The roles of cellular Factor XIII-A in osteoblasts

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June, 2015

A thesis submitted to McGill University in partial fulfillment of the requirements

of the degree of Doctor of Philosophy in Dental Sciences

CONTENTS

Abstract

Transglutaminases (TG) enzymes are expressed in wide variety of tissues and found in different cellular compartments, where they participate in various biological functions ranging from tissue stabilization to cell signaling. We have shown previously that MC3T3-E1/C14 osteoblasts express both transglutaminase 2 and Factor XIII-A (FXIII-A), the latter one appearing to be active as a transglutaminase during osteoblast differentiation. While data exists on the roles of FXIII-A as an extracellular stabilizer of matrix molecules in blood clots, during wound healing and during bone formation *in vitro*, the potential cellular functions of FXIII-A are not well understood. This thesis focuses on investigating the role of FXIII-A in osteoblasts and reports two new potential functions for FXIII-A. First contribution shows that in osteoblast FXIII-A activity targets the detyrosinated tubulin (Glu-tubulin) and promotes the formation of a high 150 kDa Glu-tubulin which is more stable in osteoblasts than the monomer form. This crosslinked Glu-tubulin is also membrane associated and found on the cell surface of osteoblasts where its presence is linked to secretion and deposition of extracellular matrix during osteoblast differentiation. We show that only α-tubulin, which gives rise to Glu-tubulin, is a TG substrate *in vitro* activity assay. The data in this thesis also shows that previously observed FXIII-A patches represent a pool of FXIII-A in caveolae, which are specialized membrane invaginations that have long been implicated in vesicular transport and signal transduction. We show FXIII-A co-localizes with caveolin-1 on the inner leaflet of plasma membrane in differentiating osteoblasts. Despite the presence of FXIII-A, caveolae had no detectable TG activity suggesting that FXIII-A may have a non-crosslinking function in caveolae. An irreversible TG inhibitor, NC9, which is capable of also altering TG enzyme conformation, displaced FXIII-A from caveolae which is linked to increased c-Src activation and increased caveolin-1 phosphorylation and homo-oligomerization. This suggests that cellular FXIII-A in osteoblasts has a role on regulating c-Src signaling. In summary, results in this thesis suggest that FXIII-A has both crosslinking and non-catalytic functions which regulate extracellular matrix accumulation and signaling pathways in osteoblasts, respectively.

Résume

Les transglutaminases (TG) sont des enzymes exprimées dans une grande variété de tissus. On les trouve dans différents compartiments cellulaires, où elles participent à plusieurs fonctions biologiques allant de la stabilisation cellulaire à la signalisation cellulaire. Nous avons précédemment montré que les ostéoblastes MC3T3-E1/C14 exprimaient la transglutaminase 2 et le facteur XIII-A (FXIII-A), ce dernier agissant comme une transglutaminase pendant la différentiation ostéoblastique. Alors que des donnees existent sur les roles de FXIII-A en tant que stabilisateur extracellulaire des molécules matricielles dans les caillots sanguins pendant la cicatrisation et la formation osseuse *in vitro,* ses fonctions cellulaires potentielles sont encore peu comprises. Ce travail de thèse porte sur le rôle de FXIII-A dans les ostéoblastes et démontre deux nouvelles fonctions potentielles pour FXIII-A.D'une part, au niveau des ostéoblastes, l'activité FXIII-A cible la tubuline détyrosinée (Glu-tubulin) et favorise la formation d'une Glu-tubuline de haut poids moléculaire 150 kDa qui est plus stable que la forme monomérique. Ce complexe Glu-tubuline est aussi associé à la membrane et est retrouvé à la surface cellulaire des ostéoblastes où sa présence est liée à la sécrétion et au dépôt de matrice extracellulaire lors de la différentiation ostéoblastique. Nous avons montré que seule l' α-tubuline qui engendre la Glu-tubuline est un substrat de TG *in vitro.* D'autre part, les données de cette thèse montrent aussi que FXIII-A précédemment observé provient d'un pool de FXIII-A dans les cavéoles. Les cavéoles sont des vésicules provenant de l'invagination de la membrane plasmique, impliquées dans le transport vésiculaire et la transduction du signal. Nous avons montré que FXIII-A est co-localisée avec la cavéoline-1 au niveau du bord interne de la membrane plasmique des ostéoblastes en cours de différentiation. Malgré la présence de FXIII-A, les cavéoles n'ont pas d'activité TG détectable, ce qui suggére que FXIII-A peut avoir une fonction non réticulante dans les cavéoles. Un inhibiteur irréversible de TG, NC9 qui est capable d'altérer aussi la conformation de l'enzyme TG déplace FXIII-A de la cavéole ; ceci est liée à une augmentation de l'activation de c-Src, de la phosphorylation et l'homo-oligomérisation de la cavéoline-1.Ceci suggère que FXIII-A cellulaire a un rôle

