine-6-phosphate isomerase (GPI) and thereby reduce the pool of UDP-HexNAc (i.e. UDP-GlcNAc and UDP-GalNAc) in Baby Hamster Kidney (BHK) fibroblasts (Cayli et al, 1999). Glucosamine treatment increases UDP-GlcNAc content as it skips the rate-limiting step of fructose-6-phosphate conversion to glucosamine-6-phosphate, and instead is directly phosphorylated to glucosamine-6-phosphate. Glucosamine-mediated increase of UDP-GlcNAc was first reported by Marshall and group in adipocytes (Marshall et al, 2005a, Marshall et al, 2005b), and a similar increase in the UDP-GlcNAc synthesis was also reported by GFAT overexpression in cultured mesangial cells and vascular smooth muscle cells (Schleicher & Weigert, 2000). Ammonium chloride increases UDP-GlcNAc in BHK fibroblasts and Chinese Hamster Ovary (CHO) cells (Ryll et al, 1994), which is probably mediated by GNPDA (Cayli et al, 1999).

2.3 TURNOVER OF HYALURONAN

Catabolism of hyaluronan is equally important as synthesis in the maintenance of tissue homeostasis, embryonic development, wound healing and regeneration (Stern & Jedrzejas, 2008). Hyaluronan is degraded by hyaluronidases (Hyals), which can be divided into distinct groups based on their end products. According to Karl Meyer (Meyer & Palmer, 1934, Meyer et al, 1941), there are vertebrate endo-β-N-acetylhexosaminidases that hydrolyse the glycosidic bond, and bacterial endo-β-N-acetylhexosaminidases that function by β-elimination (Stern & Jedrzejas, 2006). Another hyaluronidase group consists of endo-β-glucuronidases, found in leeches and crustaceans. Their mechanism of action is still largely unknown though they resemble the vertebrate version of Hyals (Karlstam et al, 1991, Yuki & Fishman, 1963). In human genome, there are 6 *HYAL* genes located on two different chromosomes; 1) *HYAL1–3* are found in chromosome 3p21.3, 2) *HYAL4*, Hyaluronidase pseudogene 1 (*PHYAL1*) and Sperm adhesion molecule 1 (*SPAM1*) are located in chromosome 7q31.3. In humans, HYAL1–3 are the most ubiquitous enzymes involved in hyaluronan degradation (Csoka et al, 1999, Stern, 2005a, Stern & Jedrzejas, 2006). HYAL1, an acid-active lysosomal enzyme, is the main hyaluronidase found in plasma and urine (Csoka et al, 1999, Frost et al, 1997). HYAL2 is mainly found in somatic tissues and it is located in the plasma membrane with a GPI anchor. HYAL2 is also active in acidic pH and degrades hyaluronan down to about 20 kDa in size (Lepperdinger et al, 1998, Stern, 2004). The rate of hyaluronan degradation is tissue-specific, as the half-life of hyaluronan varies from about 2.5–4.5 minutes in plasma (Fraser et al, 1981), to one day in the skin (Tammi et

al, 1991), and to a whole three weeks in cartilage (Morales & Hascall, 1988). After HYAL2 degradation, the hyaluronan fragments are taken up by the cells into lysosomes for complete degradation, probably by HYAL1 and two exoglycosidases, β-glucuronidase and β-N-acetylglucosaminidases (Stern, 2003). HYAL3 is widely distributed in human body, although it is predominantly found in testis and bone marrow, suggesting that HYAL3 has a role in stem cell regulation (Csoka et al, 1999, Csoka et al, 2001). Though there is a report stating that *HYAL3* knockout mouse showed no accumulation of hyaluronan, it is believed to have an activating effect on HYAL1 (Hemming et al, 2008). Deficiency of HYAL1 leads to a lysosomal storage disease called as mucopolysaccharidosis IX, with cutaneous swelling, painful soft tissue masses, disproportionate stature etc. (Natowicz et al, 1996, Triggs-Raine et al, 1999). Mice with HYAL1 knockout display osteoarthritis with accumulation of hyaluronan in joints (Martin et al, 2008).

Recently, a new hyaluronidase-like enzyme, KIAA1199 was reported by Yoshida et al (Yoshida et al, 2013). KIAA1199 was initially thought to be an inner-ear protein in Deiters cells and fibrocytes, and associated to deafness (Abe et al, 2003). Yoshida et al (2013) discovered that in human skin fibroblasts, KIAA1199 binds and catabolizes hyaluronan in an endo-β-N-acetylglucosaminidase type manner. In synovial fibroblasts isolated from osteoarthritis and rheumatoid arthritis patients, there is an increased expression of KIAA1199 (Yoshida et al, 2013). Another study points out that KIAA1199 is induced by human papillomavirus infection in cervical neoplastic lesions. KIAA1199 binds to and promotes EGFR signaling and results in EMT in carcinogenesis (Shostak et al, 2014). KIAA1199 expression is upregulated in colorectal and breast cancer (Evensen et al, 2013, Tiwari et al, 2013, Xu et al, 2015b) and it binds to glycogen phosphorylase kinase-β subunit (PHKB) and promotes glycogen breakdown, which is essential for survival of cancer cells (Terashima et al, 2014).

2.4 HYALADHERINS

Hyaluronan binds to many proteins, some of which are cell surface receptors and proteins involved in signaling. The link module superfamily of hyaladherins include aggrecan, neurocan, link proteins, LYVE-1, CD44, versican, brevican, neurocan, TSG-6, HARE and the 4 link proteins (Toole, 2004). Common to all these proteins is a 100 amino acids hyaluronan-binding domain (Day & Prestwich, 2002). Other molecules able to bind hyaluronan include IαI heavy chains, CDC37, hyaluronan binding protein (HABP), CD38, receptor for hyaluronan-mediated motility (RHAMM), and layilin (Bono et al, 2001, Day & Prestwich, 2002). A minimum of 10 sugar units of hyaluronan chain (HA10) is required for its binding to those members of the family with two link domains in tandem (Hascall & Heinegard, 1974a, Hascall & Heinegard, 1974b, Seyfried et al, 2005). However, hyaluronan oligosaccharides of < 10 sugars i.e. HA6 and HA8 suffice to displace high molecular weight

hyaluronan and act as antagonists for binding of hyaluronan to its receptors, or other hyaladherins, such as CD44 (Knudson & Knudson, 1993, Lesley et al, 2000, Tammi et al, 1998, Teriete et al, 2004, Underhill & Toole, 1979).

2.5 BIOLOGICAL FUNCTIONS OF HYALURONAN

Initially hyaluronan was thought to be just a space-filler in tissues but decades of research have revealed the importance of hyaluronan in the extracellular matrix for several biological functions such as inflammation, cell proliferation, general homeostasis, wound healing and tissue regeneration, to name but a few (Tammi et al, 2008, Tammi et al, 2011, Toole, 2000, Toole, 2004) (Fig. 2). While hyaluronan is synthesized and secreted by the cells into the surrounding medium, binding to cell surface receptors such as CD44 can also retain some of it in the pericellular space (Fig. 2). This formation of pericellular hyaluronan coat was first described in 1970's (Clarris & Fraser, 1968), using a test called as "red blood cell exclusion", where a suspension of red blood cells allowed to settle on cell cultures are excluded by the space-filling hyaluronan and other proteoglycans. The pericellular hyaluronan coat thus influences the shape and space occupied by the cells in tissues. Apart from endogenous hyaluronan coat produced by different types of cells such as dividing vascular smooth muscle cells, chondrocytes and bone-marrow derived mesenchymal stem cells (Heldin & Pertoft, 1993, Knudson & Knudson, 1993, Rilla et al, 2008), overexpression of exogenously added hyaluronan synthases can also induce pericellular hyaluronan coats (Itano et al, 1999, Kultti et al, 2006, Rilla et al, 2008, Siiskonen et al, 2013b).

2.5.1 Cell proliferation

Hyaluronan influences cell growth and proliferation, but depending on the cell type the effect varies. Hyaluronan is involved in activating signaling events related to cell proliferation, such as activation of mitogen-activated protein kinase (MAPK) cascade, in particular ERK kinase, by interaction of CD44 and epidermal growth factor receptor (EGFR), in addition to providing a favorable matrix to promote cell division (Brecht et al, 1986, Meran et al, 2011). In keratinocytes, hyaluronan accumulates in the cleavage furrow of mitotic cells during cell division (Tammi et al, 1991). Accumulation of hyaluronan is essential in cell proliferation and migration during the development of limbs (Li et al, 2007b). Hyaluronan is also required for the expansion of the cumulus cell-oocyte complex, and extrusion of the oocyte (Salustri et al, 1989, Salustri et al, 1999). Growth factors such as TGF-β and basic fibroblast growth factor (bFGF) induce hyaluronan synthesis to stimulate cell proliferation in embryonic mesoderm (Toole et al, 1989).

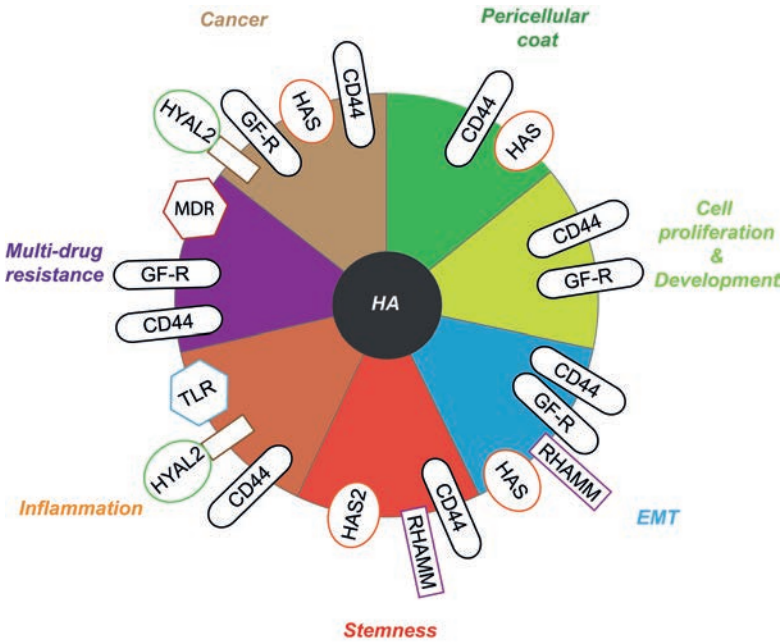

Figure 2. Functions of hyaluronan. Hyaluronan and its interaction with partner molecules like growth factor receptors (GFR), CD44, RHAMM, HAS, toll-like receptors (TLR), HYAL2 and multidrug resistance proteins (MDR), associated with several cellular functions and implications are highlighted; EMT = epithelial-to-mesenchymal transition.

4MU and mannose decrease hyaluronan synthesis by reducing the cytosolic levels of UDP-sugar substrates, and inhibit cell proliferation (Jokela et al, 2008, Rilla et al, 2004). On the other hand, inhibition of hyaluronan synthesis is required for pre-cartilage condensation of skeletal elements (Li et al, 2007b). Increased hyaluronan synthesis by secretion of growth factors is considered an adaptation by melanoma cells to promote cell proliferation (Willenberg et al, 2012a). However, contrary to the previous report, HAS3 overexpression and increased hyaluronan synthesis slows proliferation of cultured melanoma cells (Takabe et al, 2015).