sur la régulation de la signalisation de c-Src. En résumé, les résultats de cette thèse suggèrent que FXIII-A possède une fonction de réticulation qui régule l'accumulation de la matrice extracellulaire et une fonction non catalytique qui régule les voies de signalisation.

ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. Mari T. Kaartinen for her mentorship and invaluable guidance. I am sincerely grateful for all the instructive feedback, as well as her patience that made this research project a truly memorable experience.

I would also like to thank the past and present members of the Kaartinen and McKee Laboratories for their advice, kindness and selfless assistance during my study. This thesis would not have been complete without your combined contributions. I especially thank Tchilalo Boukpessi for the translation of French abstact.

I would like to thank Mrs. Aisha Mousa for the technique assistance in all phases of my research work, Dr. Erika Wee in the Advanced BioImaging Facility (ABIF) of McGill University's Life Science Complex for excellent assistance in using TIRF microscopy and Mr. Liu Younan in Dr. Simon Trans lab for the assistance with their EPI fluorescence microscope.

To the members of my advisory/thesis committee, Dr. Dieter Reinhardt, Dr. Monzur Murshed, Dr. John Presley, I thank you for your expertise, time and insightful advices to better my research projects.

I would also like to thank Dr. Driss Mountassif in Dr. Isabelle Rouiller lab for the demonstration and guidance of ultracentrifuge usage.

Finally, I would like to express my sincere gratefulness to my parents and sisters. Without your unconditional love and endless support, I cannot imagine what my life would be. I wish you all the happiness and joy in life.

CONTRIBUTIONS OF AUTHORS

This thesis includes published paper and an unpublished manuscript of which the candidate is either the primary author. Individual contributions of all authors are listed as below:

1. Wang S, Cui C, Hitomi K, Kaartinen M (2014) Detyrosinated Glu-tubulin is a substrate for cellular Factor XIIIA transglutaminase in differentiating osteoblasts. Amino Acids 46 (6):1513-1526.

- S Wang designed and performed all experiments and data analysis, and wrote the manuscript.
- C Cui helped with preliminary study of the bF11 peptide localization by immunofluorescence finally presented in Figure. 4a.
- K Hitomi supplied peptide reagents (bF11, bF11QN) for the study.
- MT Kaartinen assisted in designing the experiments, supervised the study and edited the manuscript.

2. Wang S, Kaartinen M (2015) Cellular Factor XIIIA transglutaminase localizes in caveolae and regulates caveolin-1 phosphorylation, homo-oligomerization and c-Src signaling in osteoblasts. Journal of Histochemistry & Cytochemistry (resubmitted).

- S Wang designed and performed all experiments and data analysis, and wrote the manuscript.
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ABBREVIATIONS

AA	Ascorbic acid
βGP	β-glycerophosphate
bPA	5-(biotinamido)pentylamine
Cbz-Gln-Gly	N-α-benzyloxycarbonyl-L-glutaminylglycine
COL I	Type I collagen
ECM	Extracellular matrix
FN	Fibronectin
FXIII-A	Factor XIII A
MDC	Monodansyl cadavarine
MTs	Microtubules
TG	Transglutaminase
TG2	Transglutaminase 2
TIRF	Total Internal Reflection Fluorescence
Syt	Synaptotagmin

Chapter 1–Introduction

Bone

The human skeleton is composed of 206 bones of different sizes and shapes which operate together to fulfill locomotive and protective functions. The skeleton also acts as calcium and phosphate storage as well as regulates energy metabolism (1-3). Bone is a highly dynamic and specialized connective tissue that is remodeled throughout life in a process that maintains bone mass and quality. Any defects in this remodeling cycle lead to bone disorders such as osteoporosis and osteopetrosis. The cells responsible for maintaining bone mass are: osteoblasts, osteoclasts, and osteocytes which reside in different locations in the bone. Bone is formed by osteoblasts and resorbed by osteoclasts. The activity of these two cell types constitutes bone remodeling cycle which maintains bone in a constant state of renewal (4,5).

Bone tissue is formed during fetal mesenchymal development by two mechanisms: intramembranous and endochondral ossification. During intramembranous ossification, the mesenchymal stem cells condense and subsequently differentiate into osteoblasts and begin to secrete osteoid that eventually mineralizes and thus becomes bone. Some osteoblasts get trapped within the bone matrix and differentiate into osteocytes which serve a mechanosensory function and participate in the maintenance of bone mass by regulating the function of osteoblasts and osteoclasts (6,7). Most of the flat bones such as the skull, parts of the clavicle and the scapula are developed through intramembranous bone formation (8). In

endochondral ossification, bone is formed via cartilaginous template which is removed and replaced by bone matrix created by osteoblast activity (9). The majority of long bones are formed through endochondral formation.

Bone matrix and mineralization

Bone is mainly composed of inorganic mineral (hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ (HA), 69–80% by weight), organic matrix (primarily type I collagen (COL I), 17–20%) and water (not in free form, 9-10%) (10.11). Among the 28 types of collagen, that have been identified so far in vertebrates (12), COL I is the most abundant one. In bone, COL I provides tensile strength to the tissue and functions as a scaffold for mineralization, i.e., bone can be considered in a simplistic manner as matrix of COL I nanofibrils which has HA crystals precipitated within and between COL I fibrils (13). Before the process of matrix mineralization, osteoblasts first secrete a COL I rich extracellular matrix (ECM). The ECM also includes other non-collagenous ECM proteins such as fibronectin, osteocalcin, bone sialoprotein and osteopontin, which are thought to be able to regulate various protein-protein interactions and modulate the process of mineralization (14). The mineralization is generally thought to be initiated by the matrix vesicles that bud off the plasma membrane of osteoblasts to create an environment of high calcium and phosphate that allows the formation of HA nanocrystals. These crystals begin their growth inside the matrix vesicles and grow larger in the ECM upon their release from the vesicles (15,16). The propagation of mineralization requires an increase in phosphate concentration that is created by

phosphatase enzymes. One such enzyme is tissue-nonspecific alkaline phosphatase (TNAP) that cleaves the mineralization inhibitor pyrophosphate into two phosphate molecules. It has been suggested that the matrix vesicle constituents, especially the TNAP and nucleotide pyrophosphatase phosphodiesterase play important roles in creating nanocrystals in the confined vesicle space (17).

Collagen type I

In tendons, bone and skin, COL I is the major type of collagen (12). The COL I molecule consists of two α1-chains and one α2-chain ($\alpha1(I)_2\alpha2(I)$), which are encoded by COL1A1 and COL1A2 genes, respectively (18). The three α–chains organize around each other into a right-handed coiled coil, i.e., a triple helix (19). Like all fibrillar collagens, COL I is a triple-helical protein that organizes and assembles into larger molecular assemblies, i.e., fibrils in the ECM. These fibrils have an approximate length of 300 nm and a diameter of 1.5 nm (20).

Biosynthesis and secretion of collagen type I

COL I biosynthesis is a complex multi-step process that involves number of chaperones and modifying, folding and processing enzymes that catalyze COL I α-chain post-translational modifications (PTMs) both intra- and extracellularly (21-23). COL I synthesis starts from the synthesis of the precursor, type I procollagen. The ribosome -bound mRNA is translated into preprocollagen molecules (polypeptidechains) which are then translocated into the lumen of the rough

endoplasmatic reticulum (ER). After cleavage of the signal peptide, the procollagen type I chains undergo multiple steps of post-translational modifications in the ER which include hydroxylation of proline and lysine residues and glycosylation of some of the hydroxylysine and asparagine residues (12,22). The interaction of the C-propeptidic ends of the three α-chains brings the three procollagen chains together and initiates the formation of triple helix folding towards the N-terminus (21).