2.5.2 Epithelial to mesenchymal transition

Hyaluronan plays a significant role in the epithelial-to-mesenchymal transition (EMT) of cells during tissue development, wound healing and cancer progression (i.e. invasion and metastasis). In a recent study on cardiac regeneration in a zebrafish model, expression of RHAMM, HASs and hyaluronan play an essential role in epicardial cell EMT and migration, and the whole signaling cascade involves FAK and Src kinases as downstream effectors for RHAMM. Also, in a mouse model, hyaluronan and RHAMM are upregulated during cardiac infarction. This suggests that hyaluronan is an important molecule in cardiac repair, which involves EMT and cell migration (Missinato et al, 2015). TGF-β is one of the stimulants for EMT cell morphogenesis and motility, as reported by several investigators (Brockhausen et al, 2015, Chanmee et al, 2014, Sengupta et al, 2013), and

hyaluronan is one of the downstream signaling molecules in EMT activation. When a mouse mammary epithelial cell line (NMuMg) is induced with TGF-β, expression of HAS2 is upregulated by the Smad/p38 mitogen-activated protein kinase pathway and eventually results in hyaluronan synthesis. Suppression of *HAS2* expression inhibits the TGF-β mediated EMT of the mammary epithelial cells (Porsch et al, 2013). Yet another study on lung and breast cancer cell lines points out that TGF-β1-mediated induction of *HAS1–3* expression and hyaluronan synthesis activates CD44-EGFR interaction and leads to upregulation of the downstream effectors AKT and ERK, and finally to EMT (Li et al, 2015). Interestingly, excessive hyaluronan production in mammary tumors of a HAS2 transgenic mouse model upregulates TGF-β expression and activates the transcription factors Snail and Twist, finally leading to EMT (Chanmee et al, 2014). HAS2 overexpression in Madin-Darby canine kidney and human mammary epithelial cells results in phenotypical changes corresponding to EMT (Zoltan-Jones et al, 2003). Several other growth factors and cytokines such as TNF-α and IL-1β are reported to stimulate hyaluronan-mediated induction of EMT in cancer and normal epithelial cells (Chow et al, 2010, Takahashi et al, 2010). In colon cancer cells, overexpression and suppression of CD44 increases and decreases EMT, respectively (Cho et al, 2012).

2.5.3 Support of stemness

A stem cell niche is formed by the surrounding cellular and extracellular factors in the microenvironment. The balance of these regulatory factors facilitate the ratio between cells that undergo self-renewal and differentiation (Jha et al, 2011, Li & Xie, 2005). During embryogenesis, hyaluronan mediates the EMT of progenitor cells to mesenchymal stem cells (MSCs) for the development of various tissues and organs (Shukla et al, 2010, Solis et al, 2012). Hyaluronan plays a vital role in the differentiation of human embryonic stem cells (hESCs) into hematopoietic stem cell lineage (HSCs) by regulating the expression of several marker genes. Using embryoid bodies from ESCs, grown as suspension, Schraufstatter *et al* (Schraufstatter et al, 2010) show that hyaluronan deprivation by hyaluronidase treatment results in a blockade of growth of CD45+ HSCs. Also, removal of hyaluronan in embryonic bodies by 4MU results in decreased expression of the early and late mesodermal markers *BRY* and *BMP2,* which leads to poor mesodermal differentiation (Schraufstatter et al, 2010). HAS2 acts as a significant source of hyaluronan during embryogenesis (Camenisch et al, 2000). CD44 and RHAMM interactions with hyaluronan establish cell migration and EMT during embryonic development (Craig et al, 2010, Hatano et al, 2012, Matrosova et al, 2004). Signaling cascade events due to CD44-hyaluronan interactions result in the activation of MEKK1 and ERK to promote cell proliferation, differentiation and EMT in embryonic stem cells (ESCs) (Craig et al, 2010, Hatano et al, 2011, Kothapalli et al, 2008). When primary human chondrocytes from osteoarthritis (OA) patients were cultured in a hyaluronan-rich medium, the effects were surprising; increased mitochondrial DNA integrity, improved ATP production, and better cell viability were observed (Grishko et al, 2009). This is one of

the studies that emphasises the role of hyaluronan on stemness. In mouse adipose derived stem cells, introduction of hyaluronan in culture medium drastically increases the growth rate of the cells in early passages, and significantly reduces cellular senescence (Chen et al, 2007). Culturing ESCs on a hyaluronan–coated surface instead of feeder layers resulted in the maintenance of pluripotency of the cells (Lutolf et al 2009). In support of this contention, undifferentiated stem cells during embryogenesis possess higher hyaluronan content than their differentiated counterparts (Toole, 1997). High molecular weight hyaluronan stimulates differentiation and invasion of epicardial cells, which are key steps in the formation of the coronary vasculature during embryonic development. To enable this process, hyaluronan initiates the association of CD44 with MEKK1 and promotes MEKK1 phosphorylation, in addition to persuading ERK- and NFκB-dependent pathways (Craig et al, 2010). Hyaluronan is also involved in enhanced proliferation, self-renewal and differentiation of neural precursor astrocytes through enhanced expression of connexin-26, -32, and -43 (Ahmed et al, 2009). In a 3D culture model of MSCs in a hyaluronan matrix, the expression of several inflammatory chemokines such as CXCL-4, -13, chemokine receptor CXCR5 and matrix metalloproteinases (MMPs) are changed (Lisignoli et al, 2006). This study also demonstrates that hyaluronan could act as a signaling molecule to activate MSCs in tissue regeneration, which involves active proliferation, self-renewal and differentiation of the stem cells (Lisignoli et al, 2006).

2.5.4 Role of hyaluronan in inflammation

Hyaluronan, based on its molecular size i.e. high and low molecular weight, has distinct functions in inflammation. High molecular weight hyaluronan is usually anti-inflammatory (Delmage et al, 1986). On the other hand, low molecular weight oligosaccharides of hyaluronan are pro-inflammatory (Rayahin et al, 2015, Stern et al, 2006). This size-dependent effect of hyaluronan makes it an adaptable molecule in several contexts i.e. tumor growth, gene expression, drug resistance, inflammation, angiogenesis etc. In a LPS (lipopolysaccharide) – induced lung inflammation model studied in mice, high molecular weight hyaluronan activates TLR4 in lung epithelial cells, which then inhibits nuclear translocation of NF-κB p65 and suppresses the secretion of inflammatory cytokines, thereby preventing the recruitment of inflammatory cells (Xu et al, 2015a). Hyaluronan level is amplified in inflammatory conditions such as skin and lung injury (Jiang et al, 2005, Tammi et al, 2005), arthritis (Goldberg et al, 1991) and asthma (Cheng et al, 2011). Hyaluronan forms cable-like structures during inflammatory conditions, which helps the attachment of inflammatory cells such as monocytes and other leukocytes in the affected sites (de la Motte et al, 2003, Jokela et al, 2015, Jokela et al, 2008). Hyaluronan can also influence inflammation indirectly by promoting cell proliferation and migration (Jokela et al, 2008, Jokela et al, 2013). During skin injury, inflammatory T cells release cytokines that enhance the expression of *HAS2 and 3*, and thereby increase hyaluronan synthesis by keratinocytes (Jameson et al, 2005). During inflammation, hyaluronan is degraded by hyaluronidase

HYAL2 or reactive oxygen species (ROS), resulting in fragmentation, which then acts as a stimulant for the expression of inflammation related genes such as IL12, IL-1β, TNFα and matrix metalloproteinases (MMPs) (Do et al, 2004, Horton et al, 1998, Iacob & Knudson, 2006, Taylor et al, 2007, Termeer et al, 2002). TLR2 and TLR4 are reported to activate hyaluronan-mediated inflammatory responses in lung injury (Jiang et al, 2005). Interestingly, low molecular weight hyaluronan downregulates adenosine A2a receptor (A2aR) in lung inflammation via a CD44-mediated signaling cascade and protein kinase C signaling (Collins et al, 2011).

2.5.5 Hyaluronan in multidrug resistance

The family of multidrug resistance proteins such as MDR1 (multidrug resistance transporter 1), MRP2 (multidrug resistance protein 2), and ABC transporter proteins are widely believed to mediate multidrug resistance (Guan et al, 2015, Moitra, 2015). It is interesting to note that hyaluronan is one of the agonists in the activation of these drug transporters (Misra et al, 2005, Ohashi et al, 2007). Several receptor tyrosine kinases (RTKs) like EGFR, IGIF1Rβ, c-MET, PDGFRβ and their phosphorylation of tyrosine residues are "activation" signals for ABC transporters (Shukla et al, 2012, Sun et al, 2012). Hyaluronan and CD44 interactions are the first and foremost event in the activation of RTKs and thereby ABC transporters (Chanmee et al, 2015). Hyaluronan-mediated activation of ErbB2 and the downstream signaling cascade involving the formation of CD44-phosphoinositide-3-kinase-ezrin-chaperones-cdc37 complex and Akt/MAPK pathways have been assigned a significant role in multi-drug resistance of cancer cells (Ghatak et al, 2005, Misra et al, 2003, Misra et al, 2005). Increased expression of Emmprin, a cell surface receptor, which increases hyaluronan production in cancer cells also promotes multi-drug resistance (Misra et al, 2003). In ovarian cancer patients, platinum-based chemotherapy increases serum hyaluronan levels and correlates with drug resistance. Cultured ovarian cancer cells also bestowed drug resistance and showed increased cell survival due to increased hyaluronan synthesis and CD44 and ABCC2 expression on the cell surface (Ricciardelli et al, 2013). Cultured human mesenchymal stem cells derived from placenta also showed drug resistance to doxorubicin by hyaluronan-mediated CD44 signaling, which then activates the PI3K/Akt pathway (Liu et al, 2009). Similarly, exogenously added hyaluronan increases multidrug resistance in head and neck squamous cell carcinoma cells (Wang & Bourguignon, 2006). Interestingly, low molecular weight hyaluronan oligosaccharides show the opposite effect and inhibit the chemoresistance of cancer cells (Misra et al, 2003).

2.6 Hyaluronan in cancer

Otto Warburg was the first to describe that cancer cells undergo a metabolic switch in which they, even in the presence of oxygen, favor energy production through glycolysis instead of oxidative phosphorylation (Warburg et al, 1924). This is logical because cancer cells divide rapidly and need a robust mode of energy production and a good supply of

building blocks provided by glycolysis intermediates. In cancer cells, glucose is readily available through upregulation of various glucose transporters, and metabolic enzymes in HBP are also activated (Gitenay et al, 2014), providing substrates for hyaluronan synthesis. Indeed, hyaluronan contents in most tumors differ from the corresponding normal tissues and depend on the specific tissue and the stage of tumor progression (Sironen et al, 2011). Tumor cells originating from stratified epithelia tend to produce less hyaluronan with advancing dedifferentiation of the tumor, while those originating from simple epithelia can show increased hyaluronan (Tammi et al, 2008). Apart from malignant cells, hyaluronan content in the surrounding stroma also has a significant role in carcinogenesis and metastasis. Indeed, in tumors originating from simple epithelia the degree of the peritumoral stromal hyaluronan increase determines the prognosis of the disease.

As an example of the interactions between tumor cells and stroma, and hyaluronan changes in tumors, melanoma cells produce PDGF and induce hyaluronan synthesis in stromal fibroblasts by increasing *HAS2* expression (Pasonen-Seppanen et al, 2012, Willenberg et al, 2012b). Hyaluronan content in the tumor varies according to the stage of progression, i.e. it increases from benign to localized melanoma tissues and then declines in more invasive tumors and metastases (Karjalainen et al, 2000). Interestingly, CD44 and HAS1–3 expression levels follow a similar pattern to that of hyaluronan in different stages of melanomas. On the other hand, hyaluronidase 2 (HYAL2) expression is increased in invasive and metastatic melanoma lesions, suggesting an inverse relationship between hyaluronan content and HYAL2 (Siiskonen et al, 2013a). The reduced expression of CD44 and hyaluronan thus correlate with poor patient survival (Karjalainen et al, 2000). A similar trend in hyaluronan and CD44 stainings, i.e. increased and reduced levels in well-differentiated and poorly differentiated tissues, respectively, is seen in many squamous cell carcinomas, like those in skin, mouth, esophagus, larynx and lung (Hirvikoski et al, 1999, Karvinen et al, 2003a, Kosunen et al, 2004, Pirinen et al, 1998, Wang et al, 1996).