After procollagen processing and assembly, the triple-helical molecules undergo further post-translational modification in the Golgi and are subsequently packaged into secretory vesicles, which are then transported to the plasma membrane. Although various procollagen carriers of different sizes and properties have been reported during procollagen trafficking (23-26), it is generally accepted that procollagen transport from ER-to-Golgi and Golgi-to-PM are both microtubules associated processes (26-28). However, the majority of researches on procollagen trafficking has been done on fibroblasts. How secretory vesicles reach and fuse with the plasma membrane in osteoblasts is not well understood. Recently, synaptotagmin VII and Rab GTPases have been linked to regulating procollagen secretion in osteoblast (29,30). It has been proposed that microtubules play a key role in procollagen-containing vesicle transport by serving as tracks to move them from Golgi to the plasma membrane with the aid of motor proteins dynein and/or kinesin family members (31,32)

Microtubule dynamics and post-translational modifications of tubulin

Microtubules are major components of the eukaryotic cytoskeleton and they are essential for a number of cellular processes, such as cell division, motility and intracellular transport (33,34). Microtubules are comprised of tubulin heterodimers, which consist of α- and β-tubulin units. Primary sequences of tubulins are well conserved in eukaryotes due to the functional significance of tubulins in various cellular events, however, the tubulin gene family has multiple isotypes in vertebrates (7 α- and 8 β-tubulins in humans) (35). Microtubules are dynamic structures that grow and shrink rapidly in a guanosine triphosphate (GTP) -powered process. The microtubule network can be rearranged quickly by polymerization–depolymerization events. Certain cellular events, such as protein secretion and differentiation, require stabilization of microtubules, i.e., prevention of their depolymerization event (36-38). This is orchestrated by number of microtubule stabilizers among which are the microtubule associated proteins (MAPs), such as MAP1, MAP2 and Tau (39,40).

Tubulin PTMs are also involved the stabilization of microtubules, in fact, it is believed that the regulation of microtubule dynamics remains a key aspect of tubulin PTMs (33). Both α- and β-tubulin undergo many PTMs that create subpopulations of tubulins with different properties that appear to regulate microtubule function in various cellular processes (41-44). Tubulins have been reported to undergo acetylation, phosphorylation, detyrosination, deglutamylation, glutamylation, glycylation, palmitoylation and polyamination/transamidation (**TABLE I**). Nearly all of the amino acid sequence differences, as well as tubulin PTMs (except for acetylation) are

confined to the C-terminal tails of α-terminal and β-tubulin since these tails are exposed at the outer surface of microtubules, and offer key interaction sites for MAPs (43, 45). In this thesis, we focus on detyrosination and modification by transglutaminases (polyamination/ transmidation reaction).

TABLE I. Post-translational modifications (PTMs) identified in tubulins, summarized from (46).

PTMs	Description	Sites
Acetylation	Addition of acetyl group on α-tubulin.	Lys40 of α1A/B
Phosphorylation	Addition of phosphate	Ser444 and Tyr437 of βIII Ser441 of βVI Ser172
Detyrosination	Removal of the C-terminal tyrosine.	C-terminus of α1A/B α 4
Δ2 deglutamylation	Removal of the penultimate glutamate from detyrosinated α-tubulin.	C-terminus
Glutamylation	Addition of one or multiple glutamate as a side chain on α- and β-tubulins. Multiples sites possible. Up to 12–20 additional glutamates.	Glu445 of α1A/B Glu443 and Glu 445 of α4A Glu441 of βI, Glu435 of βII Glu438 of βIII , Glu433 of βIVa
Glycylation	Addition of one or more glycines as a side chain on α- and β-tubulins. Multiple sites possible. Up to 30–40 additional glycines.	Glu445 of α3 A/B Glu437 of βIV
Palmitoylation	Addition of palmitate on α-tubulin.	Cys376 of α-tubulin
Polyamination	Covalent addition of a polyamine, such as putrescine, spermidine and spermine, to a protein bound glutamine residue.	β-tubulins Q15