In malignancies originating from simple epithelia, such as breast, colon and gastric cancers, a high level of cell-associated hyaluronan content is often associated with poor prognosis and tumor relapse (Auvinen et al, 2000, Kobel et al, 2004, Ropponen et al, 1998, Setala et al, 1999). In cancers of simple epithelia, stromal hyaluronan levels are also increased and are often associated with more invasive stages with breast, lung, prostate, ovarian, bladder and thyroid carcinomas as examples (Sironen et al, 2011, Tammi et al, 2008, Toole, 2004). Expression of all HAS (HAS1–3) isoforms in the stromal cells of breast cancer correlates with increased hyaluronan levels and also associates with poor survival rate of the patients (Auvinen et al, 2014). In another study on breast cancer, increased HAS2 expression correlates with triple-negativity of invasive ductal carcinomas (IDCs) and metaplastic carcinomas of the breast (MCB) (Lien et al, 2014).

Overexpression of *HAS2* is associated with increased tumor growth in a rat model of colon carcinoma (Jacobson et al, 2002). Increased *HAS3* expression is observed in metastatic colon

carcinoma cells, isolated from lymph nodes, and the cells also possess increased pericellular hyaluronan. When *HAS3* expression is inhibited with anti-sense oligonucleotides, the colon cancer cells showed significantly reduced anchorage-independent growth (Bullard et al, 2003). Overexpression of HAS3 in pancreatic cancer cells induces loss of cell adhesion by removal of E-cadherin in plasma membrane and this, in part, is due to accumulation of hyaluronan, while the epithelial cell adhesion is restored following treatment with polyethylene glycolylated (PEGylated) human recombinant hyaluronidase (PEGPH20) (Kultti et al, 2014).

2.7 Rab GTPases and vesicular trafficking

Vesicular trafficking delivers proteins and any other molecules to the desired subcellular destination, which is vital to continued functioning of the cellular machinery (Stenmark, 2009). A family of small GTPase proteins like Rabs and Arfs controls vesicular trafficking in eukaryotic cells. The family of Rab GTPases, comprising more than 60 proteins, is the largest among the small GTPases that belong to the Ras superfamily of monomeric G-proteins (Schwartz et al, 2007).

During vesicular trafficking, Rab-GTPases control 5 key steps: 1) sorting – by activating a cargo-associated sorting adaptor in the budding vesicle, 2) uncoating – by recruiting specific PI3K or phosphatases the PI composition is altered to remove the coat proteins, 3) motility – by recruiting adaptors for motor proteins or by directly binding to motor proteins to facilitate the movement of vesicles on the cytoskeletal "tracks", 4) tethering – by recruiting rod-shaped tethering factors to initiate contact with acceptor membranes and 5) fusion – by activating SNARE (soluble N-ethylmaleinide-sensitive factor (NSF) attachment protein receptor) complexes to mediate vesicle fusion (Stenmark, 2009). The Rab GTPases are master regulators of the intracellular traffic of proteins, lipids and sometimes a whole organelle like Golgi and mitochondria (Hutagalung & Novick, 2011, Schwartz et al, 2008, Stenmark, 2009). For example, during the sorting of mannose-6-phosphate receptors (M6PRs) from late endosomes to trans-Golgi network, Rab9 recruits a sorting adaptor TIP47 to facilitate the proper distribution of M6PR into the recycling buds (Carroll et al, 2001). Rab proteins are activated ("on" state) when bound to GTP and come back to resting "off" state with the GDP bound state (Hutagalung & Novick, 2011). Guanine exchange factors (GEFs) activate the GTP binding of Rabs and the reverse mechanism of inactivation is mediated by GTPase-activating proteins (GAPs) by removing an inorganic phosphate (Pi) (Boucrot et al, 2010, Schwartz et al, 2008).

Specific Rab proteins are associated with different itineraries of vesicular traffic and in some cases their functions can be redundant. For example, Rab5 and Rab7 are involved in vesicular traffic of early and late endosomes, respectively (Kummel & Ungermann, 2014, Mottola, 2014). However, Rab10 is involved with transportation of cargos from the trans-golgi network to the plasma membrane, i.e. on a secretory vesicle, and also in recycling

vesicles from early endosomes back to the plasma membrane (Babbey et al, 2006, Babbey et al, 2010, Chen et al, 2012). Rab GTPases recruit specific effectors to a particular membrane micro-domain and thereby specify membrane identity. For example, Rab5 recruits the enzyme PI3K-C2gamma in initiating Akt2 endocytosis (Braccini et al, 2015). Crosstalk between different Rabs is often observed during subsequent maturation of vesicles and this mechanism has been named "Rab conversion" (Hutagalung & Novick, 2011).

There are several examples of Rab involvement in receptor signaling. Downstream of EGFR, Rab5 activates its effectors – APPL1 and APPL2, which translocate to the nucleus and control gene expression and cell proliferation via NuRD histone deacetylase complex (Miaczynska et al, 2004). Several pathogens hijack Rab-dependent internalization pathways to enter their host cells. In the case of *Salmonella typhimurium*, 18 different Rabs are reported to be exploited by the pathogen in phagosomes during their maturation (Smith et al, 2007). *Helicobacter pylori* secretes a toxin, hijacking Rab7 in the phagosomes for the intracellular survival of the bacterium (Terebiznik et al, 2006). *Mycobacterium*, on the other hand, prevents recruitment of Rab7 and thereby inhibits phagosome maturation (Via et al, 1997).

Abnormalities in the expression of Rabs are often seen in cancer. Rab25, for example, is overexpressed in breast and ovarian cancers and is correlated with poor survival (Cheng et al, 2004). Loss of Rab21, which functions in endocytic trafficking of integrins, is seen in several cancers and correlates with aneuploidy (Pellinen et al, 2008). Rab23 is frequently overexpressed in sarcoma, breast, and colorectal cancer, and is downregulated in bladder and kidney cancer (www.oncomine.org). In gastric cancer, Rab23 amplification is associated with high invasion of the primary tumor cells and it is known that Rab23 attenuates sonic hedgehog signaling (Eggenschwiler et al, 2001, Hou et al, 2008).

3 Aims of the Study

The aim of this thesis work was to address how HAS trafficking regulates hyaluronan synthesis and to identify the factors controlling the plasma membrane presence of HAS. In doing so, HAS3 was chosen to be a representative enzyme among the HAS family due to its relative abundance in the plasma membrane, compared to the other HASs. Two issues were considered most important: the proteins involved in the HAS trafficking machinery, and the metabolism of the UDP-sugar substrates of HASs, including their significance in the progression of cancer.

More specifically, the targets were:

1. HAS3 dynamics in the plasma membrane and its contribution to the initiation and termination of hyaluronan synthesis.

2. Role of UDP-sugar metabolism in HAS3 traffic, hyaluronan synthesis, basic cellular functions and progression of melanoma.

4 Materials and Methods

4.1 MATERIALS

4.1.1 Cell lines

Table 2. Cell lines used in this thesis work. Standard culture conditions used for the cells are presented in the original publications.

S.No	Origin of the cells	Name of the cell line	Original publication in this thesis	Original reference
1	Human breast adenocarcinoma	MCF7	I	(Soule et al, 1973)
2	Human breast adenocarcinoma (with EGFP-HAS3 overexpression)	MCF7-EGFP-HAS3	I	Dr. Genevieve Bart, University of Eastern Finland
3	Human melanoma	MV3	II	(van Muijen et al, 1991)
4	Human melanoma (with EGFP-HAS3 overexpression)	MV3-EGFP-HAS3	II	(Takabe et al, 2015); Dr. Genevieve Bart, University of Eastern Finland
5	Monkey kidney epithelium	COS1	II	(Gluzman, 1981)
6	Human mesothelium	LP-9	III	Institute of Clinical Medicine, University of Eastern Finland
7	Human chondrosarcoma	HCS	III	(Takigawa et al, 1989)
8	Human melanoma	C8161	III	(Welch et al, 1991)
9	Dog kidney epithelium	MDCK	III	(Gaush et al, 1966)
10	Dog kidney epithelium (with EGFP-HAS3 overexpression)	MDCK-EGFP-HAS3	I, III	(Rilla et al, 2012); Dr. Aki Manninen, Biocenter Oulu,
11	Human epidermal keratinocytes	HaCaT	IV	(Boukamp et al, 1988)

4.1.2 Human tissue specimens

Diagnostic tissue samples were obtained from Kuopio University Hospital. After the initial biopsy, the tissues were fixed in 10% buffered formaldehyde, embedded in paraffin and sectioned 5 μm thick for histological staining. The ethics committees of Kuopio University Hospital and The Finnish National Supervisory Authority for Welfare and Health (VALVIRA) have approved the study protocol. Other details are presented in the original publication (II).

4.1.3 Plasmids, Antibodies and other reagents

Detailed descriptions are presented in original articles.

4.2 METHODS

Table 3. Methods to study HAS3 traffic. Detailed protocols are in original publications.

Purpose	Method	Original Publication	Reference
To track HAS3 vesicular movement between cellular compartments	Photo-conversion with Dendra2 fusion protein	I, II	(Gurskaya et al, 2006)
To study HAS3 endocytosis	Tracking photoconverted Dendra2-HAS3 from plasma membrane (PM) to inside of cells	I, II	Method optimized in original publication I
To study HAS3 residence in PM	Analyzing EGFP-HAS3 signal in PM	I	Method optimized in original publication I
To analyze HAS3 in endosomes	Colocalization analysis of EGFP-HAS3 with endosome markers, EEA1, clathrin heavy chain, transferrin and alexafluor hydrazide	I, II	Method optimized in original publications I, II
To identify and track HAS3 recycling in PM	1) Tracking cell surface biotinylated EGFP-HAS3 and endocytosis and surface reappearance 2) TIRF imaging of HAS3 vesicle dynamics on cell surface	II	(Sun et al, 2014) Method optimized in original publication II
To evaluate HAS3 secretion in extracellular vesicles	2D and 3D cultures, confocal, TEM and SEM imaging and flow cytometry analysis	II, III	Method optimized in original publication III
To study HAS3 degradation in lysosomes	Photoconversion of whole cells and analysis of half-life of Dendra2-HAS3	II	Method optimized in original publication II
To study Golgi-to-PM traffic of HAS3	Tracking photoconverted Dendra2-HAS3 from perinuclear region to PM	II	Method optimized in original publication II

Table 4. Methods to analyze hyaluronan and UDP-sugar content.

Purpose	Method	Original Publication	Reference
To study pericellular hyaluronan coat formation	Quantification of staining with fluorescent HABC probe	I, III, IV	(Rilla et al, 2008)
To study hyaluronan secretion in culture medium	Enzyme-linked sorbent assay for hyaluronan	I, II, III, IV	(Hiltunen et al, 2002)
To measure UDP-sugar content	Anion-exchange HPLC	II, IV	(Oikari et al, 2014, Tomiya et al, 2001)

Table 5. Methods to study effect of hyaluronan synthesis on cell biological functions.

Purpose	Method	Original Publication	Reference
To study cell proliferation	Cell counting, DAPI staining	I, II, IV	Vierodt 1852 (invented the method for cell counting) & Louis-Charles Malassez (invented hemocytometer)
To study cell migration	Scratch wound assay	II, III	(Todara 1965)
To study cell adhesion to type I collagen	Colorimetric assay with MTT dye	I, II	Method optimized in original publication I

Table 6. Other standard methods used in this thesis work.

Purpose	Method	Original Publication	Reference
HAS3 overexpression	1) Lentiviral transduction of EGFP-HAS3, which is inducible by doxycycline 2) Linearization of EGFP-HAS3, followed by stable insertion and selection by neomycin resistance	I, II, and III	(Rilla et al, 2012) (Siiskonen et al, 2013) (Takabe et al, 2015)
mRNA knockdown	SiRNA transfection	I, II, IV	(Fire et al, 1998, Hamilton & Baulcombe, 1999)
To analyze proteins	Western blot	I, II, IV	(Burnette, 1981, Renart et al, 1979, Towbin et al, 1979)
To identify proteins involved in HAS3 traffic	Sucrose gradient ultracentrifugation and mass spectrometry	I	Method optimized in the original publication (I)
To identify subcellular organelles	Dyes and antibodies in western blot, live cell imaging and immunofluorescence	I, II, III	Method optimized in corresponding original publications (I-III)

Purpose	Method	Original Publication	Reference
To inhibit or enhance synthesis of UDP-sugars and hyaluronan	4MU, mannose, glucosamine, and siRNA (against GFAT1, GNPDA1 and 2, UGDH) treatments	II, IV	(Rilla et al, 2004), (Jokela et al, 2008) and optimized in original publications II and IV
To identify O-GlcNAc modification of HAS3	ThiametG and OGT siRNA to respectively increase and decrease O-GlcNAc modification; Western blot using RL2 anti-O-GlcNAc antibody	II	(Holt et al, 1987, Snow et al, 1987, Torres & Hart, 1984, Vigetti et al, 2012c, Yuzwa et al, 2008) and optimized in original publication II

5 Results

5.1 CONTROL OF HAS3 TRAFFIC BY RAB10

5.1.1 Rapid turnover of HAS3 in plasma membrane

The plasma membrane specific marker mRFP-Rpre was used as a marker to locate the EGFP-HAS3 population present in the plasma membrane (I, Fig. 5A). The pericellular hyaluronan coat, stained with the fluorescently labelled HABR probe, was used as a marker for cell surface (I, Fig. 6A). Using photoconversion of Dendra2-HAS3 from green-to-red, the traffic of HAS3 from the plasma membrane was analyzed in MCF7 cells. In control cells, the traffic of Dendra2-HAS3 was so fast that there was already a large movement of "red" HAS3 from surface to cell interior (I, Fig. 6A) at the 2 min time point. EGFP-HAS3 transport vesicles were mostly positive for EEA1, an early endosome marker (I, Fig. 9A), suggesting that endocytosis of HAS3 is rapid and very important in maintaining the plasma membrane residence of the enzyme.

5.1.2 Rab10 silencing increases the plasma membrane residence of HAS3

Analysis of HAS3 transport vesicles by mass spectrometry in MDCK cells overexpressing GFP-HAS3 led to identification of proteins related to vesicular traffic, the prominent one being Rab10 (I, Table 2). Co-immunoprecipitation and colocalization assays in MCF7 cells showed the association of Rab10 and HAS3 in transport vesicles (I, Fig. 1-2). In MCF7 cells transiently transfected with EGFP-HAS3, Rab10i showed an increased plasma membrane signal of HAS3 (I, Fig. 5A,B) without influence on the overall signal of EGFP-HAS3 in whole cells (I, Fig. 5C).

In cells with Rab10i, the traffic of Dendra2-HAS3 from the cell surface was significantly inhibited and most of the photoconverted "red" HAS3 stayed at the cell surface (I, Fig. 6A). Kinetic analysis showed that, in control cells, within ~5 min almost 50% of the photoconverted Dendra2-HAS3 was transported from the cell surface into the cell and at the end of the experiment (16 min) there was only 30% of the original signal in the cell surface, while following Rab10i about 73% of the signal was left in the cell surface (I, Fig. 6B).

5.1.3 Rab10 regulates clathrin-mediated early endocytosis of HAS3

The mass spectrometry results suggested that HAS3 vesicles contain clathrin heavy chain (Table 2 in I) and so clathrin-mediated trafficking is a likely explanation for HAS3 transportation. In MCF7 cells, clathrin heavy chain was significantly colocalized to EGFP-HAS3 with a Pearson's correlation coefficient value (*Rr*) of 0.46, when compared to empty

EGFP (mock) vector (*Rr* = 0.19) (I, Fig.7A,C), suggesting that HAS3 is associated with clathrin and that its endocytosis is likely accounted by clathrin-coated vesicles. In support of this finding, mRFP-HAS3 was partially colocalized with fluorescein-conjugated transferrin (*Rr* = 0.24), a marker for clathrin-mediated vesicular trafficking. In contrast, no colocalization was observed between EGFP-HAS3 and a fluid-phase endocytosis marker, Alexafluor hydrazide 594 (*Rr* = 0.07) (I, Fig.7B,D), indicating that HAS3 follows clathrin-mediated endocytosis.

The colocalization of mRFP-HAS3 with fluorescein-conjugated transferrin was significantly reduced in cells with Rab10 knockdown (*Rr* = 0.13), compared to control and scrambled siRNA treated cells (*Rr* = 0.24 and 0.21 respectively) (I, Fig. 8A,B). At the same time, total transferrin uptake was not changed by Rab10 siRNA (I, Fig. 8C). Moreover, EGFP-HAS3 was colocalized with an early endosome marker, EEA1 (Early Endosomal Antigen 1) with an *Rr* value of 0.37, while Rab10 knock down significantly reduced it (*Rr* = 0.21) (I, Fig. 9A,B). The results indicate that Rab10 is important for the clathrin-mediated early endocytosis of HAS3.

5.2 UDP-SUGAR AVAILABILITY CONTROLS HAS3 TRAFFIC AND HYALURONAN SYNTHESIS

5.2.1 Manipulation of cellular UDP-sugar contents and O-GlcNAcylation of HAS3

Changes in the cellular levels of UDP-GlcUA and UDP-GlcNAc were analyzed in MV3 cells stably overexpressing EGFP-HAS3 (i.e. MV3-EGFP-HAS3) following treatments by 4MU (0.5 mM), mannose (20 mM), and glucosamine (0- 2 mM), and by siRNAs against the enzymes GFAT1, GNPDA1 and 2, and UGDH. The effects of these treatments on cellular UDP-sugars and hyaluronan synthesis are presented in (II, Fig. 2A-C, E-G) and summarized in Table 7.

Western blotting of EGFP-HAS3, extracted and immunoprecipitated from MV3 cells, was positive for O-GlcNAc modification (II, Fig. 2M,N) when probed with the RL2 anti-O-GlcNAc antibody. The treatments used to modify O-GlcNAcylation of HAS3 did not influence the UDP-sugar contents of the cells (II, Fig. 2D,H). However, altering the cellular UDP-GlcNAc content of the cells affected the O-GlcNAc modification level (Table 7).

5.2.2 Endocytosis of HAS3 is regulated by UDP-sugars and O-GlcNAcylation

The perinuclear signal of Dendra2-HAS3 in MV3 cells was colocalized with the Golgi marker Golgin 97 (II, Suppl. Fig. 1C). Using the green-to-red photoconversion in the putative Golgi region, the traffic of Dendra2-HAS3 to the plasma membrane was analyzed for a time period of 1 h (II, Suppl. Fig. 2A). Compared to control, all the treatments listed in Table 7 showed a slower arrival of Dendra2-HAS3 in the plasma membrane (II, Suppl. Fig. 2C) – thus making the result difficult to interpret.

Table 7. Treatments affecting UDP-sugars, O-GlcNAcylation of HAS3 and hyaluronan content in MV3-EGFP-HAS3 cells

S.No.	Treatment	Concentration	UDP-GlcUA	UDP-GlcNAc	O-GlcNAcylation of HAS3	Hyaluronan
1	4MU	0.5 mM	↓↓	ns	ns	↓
2	UGDHi	40 nM	↓	↓	-	↓
3	Mannose	20 mM	↓	↓↓	↓↓	↓↓
4	Glucosamine	0.5-2 mM	↑	↑↑	↑	↑↑
5	GFAT1i	40 nM	ns	↓	-	↓
6	GNPDAi	40 nM	ns	↓	-	↓
7	ThiametG	20 µM	ns	ns	↑	↑
8	OGTi	40 nM	ns	ns	↓↓	↓

↓ Decrease; ↓↓ < 0.5 fold; ↑ Increase; ↑↑ > 2 fold; ns – not significant; "-" no data

Endocytosis of photoconverted Dendra2-HAS3 from the plasma membrane was analyzed in MV3 cells using deep mask red as a marker for the plasma membrane. Depletion of UDP-sugars with 4MU or mannose increased the endocytosis of Dendra2-HAS3. In a similar fashion, when GFAT1, GNPDA and UGDH were knocked down to deplete the UDP-sugars, endocytosis of Dendra2-HAS3 was increased (II, Fig 3A, B). In contrast, a surplus of UDP-GlcNAc (with 2 mM glucosamine) significantly reduced endocytosis of Dendra2-HAS3, while 1 mM glucosamine did not differ from the controls, suggesting a threshold level of UDP-GlcNAc that starts to significantly retard endocytosis.

ThiametG and knockdown of OGT decreased and increased, respectively, the endocytosis of Dendra2-HAS3 (II, Fig 3A, B). Taken together, the results suggest that plasma membrane residence of HAS3 is directly proportional to cellular UDP-sugar levels and O-GlcNAcylation of HAS3.

Interestingly, when the hyaluronan chain attached to Dendra2-HAS3 was removed by adding *Streptomyces* hyaluronidase in the culture medium, endocytosis of HAS3 was enhanced (II, Fig 3A, B). Since CD44 is one of the principal receptor for hyaluronan and is found in abundance on the surface of MV3 cells, the impact of CD44 on HAS3 endocytosis was also studied. Results showed that knocking down CD44 with a siRNA did not affect HAS3 endocytosis (II, Suppl. Fig. 3). This suggests that synthesis and elongation of hyaluronan, but not its anchorage to CD44 receptor, determine the presence of HAS3 in plasma membrane.

5.2.3 Increased UDP-GlcNAc level and O-GlcNAcylation inhibit lysosomal degradation of HAS3

Since the endocytosed HAS3 can be routed to degradation or recycling, it was important to check its rate of degradation. Dendra2-HAS3 in MV3 cells was photoconverted in the entire cell and the disappearance of the red signal was monitored over a time period of 5 h. Using transmitted light, DIC images were also taken alongside to normalize the fluorescence and control for possible errors in focusing. In control cells, the half-life of Dendra2-HAS3 was around 3 h and at the end of the 5 h observation period only ~30% of the original signal remained (II, Fig. 4A,B). When lysosomal degradation of proteins was blocked by chloroquine (100 μM), the stability of Dendra2-HAS3 was increased, while blocking proteasomal degradation by MG132 (2.5 μM) showed no effect (II, Fig. 4A,B), suggesting that HAS3 is mainly degraded in the lysosomal pathway.

Glucosamine treatment slowed down the degradation of Dendra2-HAS3. Similarly, treatment with ThiametG also significantly reduced the rate of Dendra2-HAS3 degradation (II, Fig. 4A,B). However, other treatments did not influence the degradation rate of HAS3. The results suggest that increased UDP-GlcNAc levels, perhaps through O-GlcNAcylation, shielded HAS3 from lysosomal degradation.