The detyrosination of microtubules is one typical form of PTM for tubulin. It occurs through the removal of the C-terminal tyrosine residue on α-tubulin which generates a new C-terminal end with a glutamate residue (47). This form is known as Glu-tubulin and used as a marker for microtubule stability, as it is shown to accumulate in stabilized microtubules to affects its interactions with intermediate filaments (41,48). The functions of Glu-tubulin have also been related to microtubule dynamics and the regulation of motor proteins movements (33). It has been reported that the motor protein kinesin-1 moves preferentially towards detyrosinated microtubules (49,50). Although microtubule stabilization induces microtubule detyrosination in cells, detyrosination itself may not be sufficient to enhance microtubules stability (51). How Glu-tubulin presence in microtubules result in stabilized microtubules is still not well understood, although it was proposed that a plus end capping may be involved (52).

Besides detyrosination, the acetylation of α-tubulin and polyglutamylation (both α- and β-tubulin) are also present on stable microtubules and thought to facilitate motor protein binding and cargo delivery (53-55). β-Tubulin can be also phosphorylated and polyaminated. The former modification is not frequently observed, while the latter has been suggested to stabilize neuronal microtubules and its level correlates with essential events, like brain maturation and neuronal differentiation (41,44). Most PTMs of tubulin are reversible through enzymes that are able to reverse the modification. For example, detyrosination is catalyzed by a still unknown carboxypeptidase (42), and it can be reversed by an enzyme called tubulin

tyrosine ligase (TTL) (56,57). In fact, a large number of enzymes that catalyze tubulin PTMs are still unknown. Multiple PTMs can happen on the same tubulin molecule and work in concert to modulate microtubule function (34,42,58).

Plasma membrane and cell surface tubulin

Tubulin is primarily a constituent of cytoplasmic microtubules, but a small, yet significant fraction of the total cellular tubulin is found to be integrated in the plasma membranes. In this context, integral plasma membrane protein means that a protein is normally resistant to non-ionic detergents or sodium carbonate (pH 10) extraction (59). It is frequently reported that tubulin interacts with lipid raft domains of the integral plasma membrane (60-63), however, overall, the functions of integral membrane tubulin and even how far it penetrates into the plasma membrane, is not well understood.

The presence of tubulin on the cell surface was first demonstrated in 1976, with an approach that involved labeling of the surface of pigeon erythrocytes with colchicine-sepharose beads which bind to α- and β-tubulin (64). The authors speculated a deep penetration of tubulin into the membrane, since the colchicine that binds tubulin extends at most 11–12Å into the membrane. Tubulin penetration into the outer leaflet of the plasma membrane has also been shown by surface labeling of human monocyte-like cell line, U937 (65). The authors used an indirect staining procedure and biotinylated antibodies and flow cytometry to detect actin and tubulin on the cell surface in U937 cells. Studies on cell-surface tubulin often link its role with malignancy and leukemic transformation (66,67). Relatively few studies have been

carried out to investigate this pool of tubulin in non-malignant cells.

Caveolae

Structure and functions

Caveolae were first observed in the 1950s (68) by electron microscopy in endothelial and epithelial cells. Caveolae are 50-100 nm flask-shaped or omega-shaped membraneous invagination structures that are located at or near the plasma membrane. They were originally named caveolae “small caves” (69). However, further studies reported caveolae to display variety of shapes other than the traditional membrane invaginated one. They can exist in vesicular/fully-invaginated form, rosettes, grape-like clusters and prolonged tubular like structures (69).

The function of caveolae has been of great interest since their discovery and they have long been implicated in vesicular transport such as transcytosis, potocytosis and endocytosis, where caveolae are considered to concentrate and uptake molecules or ions into the cell (69), and more importantly in signal transduction (70-72). To study the functions of caveolae, they need to be isolated from cell membranes by the sucrose gradient centrifugation. Since caveolae membranes are highly enriched in glyco-sphingolipids and cholesterol, they have a low buoyant density in sucrose density gradient (73) and they resist solubilization by non-ionic detergents. Through sucrose density gradient centrifugation after detergent solubilization, caveolae can be separated for further investigation to identify the proteins that are co-purified within caveolae. After the initial discovery of signaling molecules in caveolae enriched

membrane, such as Src tyrosine kinases and heterotrimeric G proteins (70,71), the list of signal transduction molecules associated with caveolae has been expanding. Nowadays it includes the nitric oxide synthases, estrogen receptor α, nerve growth factor receptor, epidermal growth factor receptor, signaling molecules in the mitogen-activated-protein kinase cascade, and the insulin receptor, to mention a few (74-80). It appears that caveolae are involved in the compartmentalization of various signaling molecules and it seems that caveolae are negative regulators for many of the signaling proteins they interact with, probably holding them in an inactive form (72).