5.2.4 HAS3 recycling in plasma membrane is regulated by UDP-sugars

The robust endocytosis of HAS3 that takes place in a matter of minutes, versus its slower degradation rate in hours suggest that once endocytosed a major chunk of the protein could be recycled back to the plasma membrane. When endocytosis was enhanced by depletion of UDP-sugars, inhibition of O-GlcNAcylation, or treatment with hyaluronidase, EGFP-HAS3 accumulated in early endosomes (II, Fig. 5A). Early endosomes are therefore a likely source for the possible recycling of HAS3. To confirm the idea of HAS3 recycling, the extracellular part of EGFP-HAS3 was labelled with a DTT-cleavable, hydrophilic biotin (i.e. EZ™-link Sulfo-NHS-SS-biotin; see methods in II). By studying the endocytosis of biotinylated EGFP-HAS3 into the cytoplasm and its reappearance on the cell surface, recycling of EGFP-HAS3 was confirmed. Depletion of UDP-GlcUA (4MU) and UDP-GlcNAc (mannose) resulted in more endocytosis and less recycling of EGFP-HAS3. A surplus of UDP-GlcNAc by glucosamine and increased O-GlcNAcylation by ThiametG led to more EGFP-HAS3 recycling to the plasma membrane (II, Fig. 5B). To monitor HAS3 recycling in more detail, TIRF microscopy was utilized to capture the dynamics of EGFP-HAS3 vesicles 100-200 nm under plasma membrane (i.e. TIRF zone). The appearance and disappearance of EGFP-HAS3 vesicles in the TIRF zone was recorded for a time period of 2 min with 0.5 s time intervals (II, Fig. 6) and for kinetic analysis the first 70 seconds recordings of the vesicle turnout was used (II, Fig. 7). Depletion of UDP-GlcUA (4MU and UGDHi) and UDP-GlcNAc (mannose, GFATi and GNPDAi) reduced the numbers of transient visits of the EGFP-HAS3 positive vesicles in the TIRF zone (II, Fig. 6, 7E). Inhibition of O-GlcNAcylation

also led to reduction of these vesicles, considered to reflect recycling of EGFP-HAS3. In contrast, a surplus of UDP-GlcNAc by glucosamine, and increased O-GlcNAcylation by ThiametG, increased the visits of EGFP-HAS3 positive vesicles. (II, Fig. 6, 7E). These results demonstrate that endocytosis and recycling are important for maintaining HAS3 in the plasma membrane, and that these are governed by the availability of UDP-sugars and O-GlcNAcylation.

5.3 UDP-SUGAR CONTENTS AND HAS3 RELEASE IN EXTRACELLULAR VESICLES

5.3.1 Release of hyaluronan-coated extracellular vesicles in the culture medium

Active hyaluronan production on the cell surface was accompanied by numerous long plasma membrane protrusions, and release of vesicles positive for hyaluronan in the culture medium (III, Fig. 1). In C8161 and MV3 cells with overexpression of EGFP-HAS3 and grown in an experimental 3D culture setup, the released vesicles carried HAS3 and were trapped in the collagen matrix (III, Fig. 1I). When MDCK-GFP-HAS3 cells were grown in the absence of glucose, both hyaluronan production and the release of HAS3-positive extracellular vesicles were attenuated (III, Fig. 5). The results suggest that hyaluronan synthesis stimulates the release of extracellular vesicles containing EGFP-HAS3 from the plasma membrane. Most strikingly, some of the vesicles were shed directly from the tips of the microvillus-like protrusions (II, Fig. 8A,B) – a signature organelle for hyaluronan synthesis. The matrix of cells with depleted UDP-GlcUA (4MU, UGDHi) or UDP-GlcNAc (mannose, GFATi, GNPDAi) showed significantly less extracellular vesicles containing EGFP-HAS3. On the other hand, increased UDP-GlcNAc content by glucosamine led to an overflow of the EGFP-HAS3 positive extracellular vesicles. Changes in O-GlcNAcylation caused changes in parallel to those of UDP-GlcNAc: increased O-GlcNAcylation by ThiametG enhanced vesicle shedding while its inhibition by OGTi resulted in reduced numbers of extracellular vesicles containing EGFP-HAS3 (II, Fig. 8A,B). The results suggest that any disturbance in the metabolism of UDP-sugars can influence the release of HAS3-positive extracellular vesicles.

5.3.2 Hyaluronan synthesis initiates secretion of HAS3-driven extracellular vesicles

In transmission electron microscopic analysis, the control MDCK-GFP cells showed negligible numbers of vesicles in the extracellular matrix. In contrast, MDCK-GFP-HAS3 cells showed an abundance of vesicles, especially in the size range of 50-1000 nm (III, Fig. 6). Vesicles budding from the tips of plasma membrane protrusions and the surface of the plasma membrane itself were noted (III, Fig. 6C,D). Scanning electron microscopic analysis showed long plasma membrane protrusions with tiny vesicle buds on their tips (III, Fig. 7A-C). In the 3D culture model, MDCK-GFP-HAS3 cells grown as cysts showed both basal and apical side release of vesicles, positive for GFP-HAS3, actin, hyaluronan, and CD44 (III, Fig. 4B,D,F,H,J), Treating the GFP-HAS3 vesicles with HA6 oligomers did not result in the

disappearance of the hyaluronan coat, suggesting that the hyaluronan is not bound to CD44 (III, Fig. 3D). The proportion of hyaluronan present in the purified GFP-HAS3-positive vesicles was only ~0.5% of the total secreted hyaluronan (III, Fig. 3F). HAS3 overexpression disturbed epithelial architecture and created an anomalous presence of cells in the lumen of the cyst cultures (III, Fig. 4A,C,E,G,I).

When LP9 cells were treated overnight with 4MU, an inhibitor of hyaluronan synthesis, all the plasma membrane protrusions and vesicle budding sites disappeared – correlating with reduced hyaluronan secretion in the growth medium (III, Fig. 7E,G). However, when 4MU was removed, the protrusions and vesicle budding sites reappeared alongside the recovery of hyaluronan secretion (III, Fig. 7F,G). Thus, the results indicate that hyaluronan synthesis is the activation factor for the release of HAS3-driven extracellular vesicles.

5.4 HEXOSAMINE BIOSYNTHESIS IN THE MAINTENANCE OF UDP-GLCNAC CONTENT AND HYALURONAN SYNTHESIS

5.4.1 Silencing of GFAT and GNPDA enzymes in keratinocytes and melanoma cells

In this study, the role of GFAT and GNPDA enzymes in regulating cellular UDP-GlcNAc and thereby hyaluronan synthesis was documented in the human HaCaT keratinocytes. In HaCaT cells, the mRNA level of GFAT2 enzyme remained close to the detection limit in standard culture conditions, while GFAT1, and GNPDA1 and 2 showed robust expression. SiRNA-mediated silencing reduced the mRNA levels of GFAT1 and GNPDA1 below 20% of controls, and GNPDA2 to ~40% (IV, Fig. 2A,H,K). Furthermore, there were significant reductions in the protein levels (IV, Fig. 3A,B). Interestingly, silencing GNPDA1 resulted in an approximately ten-fold upregulation of GFAT2 mRNA level, also when combined with GFAT1 siRNA, but not when combined with GNPDA2 siRNA (IV, Fig. 2D-F). Another interesting cross-regulation was a slight increase of GFAT1 mRNA and protein levels that was observed with GNPDA2i (IV, Fig.2B-C, 3A-B).

In contrast to keratinocytes, siRNA-mediated silencing of GFAT1 and GNPDA1 and 2 in MV3 melanoma cells did not show any complex feedback cross-regulation in mRNA levels of the enzymes (data not shown). The results suggest that the rate-limiting step of hexosamine biosynthetic pathway is subject to complex regulation at multiple levels, including feedback control of gene expression, in addition to post-translational regulation of enzymes and availability of substrates. Furthermore, it is possible that the function and gene expression of GFAT and GNPDA enzymes appears to vary depending on the cell type i.e. normal or cancer phenotype.

5.4.2 Maintenance of UDP-GlcNAc and HAS2 expression by GFAT and GNPDA

In keratinocytes, GFAT1i resulted in a significant decline in UDP-GlcNAc and also UDP-GalNAc (IV, Fig. 4A,B), because these two UDP-sugars exist in an equilibrium maintained by UDP-galactose-4-epimerase (IV, Fig. 4C,D). When GFAT1i was combined with

knockdown of GNPDA1, GNPDA2 or both, the levels of UDP-GlcNAc and UDP-GalNAc also declined, perhaps even more than with GFAT1i alone (IV, Fig. 4E,F). The data showed that GFAT1 is a major determinant of cellular UDP-GlcNAc. While either GNPDA1 or 2i applied alone had no effect, their combination resulted in an increasing trend in cellular UDP-GlcNAc and UDP-GalNAc content (IV, Fig. 4A-B,E-F). The cellular levels of UDP-Glucose and UDP-GlcUA were not affected by GFAT1i and/or GNPDAi (IV, Fig. 4G,H). The results suggest that when GFAT1 is suppressed, GNPDAs start to synthesize UDP-GlcNAc, while they act in the opposite direction when GFAT1 is fully active.

However, in MV3 melanoma cells, both GFAT1i and GNPDA1 and 2i resulted in a significant decrease in cellular UDP-GlcNAc (Table 7). Based on the above data, it seems that the function of GNPDAs in maintaining cellular UDP-GlcNAc content is different in keratinocytes and melanoma cells, while GFAT1 in both the cell types synthesizes UDP-GlcNAc.

In keratinocytes, GFAT1i and GNPDA2i resulted in a significant upsurge of *HAS2* mRNA expression (IV, Fig. 7A,B). Additionally, the combination of GFAT1i with GNPDA1i or GNPDA2i or both led to a similar increase in *HAS2* expression. However, GNPDA1i nullified the effect of the increased *HAS2* expression by GNPDA2i (IV, Fig. 7C). The result indicates that with a low supply of UDP-GlcNAc in the absence of GFAT or GNPDA enzymes, increased *HAS2* expression represents an attempt to maintain sufficient hyaluronan synthesis.

5.5 INFLUENCE OF HAS3 TRAFFIC AND UDP-SUGARS ON HYALURONAN SYNTHESIS

When Rab10 expression was silenced using siRNA-mediated knockdown in MCF7 cells overexpressing EGFP-HAS3 (i.e. MCF7-EGFP-HAS3 cells), hyaluronan was significantly increased in the growth medium and pericellular coat (I, Fig. 4A, D-E). An opposite effect of decreased hyaluronan secretion was observed when EGFP-Rab10 was overexpressed (I, Fig. 4B). Rab10i also increased hyaluronan secretion by endogenous, non-transfected HAS in MCF7 cells (I, Fig. 4C), indicating that Rab10 is an important component in the molecular machinery involved in HAS3-dependent hyaluronan synthesis.

Depletion of UDP-sugars with 4MU and mannose significantly reduced hyaluronan secretion in MV3-EGFP-HAS3 cells (II, Fig. 2I). On the other hand, increased UDP-GlcNAc content following glucosamine treatment in MV3-EGFP-HAS3 cells produced a significant and dose-dependent increase in hyaluronan secretion (II, Fig. 2J). Knocking down GFAT1, GNPDAs and UGDH resulted in a significant reduction of hyaluronan secretion in MV3-EGFP-HAS3 cells (II, Fig. 2K). In HaCaT keratinocytes, while GFAT1i led to a significant reduction of hyaluronan secretion (IV, Fig. 5A), GNPDA1 and 2 siRNAs applied alone tended to increase hyaluronan and their combination yielded a significant increase (IV, Fig. 5B-C). Application of GFAT1i together with GNPDA1+2i decreased hyaluronan secretion

(IV, Fig. 5C), in line with the data on UDP-GlcNAc synthesis. Pericellular hyaluronan, another facet of hyaluronan synthesis, reproduced the results of secreted hyaluronan seen with GFAT and GNPDA siRNAs (IV, Fig. 6A,C). These results indicate that changes in UDP-sugar levels have a direct influence on hyaluronan synthesis.

Hyaluronan secretion in growth medium was increased and decreased, respectively, with ThiametG and knockdown of OGT (II, Fig. 2L), implying that O-GlcNAcylation controls HAS3 activity, even without changes in UDP-sugar levels. These results are summarized in Table 7.