Aside from their specific morphology, caveolae are also identified by their protein constituents, caveolin(s). Caveolins are coat proteins that give caveolae their characteristic shape. There are three isoforms in caveolin gene family, namely caveolin-1, -2, and -3. Caveolin-1 and caveolin-2 exist in different isoforms (caveolin-1α, 1β, caveolin-2α, 2β, 2γ) (81). Here we focus on caveolin-1 (Cav-1), which is a 21 kDa phosphotyrosine containing protein that is necessary for both the structure and function of caveolae. Caveolae are absent in some cells such as erythrocytes, platelets, lymphocytes and neuroblastoma cells, which also do not express Cav-1 (82). Cav-1 self-associates to form non-covalent, high molecular mass homo-oligomers that contain 14 to 16 individual Cav-1 molecules. These Cav-1 oligomers represent the functional assembly units of caveolae (72,83).

Caveolin and bone

Since the discovery of caveolae, they have been studied in various tissues and cell types, such as adipocytes, endothelial cells, type I pneumocytes, fibroblasts, smooth muscle cells, and striated muscle cells (68,84-87). It was however, not until a decade ago, that caveolae and Cav-1 in osteoblast was reported (88). Cav-1 and Cav-2 were both found in human fetal osteoblasts and MC3T3-E1 preosteoblast cells, with Cav-2 less abundant than Cav-1. The authors concluded that osteoblasts share the clustered organization of signal transduction molecules in their surface membranes with other cell types (89). The role of caveolae as signaling organizers in osteoblasts may not be surprising since osteoblast functions are regulated by multiple signaling pathways, extracellular stimuli including soluble growth factors, ECM, contact with other cells and mechanical force (90). In order to reach the proper maintenance of the skeleton, many signals have to be integrated appropriately. Initially, the bone phenotype of Cav-1 knockout animals was overlooked by scientists (91). In 2007, skeletal phenotyping of Cav-1 knockout mice was performed and authors reported increased trabecular number and thickness (92). The authors speculated that Cav-1 deletion leads to increased osteoblast differentiation suggesting that Cav-1 helps to maintain osteoblast progenitors in a less differentiated state. Authors did not discuss the underlying mechanism for the regulation. A very recent study connected Cav-1 with osteoclastogenesis (93). Authors found that Cav-1 was dramatically up-regulated by the receptor activator of nuclear factor kappa-B ligand (RANKL)-the osteoclast differentiation factor and knockdown of Cav-1 reduced

osteoclastogenesis. This demonstrates the important role of Cav-1 as a negative regulator of bone mass.

Src and its role on bone formation

The fact that Cav-1 negatively regulates osteoblasts and promotes osteoclast function links one signaling pathway to both cell types and to Cav-1 function - the Src family tyrosine kinases, which are among the various cytoplasmic signaling molecules that Cav-1 interact with, and phosphorylates Cav-1 at Tyr14 (94). The SRC family of non-receptor tyrosine kinases consist of eleven members in humans (Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Srm, and Yes and c-Src) (95). The distributions of these kinases are cell type-dependent, while Yes, Fyn and c-Src are ubiquitously expressed (96). The discovery of Src dates back to 1911 (97), when Peyton Rous demonstrated that the injection of cell-free extracts from chicken sarcomas give rise to new sarcomas in host animals. It was 60 years later that the agent responsible for this process was identified as the viral Src gene (v-Src) (98). After that, it was reported that v-Src had a counterpart in eukaryotic cells, termed cellular Src (c-Src, also referred to as Src) (99), which contains a regulatory domain missing in v-Src. Hence, c-Src activity is tightly controlled and involved in many physiological functions of the cells, while v-Src is constitutively active and works as an oncogene.