5.6 EFFECT OF HAS3 TRAFFIC AND UDP-SUGAR LEVELS ON CELL PROLIFERATION, ADHESION AND MIGRATION

Hyaluronan has been reported to influence several basic cellular functions such as proliferation, cell division and migration. Therefore, treatments that affect hyaluronan synthesis were expected to cause these phenotypic changes. In MCF7-EGFP-HAS3 cells, doxycycline induction of EGFP-HAS3 resulted in a dose-dependent decline of cell numbers after a 48 h culture (I, Fig. 10A) but no significant change was observed in 24 h (data not shown). Similarly, Rab10i, which increased hyaluronan, also decreased cell proliferation in MCF7-EGFP-HAS3 and MCF7 parental cells (I, Fig. 10B). In MV3-EGFP-HAS3 cells, treatment with 4MU, mannose and glucosamine displayed a significant reduction in proliferation (II, Fig. 9A). GFATi and GNPDAi showed a slight increase in proliferation. Other treatments did not differ significantly from the control (II, Fig. 9A). The results suggest that increased hyaluronan synthesis, beyond a baseline, inhibits cell proliferation in these cell types and culture conditions. However, in keratinocytes, GFATi and GNPDAi did not influence cell proliferation after a 48 h culture (IV, Fig. 8A,B). One likely explanation is that, in keratinocytes, the magnitude of change in hyaluronan synthesis from the endogenous level is not enough to impact proliferation in the short term.

The effect of pericellular hyaluronan coat formation on cell adhesion to type I collagen matrix was studied in MCF7 and MV3 cells. Hyaluronidase-mediated removal of pericellular hyaluronan resulted in an increase in cell adhesion only in MCF7-EGFP-HAS3 but not in MCF7 parental cells (I, Fig. 10D). In MCF7-EGFP-HAS3 cells, Rab10i led to decreased cell adhesion to type I collagen but, when combined with hyaluronidase, the loss of cell adhesion was partially rescued (I, Fig. 10D). Exogenously added hyaluronan did not interfere with cell adhesion (I, Fig. 10F). Similarly, cell adhesion of MV3-EGFP-HAS3 cells to type I collagen was studied with altered UDP-sugar metabolism and O-GlcNAc signaling. With reduced cellular UDP-GlcUA (4MU, UGDHi), UDP-GlcNAc (GFATi, GNPDAi) and O-GlcNAcylation (OGTi), cell adhesion was significantly increased. However, with increased cellular UDP-GlcNAc (glucosamine) the cell adhesion was also elevated (II, Fig. 9B), while, increased O-GlcNAcylation by ThiametG did not show any difference from the control (II, Fig. 9B). The overall interpretation is that although cell

adhesion is partially dependent on pericellular hyaluronan, other potential factors are probably involved.

The effect of UDP-sugar content on cell migration was studied in MV3-EGFP-HAS3 cells. Compared to control, 4MU slowed whereas mannose stimulated cell migration. Glucosamine strongly inhibited the migration (II, Fig. 9C). In keratinocytes GFATi stimulated and GNPDAi slightly inhibited cell migration (IV, Fig. 8C), while mannose did not influence the migration at all. However, glucosamine significantly inhibited keratinocyte cell migration (IV, Fig. 8D). When the respective levels of UDP-GlcNAc (IV, Fig. 4E) and hyaluronan synthesis (IV, Fig. 5A,C) were compared in all these treatments, it appears that UDP-GlcNAc and hyaluronan contents are inversely proportional to the rate of keratinocyte migration.

5.7 UDP-SUGAR LEVELS ASSOCIATE WITH HYALURONAN CONTENT IN MELANOMAGENESIS

To study the expression of GFAT1 and UGDH in different stages of melanoma, mRNA levels of the enzymes were quantitated with real time PCR and compared in three different cell lines – normal primary human epidermal melanocytes (HEMa), and two metastatic melanoma cells i.e. MV3 and C8161. The mRNA expression of GFAT1 and UGDH declined in MV3 and C8161, when compared to HEMa (II, Fig. 10A). Interestingly, hyaluronan secretion in growth medium was also reduced in MV3 and C8161 when compared to HEMa cells (II, Fig. 10B). There appeared to be a correlation between the decline of GFAT1 and UGDH mRNA and hyaluronan content of the cells, reflecting increasing aggressiveness of the melanoma cells. Using human tissue samples representing different stages of melanomas, starting from benign nevus, dysplastic nevus, *in situ* melanoma, <1 mm and >4 mm deep invasive melanomas, GFAT1 expression was scored by immunohistochemical staining. In the tissues, GFAT1 staining was significantly increased in dysplastic nevus and *in situ* melanoma, when compared to benign nevus, while the expression declined through <1 mm to >4 mm deep melanoma tissues (II, Fig. 10C,E). The protein level of GFAT1 thus closely correlated with hyaluronan content of the tissues (Fig. 10C-F). The results suggest that biosynthesis of UDP-sugars and hyaluronan content correlate with each other and possibly with the progression of melanoma in its different stages.

6 Discussion and Conclusion

6.1 HAS endocytosis as a novel checkpoint for hyaluronan synthesis

While it is well-established that numerous factors influence hyaluronan synthesis through modulation of *HAS* gene expression (TNFα, IL-1β and EGF etc.), less is known about the role of post-translational regulation of HAS (Tammi et al, 2011). In this thesis work, it was shown for the first time that the rate of HAS3 endocytosis can control its ability to synthesize hyaluronan. Rab10 was shown to be the first protein known to regulate HAS3 endocytosis, and therefore influence hyaluronan synthesis (I) (Fig. 3). A similar case is seen in another polysaccharide, chitin, in *Drosophila*, where the Rab protein Ypt32p regulates the movement of chitin synthase towards the cell surface, and any change in the trafficking affects chitin synthesis (Ortiz & Novick, 2006). Rab10 is one of the Rab GTPases, a family of approximately 60 members acting as multi-functional regulators of intracellular vesicular traffic such as recycling endocytosis, transfer of proteins and cargos from trans-golgi network to plasma membrane, and exocytosis (Hutagalung & Novick, 2011, Schwartz et al, 2007). A recent study has shown that Rab10 is coupled to phospholipid biosynthesis and regulates the formation and dynamics of ER tubules (English & Voeltz, 2013). In *C. elegans* Rab10 has been found to enhance the formation of the recycling endosomes to plasma membrane by regulating phosphatidylinositol-4,5-bisphosphate levels (Shi et al, 2012). Interestingly, none of these studies have shown a role for Rab10 in retrograde transport from the plasma membrane, or in maintaining the plasma membrane residence of proteins. Therefore, the present work on HAS3 opens a new area of Rab10 functions.

Post-translational modifications of HAS, like di- or oligomerization, monoubiquitination, O-GlcNAc modification, and phosphorylation (Bart et al, 2015, Karousou et al, 2010, Vigetti et al, 2011, Vigetti et al, 2012) have been shown to play important roles in HAS activity and hyaluronan synthesis. However, it is not clear whether these post-translational changes actually affect HAS endocytosis at the cell surface, and therefore hyaluronan synthesis. The importance of HAS di- or oligomerization is partially supported by the fact that all Golgi glycosyltransferases form homo- and heteromers, and their functions and trafficking between ER and Golgi are regulated by homo- and heteromerization (Hassinen et al, 2010). Also, monoubiquitination, a known modifier of HAS2 protein, can regulate trafficking, especially plasma membrane maintenance of proteins such as EGFR and H-Ras (Fallon et al, 2006, Jura et al, 2006).

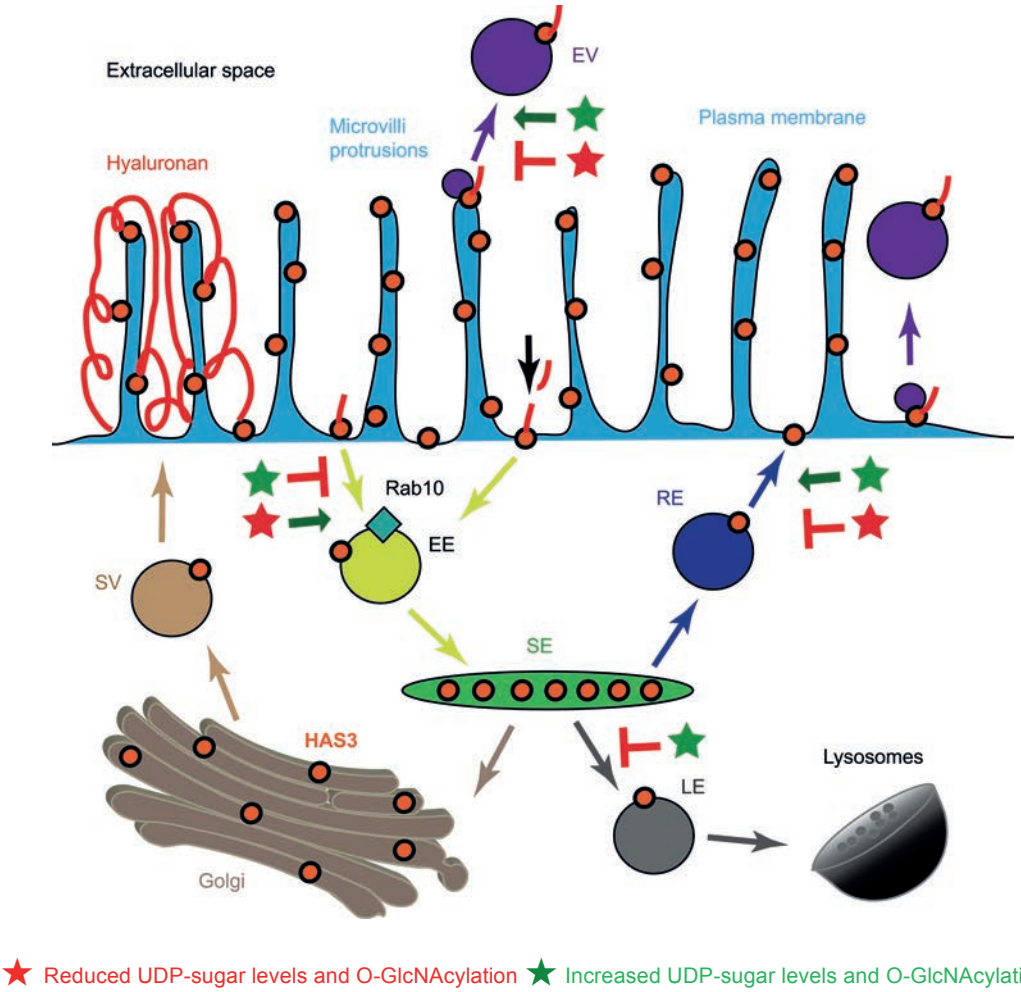

Figure 3. HAS3 traffic and hyaluronan synthesis. HAS3 follows the usual ER-Golgi secretion pathway and is activated in the plasma membrane to produce pericellular and extracellular hyaluronan around microvillus-like plasma membrane protrusions. HAS3 is transported in secretory vesicles (SV) from the Golgi to the plasma membrane but the intermediate machinery involved in storage and sorting of the protein between the organelles is yet to be identified. Rab10 regulates HAS3 endocytosis in an early step using clathrin-coated trafficking machinery. If the attached hyaluronan chain is removed using hyaluronidase, HAS3 endocytosis is triggered, implying that HAS3 turnover in the plasma membrane is associated with the probability of initiation and continuation of hyaluronan synthesis. The level of cellular UDP-sugars (UDP-GlcNAc and UDP-GlcUA) and O-GlcNAcylation, when declined, activates HAS3 endocytosis. In contrast, increased UDP-GlcNAc and O-GlcNAcylation results in inhibition of HAS3 endocytosis. In a similar fashion, HAS3 recycling is enhanced and reduced, respectively, with increased and decreased UDP-sugar levels and O-GlcNAcylation. Correspondingly, a surplus of UDP-GlcNAc and O-GlcNAcylation slows down lysosomal degradation of HAS3. Followed by a stimulus of hyaluronan synthesis, HAS3 is released in extracellular vesicles (Evs) either from the tip of microvilli-like protrusions or directly from the surface of the plasma membrane. HAS3 secretion in Evs is directly proportional to the availability of UDP-sugars and level of O-GlcNAcylation. Abbreviations: EE – early endosomes; SE – sorting endosomes; RE – recycling endosomes; LE – late endosomes.

6.2 HAS3 traffic is regulated by the availability of substrate sugars

The data of Itano et al (Itano et al, 1999) on the K_m values of HAS enzymes, and the estimated cytosolic levels of UDP-sugars (Jokela et al, 2008) approximately match with the hyaluronan synthase activity of HAS3. The present work shows that cellular levels of UDP-GlcUA and UDP-GlcNAc also control HAS3 traffic, implying that the metabolic state of the cells influences hyaluronan synthesis, and that this occurs through the transport of the HAS3 enzyme (II). The most important finding with regards to trafficking was that both UDP-GlcUA and UDP-GlcNAc controlled the plasma membrane levels of the enzyme.

HAS3 can also be O-GlcNAc modified, and the level of modification depends on UDP-GlcNAc availability. While the level of HAS3 O-GlcNAcylation parallels the cellular concentration of UDP-GlcNAc, O-GlcNAcylation influences HAS3 trafficking and activity also without concomitant changes in UDP-GlcNAc supply, as shown by the experimental suppression of OGT and O-GlcNAcase. These findings fit with the idea that O-GlcNAcylation is a mechanism to amplify the concentration effects of this UDP-sugar substrate on HAS3 activity. Indeed, there are previous examples showing that O-GlcNAcylation can influence vesicular trafficking of cargo proteins (Cole & Hart, 1999, Geng et al, 2012, Zhu et al, 2001), like other post-translational modifications such as phosphorylation (Chamberlain et al, 2012, Ko et al, 2012), acetylation (Gao et al, 2010, Lee et al, 2015) and SUMOylation (Chamberlain et al, 2012, Dai et al, 2011). It is difficult to tell whether the UDP-GlcNAc effects on HAS3 trafficking and activity are mediated entirely by O-GlcNAcylation. However, direct substrate effects on HAS3 trafficking and activity are evident, as demonstrated by depletion of UDP-GlcUA, the other precursor.

That being said, UDP-GlcUA and UDP-GlcNAc slightly differ in their effects. While excess UDP-GlcNAc protects HAS3 from lysosomal degradation, UDP-GlcUA seems to have no influence (II) (Fig. 3). This may be due to UDP-GlcNAc-induced O-GlcNAcylation of HAS3 (II), a result similar to UDP-GlcNAc protection of HAS2 from proteasomal degradation (Vigetti et al, 2012) through O-GlcNAc modification of HAS2 on the serine amino acid in position 221.

In aggregate, the experiments on the effects of UDP-sugars on multiple key points in HAS3 activity, including its endocytosis, shedding in microvesicles, lysosomal turnover, and also HAS2 gene expression, indicate that these substrates exert a major influence on hyaluronan.

6.3 Hyaluronan chain guides HAS trafficking

A growing hyaluronan chain is tightly bound to the processive hyaluronan synthase (Hubbard et al, 2012, Weigel & DeAngelis, 2007), and the maturation of full-length hyaluronan with an average size of > 1 MDa takes up to 3-4 h in fibroblasts and in yeast cells introduced with Xenopus HAS (Kitchen & Cysyk, 1995, Pummill et al, 1998). Hyaluronidase-mediated removal of the growing hyaluronan chain induces HAS3

endocytosis (II), thus supporting the claim that the hyaluronan chain under synthesis impedes HAS3 endocytosis (Fig. 3). However, disturbing the interaction of hyaluronan with its receptor, CD44, has no effect on HAS3 endocytosis (II) – suggesting that it is only the growing hyaluronan chain, and not the one attached to its receptor(s), that is involved in keeping HAS3 on the cell surface. The relatively long-standing bond between HAS3 and the growing hyaluronan chain is important since it appears to initiate and support the microvillous cell surface protrusions (Kultti et al, 2006, Rilla et al, 2008).

On the other hand, cellular availability of UDP-sugars can increase the likelihood of initiation of the hyaluronan chain. The likelihood of chain initiation can also be increased by reduction of the rate of HAS3 endocytosis, as demonstrated by the knockdown of Rab10 and high level of O-GlcNAc modification.

Recently published work by Weigel *et al*, (Weigel et al, 2015, Weigel, 2015) points out that in the presence of ample amounts of UDP-GlcNAc relative to UDP-GlcUA, *Se*HAS is able to synthesize chitin oligomers in the reducing-end of hyaluronan, where the synthesis begins. The authors speculate that chitin oligomers could thus prime hyaluronan synthesis which demonstrates the importance of UDP-GlcNAc in controlling HAS activity. The study also supports the notion that there could be additional functions for the substrate sugars in the initiation and elongation of hyaluronan chain. It is not known whether this kind of chitin priming can take place *in vivo*, or in vertebrate HASs, but this thesis work shows that a surplus of UDP-GlcNAc sustains HAS3 in the plasma membrane which stimulates hyaluronan synthesis (II).

Structural studies on bacterial cellulose synthase, another membrane-associated glycosyltransferase, show that its transmembrane domains produce a "pore" that could translocate the growing cellulose polymer into the extracellular space in a processive manner (Bi et al, 2015). It was recently demonstrated that hyaluronan can also be synthesized and translocated through the membrane by reconstituted *Streptococcus equisimilis Se*HAS in proteoliposomes (Hubbard et al, 2012). It is not known if *Se*HAS acts as monomers or oligomers in this model, but mammalian HASs can form both homo- and heteromers in live cells (Bart et al, 2015, Karousou et al, 2010). Dimerization or oligomerization could aid in pore formation and membrane translocation of the growing hyaluronan chain. Whether hyaluronidase-mediated truncation of the attached hyaluronan chain disrupts the oligomerization of HASs in the membrane, or just favors endocytosis of HAS, is still unexplored. Yet another interesting puzzle to be solved is the relationship between HAS oligomerization and traffic.

6.4 Dynamic recycling of HAS3 between endosomes and plasma membrane

The effects of UDP-sugars on HAS3 traffic between the Golgi to the plasma membrane appeared less significant than their regulation of HAS3 plasma membrane residence. This

could be speculated to be due to HAS3 trafficking from Golgi to an intermediate "recycling vesicles" compartment during the traffic towards the plasma membrane. This is apparently the case with some other proteins, for example Interleukin 6 (IL6) and TNFα (Manderson et al, 2007, Murray et al, 2005). In any case, continuous HAS3 recycling is seen between the plasma membrane and endosomes, indicating that this is an important process for the maintenance of HAS3 in the plasma membrane, and continued hyaluronan synthesis.

The recycling of HAS3 from endosomes to the plasma membrane is directly proportional to the availability of UDP-sugars in the cytosol (II) (Fig. 3). Probably a related event is that when the supply of substrate sugars declines, HAS3 accumulates in early endosomes (II). Endosomal accumulation was also observed with inhibited O-GlcNAcylation and hyaluronidase-mediated removal of the growing hyaluronan chain from the cell surface, as discussed before. This further supports the above suggestion that recycling endosomes act as an intermediate storage compartment during HAS3 traffic. Additionally, brefeldin-A treatment, which disturbs the Golgi-to-plasma membrane traffic of proteins, inhibits hyaluronan production, and this is accompanied by reduced HAS2 and HAS3 in keratinocyte plasma membrane (Rilla et al, 2005). Although this could be due to subdued Golgi-to-plasma membrane traffic of HAS, brefeldin A has an additional function of disrupting the organization of microtubules and actin, so that any vesicular transportation utilizing these cytoskeletal elements will be influenced, perhaps including recycling endosomes as a step in the HAS trafficking itinerary. This partly undefined, yet important route of HAS trafficking should be studied in more detail in the future to gain more insight into the molecular mechanisms of HAS trafficking.

6.5 Release of HAS3 vesicles in extracellular space

There is a constant flux of HAS3 to the plasma membrane, but since the average half-life of HAS3 in plasma membrane is ~5-6 min (I), not every HAS3 molecule reaching the plasma membrane initiates hyaluronan synthesis. However, a part of the HAS3 can, and must, stay longer to mature the growing hyaluronan chain. Except for the HAS3 molecules that begin hyaluronan synthesis, others apparently just depart from the plasma membrane and are destined for recycling to the plasma membrane or lysosomal degradation. In addition, the HAS3 in the plasma membrane has the option to be secreted into the extracellular space in vesicles budding from the plasma membrane (III) (Fig. 3). Examples of other proteins with similar behavior include cell surface receptors like integrins (Fedele et al, 2015) and EGFR (Adamczyk et al, 2011), matrix metalloproteinases (Hakulinen et al, 2008), cytokines (Konadu et al, 2015), and secreted proteins like Wnt (Gross et al, 2012). CD44 and actin are also released with HAS3 as fellow travelers in the extracellular vesicles, following a surge of hyaluronan synthesis (III). Endocytosis and recycling can actually favor the release of proteins into extracellular vesicles (Fang et al, 2007, Muntasell et al, 2007, Vidal et al, 1997).

Taken together, a part of HAS3 is secreted out into the extracellular space via an unknown mechanism. Also, the exact function of extracellular vesicles carrying HAS3 and hyaluronan is not understood. It is possible that hyaluronan binds to its cell surface receptors such as CD44 in the recipient cells and elicits a signal downstream of CD44 to communicate a message from the donor cells. HAS3 present in the extracellular vesicles could also carry HAS oligomers and hyaluronan from the donor to recipient cells and trigger hyaluronan synthesis. In fact, hyaluronan present in the extracellular vesicles may be one of the molecules responsible for docking the cargos onto the cell surface to deliver the contents to specific "target" cells. One could speculate that the contents of extracellular vesicles carrying hyaluronan are distinct from other vesicles of a similar nature. Clearly, these issues should be studied carefully in the near future.

In this thesis work, UDP-sugars and O-GlcNAcylation were shown to have a major influence on the shedding of HAS3-positive extracellular vesicles, the secretion of which correlates with a high level of HAS3 in plasma membrane and a high rate of hyaluronan synthesis (II, III) (Fig. 3). Surplus of UDP-GlcNAc and O-GlcNAcylation circumvents lysosomal degradation of HAS3, which could be the likely reason for the increased recycling of HAS3 to the plasma membrane and its subsequent vesicular release into the extracellular space. Although it is difficult to quantify the ratio of HAS3 undergoing endocytosis and shedding out of the cell, the meagre amount of HAS3 in the extracellular vesicles is assumed not to significantly influence its total turnover rate. Moreover, the accumulation of extracellular HAS3 takes a considerable amount of time i.e., 24-48 h (II, III), compared to endocytosis, which happens in a matter of minutes (I, II).

6.6 Synthesis of UDP-sugars and control of UDP-GlcNAc concentration

A single enzyme (UGDH) is considered to control the synthesis pathway to UDP-GlcUA, while the metabolism of UDP-GlcNAc (including UDP-GalNAc) is more complicated because four different enzymes, i.e. GFAT1 and 2, and GNPDA1 and 2, can catalyze the rate-limiting step in its synthesis pathway. UDP-GlcNAc is the end product of the hexosamine biosynthesis, and GFAT1 is the most studied enzyme in this pathway, and is also regarded as the principal enzyme governing the level of UDP-GlcNAc.

Both GFAT1 and 2 are subjected to regulation by phosphorylation, which is inhibitory in the former and stimulatory in the latter (Eguchi et al, 2009, Graack et al, 2001, Hu et al, 2004). GNPDAs can switch their catalytic role from the conversion of fructose-6-phosphate to glucosamine-6-phosphate to the reverse direction, depending on cell type, and the concentrations of their substrates like ammonia and glucosamine-6-phosphate (Alvarez-Anorve et al, 2011, Cayli et al, 1999). In this thesis work, knocking down GNPDA1+2 in keratinocytes resulted in an enhancement of cellular UDP-GlcNAc content, implying that keratinocyte GNPDAs catalyze the conversion of hexosamines (and UDP-GlcNAc) back to fructose-6-phosphate (IV). However, the same GNPDA1+2 knock down in melanoma cells

showed a drop in UDP-GlcNAc content, which means that melanoma GNPDAs act in catalyzing fructose-6-phosphate in the direction of UDP-GlcNAc synthesis (II). This is an interesting difference to note as it demonstrates the plasticity of GNPDAs in different cell types (Fig. 4).

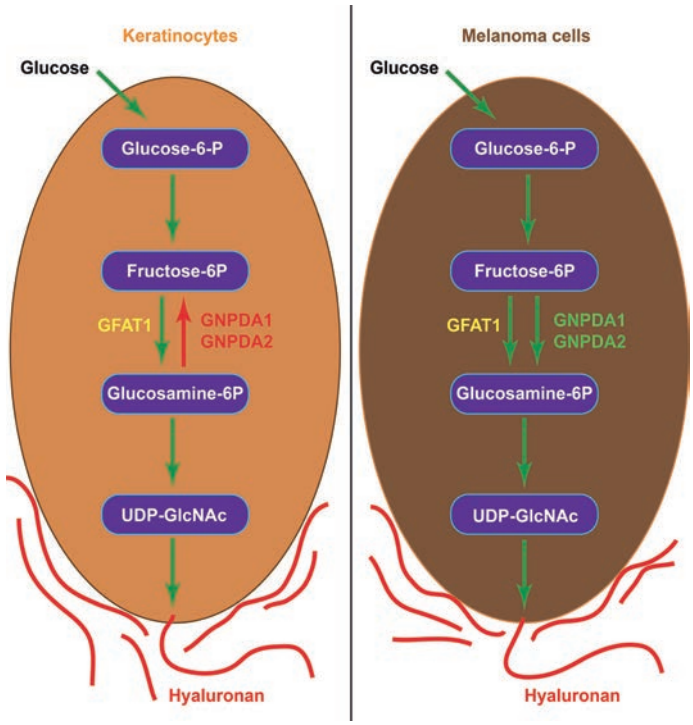

Figure 4. Hexosamine biosynthetic pathway in keratinocytes and melanoma cells in basal culture conditions. GFAT1 is the vital enzyme in catalysis of fructose-6-P to glucosamine-6-P and works in the same direction in both cell types. In keratinocytes, GNPDA1 and 2 convert glucosamine-6-P to fructose-6-P but in melanoma cells they work in reverse to convert fructose-6-P to glucosamine-6-P. Finally, glucosamine-6-P is converted to UDP-GlcNAc, which along with the other substrate, UDP-GlcUA, serve as building units of hyaluronan.

In keratinocytes, GFAT1 is the major enzyme catalyzing the formation of UDP-GlcNAc, while GNPDAs act together with GFATs in regulating the cellular UDP-GlcNAc content. This fine regulation is probably necessary because a certain level of UDP-GlcNAc is important for several functions – especially hyaluronan synthesis and O-GlcNAc signaling. For example, increased UDP-GlcNAc content hinders cell migration, as seen with the suppression of GNPDAs in keratinocytes (IV). Similarly increased UDP-GlcNAc content with glucosamine supply inhibits cell migration in both keratinocytes and melanoma cells (II, IV). On the other hand, decreased UDP-GlcNAc favors enhanced cell migration in keratinocytes and melanoma cells, as seen with suppression of GFAT1 in the former (IV) and mannose treatment in the latter (II).

Interestingly, there is also crosstalk between GFAT and GNPDA enzymes in transcriptional level, as knockdown of GFAT1 leads to an increased GNPDA2 mRNA level, and

knockdown of GNPDA1 results in a rise of GFAT2 mRNA. This again emphasizes the importance of maintaining a proper UDP-GlcNAc content in the cells.

6.7 Hyaluronan synthesis and UDP-sugars in cancer

This thesis work shows that hyaluronan synthesis is closely correlated with HAS trafficking and cellular content of UDP-sugars (i.e. UDP-GlcUA and UDP-GlcNAc), and that an upregulation of hyaluronan synthesis leads to an outburst of extracellular vesicles carrying part of the pericellular hyaluronan coat along with HASs, CD44 and actin in the cargo. In recent years, the role of extracellular vesicles carrying messages in the form of RNAs, proteins and signaling molecules, originating from different types of cancer cells has been studied and discussed in detail (Le et al, 2014, Minciacchi et al, 2015, Nishida-Aoki & Ochiya, 2015, Webber et al, 2015a, Webber et al, 2015b). However, the role of hyaluronan in the extracellular vesicles derived from cancer cells is still largely unexplored (Rilla et al, 2013b, Rilla et al, 2014). This thesis work shows that shedding of hyaluronan coated extracellular vesicles in cancer and normal cells are closely related to the synthesis rate of hyaluronan, and that the same extracellular vesicles also carry HAS enzymes (II, III). The type of inter-cellular messages carried by these hyaluronan-coated extracellular vesicles is an attractive topic to study in the near future.

Hyaluronan is involved in cell signaling, and cellular processes like migration, development, and proliferation (Toole, 2004), which are important properties in the progression of malignancies (Heldin et al, 2013, Jiang et al, 2011c, Schmaus et al, 2014, Stern, 2005b, Stern, 2008, Tammi et al, 2011a, Wu et al, 2015). In most of the cancers from epithelial origin, the amount of hyaluronan in cancer cells differs significantly from normal cells and correlates with the tumor grade and clinical prognosis of the patients (Sironen et al, 2011, Tammi et al, 2011). Thus, any information on factors affecting hyaluronan synthesis in cancer is important in solving the question why and how hyaluronan content changes in different types of cancer. In this thesis work, modulation of hyaluronan synthesis by manipulations of Rab10 and UDP-sugar concentrations showed profound effects on cell adhesion to type I collagen (I, II), and cell migration and proliferation were also affected (II, IV). However, some of the chemicals used in this study to alter UDP-sugar metabolism can have effects on cellular functions through mechanisms other than hyaluronan synthesis. For example, 4MU and glucosamine are known to inhibit cell proliferation, migration and invasion through signaling mechanisms beyond hyaluronan synthesis (Chou et al, 2015, Qin et al, 2016). Nevertheless, the role of hyaluronan on several cellular functions has been shown earlier by several independent investigations, and several approaches were used also in this study to modulate hyaluronan. This thesis shows that any change in hyaluronan synthesis and UDP-sugar metabolism influences cellular behavior with respect to its proliferation, migration and adhesion, cell biological processes often correlated with carcinogenesis.

Rab10 knockdown in a kidney cell line caused a disturbance in the epithelial cell polarity, resulting in a disorganized layer of cells in the formation of 3D tubular structures (Schuck et al, 2007) that could predispose to malignant growth. Interestingly, HAS3 overexpression in MDCK kidney epithelial cells with enhanced hyaluronan synthesis reproduced a similar effect of a deranged epithelial layer (Rilla et al, 2012). Also, Rab10 knockdown in MCF7 breast cancer cells with HAS3 overexpression led to reduced adhesion and significant reduction in cell proliferation (I). Data mining for differences in mRNA expression in cancer cells and tissues pointed towards an upregulation in Rab10 in cancers such as breast, kidney, lung and lymphoma, and a downregulation in esophageal cancer and leukemia (www.oncomine.org). The above mentioned observations suggest that Rab10's control of hyaluronan synthesis may be involved in cell adhesion to extracellular matrix proteins, which is a key process in cell polarity and epithelial-to-mesenchymal transition in carcinogenesis.

To date, there are very few reports available on UDP-sugar contents in cancers. However, mRNA expression profiles for the enzymes related to the biosynthesis of UDP-sugars are available in public databases like Oncomine™ Research Edition (www.oncomine.org). For example, mRNA expressions of GFAT1 and UGDH are elevated in breast cancer; GNPDA1 and UGDH are declined in kidney cancer. Increased GFAT2 expression is seen in breast cancer cells that are highly invasive (Simpson et al, 2012). Data on protein expression of these enzymes in tumors are very limited and correlation of their expression with hyaluronan content is not available so far. The present study on melanomas gives further support to the above literature suggesting that the expression of enzymes in UDP-sugar synthesis correlate with carcinogenesis, but studies on larger patient materials are needed to confirm and define the associations. There is a possibility that UDP-sugar metabolism contributes to the initiation and progression of melanoma and also to hyaluronan synthesis and its deleterious effects in other malignant tumors.

6.8 Conclusion and future directions

This thesis work has pointed out the regulation of hyaluronan synthesis by HAS3 endocytosis, which is in turn controlled by factors like Rab10-GTPase, UDP-sugar metabolism and O-GlcNAcylation. Additionally, HAS3 is also secreted into the extracellular space in vesicles, which is also regulated by UDP-sugar metabolism and O-GlcNAcylation. Disturbances in hyaluronan synthesis, caused by interference of UDP-sugar metabolism, Rab10 function, or O-GlcNAcylation, affect basic cellular functions such as proliferation, migration and adhesion to type I collagen. However, the effect varies in a complex manner depending on the cell type and magnitude of change in hyaluronan synthesis. Cellular expression of GFAT1 and UGDH mRNAs gradually declines from primary melanocytes to metastatic melanoma cells and correlates with the ability of the cells to synthesize hyaluronan. In human tissue samples, GFAT1 protein expression is increased in local (in situ) melanoma but then declines in advanced and invasive melanomas, again correlating with hyaluronan content and making these enzymes as candidate prognostic markers in melanoma and perhaps in other cancers.

In the future, molecular mechanisms of factors controlling HAS endocytosis should be investigated in detail. The possible machineries assembled by Rab10 to control the fate of HAS3 in the plasma membrane will be a crucial step to uncover how HAS dynamics in the plasma membrane controls the initiation and termination of the hyaluronan chain. Moreover, the role of Rab10 in HAS oligomerization is an interesting question to address.

Although this thesis work uncovers the importance of UDP-sugar substrates in the regulation of hyaluronan synthesis by controlling HAS3 traffic, many new questions arise concerning the molecular steps in further detail. One way for UDP-sugars to dictate the traffic is O-GlcNAc modification of HAS in its serine/threonine residues but their exact position in HAS sequence needs to be identified. A general difficulty in purification of HAS proteins for the purpose of mass spectrometry analysis has turned out to be a major roadblock for studying the post-translational modifications.

Other possible molecular “targets” of UDP-sugars in governing HAS traffic should be studied, perhaps by utilizing high-throughput techniques. Another key issue to be discussed, and conceivably investigated in the future, is to find the link between UDP-sugar metabolism and carcinogenesis. Molecular targets of UDP-sugar metabolism in the initial steps of carcinogenesis, such as changes in cell polarization, loss of cellular contact to extracellular matrix, and commencement of epithelial-to-mesenchymal transition should be revealed in more detail.

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The thesis showed the regulation of hyaluronan synthesis by HAS3 trafficking, which is in turn controlled by factors like Rab10-GTPase, UDP-sugar metabolism and O-GlcNAcylation. Additionally, HAS3 is also secreted into the extracellular space in vesicles, regulated by UDP-sugar metabolism and O-GlcNAcylation. Relationship between GFAT1 and GNPDA enzymes in regulating UDP-GlcNAc synthesis is also studied. In human tissue samples, GFAT1 protein expression correlates with hyaluronan content during melanoma progression, making these enzymes as candidate prognostic markers in melanoma and perhaps in other cancers.

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**PUBLICATIONS OF
THE UNIVERSITY OF EASTERN FINLAND**
Dissertations in Health Sciences

ISBN 978-952-61-2071-3
ISSN 1798-5706