The functions of Src has been implicated in cell differentiation, proliferation, and survival (100-102), and linked to the inhibition of osteoblast differentiation recently (103). It maintains the bone resorbing function of osteoclasts, which express high

protein levels of c-Src compared with most other cells types (104-105). Prominent bone phenotypes have been reported in c-Src null mice (106). Soriano and coworkers first reported that targeted disruption of the c-Src gene in mice causes osteopetrosis, a bone remodeling disorder characterized by increased bone density due to defective bone resorption in osteoclasts (6). Indeed, c-Src kinase activity and the c-Src protein are both required for osteoclast actin ring formation and thus for bone resorption (107). On the other hand, c-Src deletion in osteoblasts leads to increased osteoblast numbers and differentiation (108). c-Src also maintains osteoblasts in a less mature stage through the induction of insulin-like growth factor 5 (IGFBP5) (103). One possible molecular mechanism behind this process might be the regulation of Runt-related transcription factor 2 (Runx2) by c-Src. Runx2 is a transcription factor for osteoblast differentiation that also controls the expression of IGFBP5. It has been shown that the c-Src substrate, YAP (Yes associated protein) gets phosphorylated and binds to Runx2 in the c-Src/Yes signaling pathway which results in the repression of Runx2 activity and downstream transcription events linked to bone formation (109,110). In summary, c-Src acts as a negative regulator for bone mass.

Caveolin and c-Src

Since the initial discovery of the signaling transduction function of caveolae, Src family tyrosine kinases are among the well characterized category for caveolae-associated proteins (69,70). It has been suggested caveolin has a role in regulating c-Src activation. A peptide derived from Cav-1 (residues 82-101,

scaffolding domain) strongly inhibits c-Src auto-phosphorylation and activity (110). Perhaps individual Cav-1 molecule binds to c-Src and maintains it in an inactive configuration while Cav-1 homo-oligomer formation is likely to change the physical interaction between Cav-1 and c-Src, which makes the kinases more accessible to their substrates (111). However, follow-up studies showed different and controversial conclusions on c-Src and phospho-Cav-1 (112-114) and so the essence of the c-Src and Cav-1 interaction remains unclear. A recent study showed phospho-Cav-1 binds to the SH2 domain of activated c-Src and promotes the accumulation of the latter one to focal adhesions (115), where phospho-Cav-1 was also localized in several studies (116,117).

Role of caveolae in matrix turnover

It is well established that ECM plays an important role in the morphogenesis and cellular metabolism of tissues, conferring and linking mechanical properties and biochemical signals (12). Degradation and removal of ECM proteins is a cell-mediated process, which is integral to tissue remodeling and involved in a number of physiological processes. Extracellular proteases such as matrix metalloproteinases can degrade ECM proteins. ECM turnover is also regulated by endocytosis (118). It is known Cav-1 can mediate ECM turnover and cell-surface receptor recycling such as integrin-β1 through endocytosis (119-120).

Endocytosis refers to a biological process whereby cells take up and internalize molecules and particles from outside the cell membrane into the cytoplasm, which

allows exchanges between the cell and its ECM environment (121). The cells have developed different ways of endocytosis, and in general they include pinocytosis, phagocytosis, clathrin-dependent endocytosis, and clathrin-independent endocytosis. And caveolae-mediated endocytosis is most frequently reported clathrin-independent endocytosis mode (122,123). As caveolae also exist on the surface of osteoblasts, it is possible that caveolae-mediated endocytosis may play a role in ECM turnover in osteoblast. However, unlike the well characterized clathrin-mediated endocytosis, the caveolae-mediated one is not well understood in osteoblasts (124,125).

In addition to COL I fibrils, pre-osteoblasts initially elaborate a provisional fibronectin (FN) matrix. FN, a high-molecular weight glycoprotein is assembled on the cell surface via a β1-integrin mediated process (126). FN matrix is assembled into matrix to act as a scaffold for other matrix constituents including COL I. As an important ECM component, its turnover also plays an important role in governing ECM turnover. FN matrix is constantly internalized via caveolae-mediated endocytosis and followed by lysosomal degradation. It has been shown that FN matrix can be disassembled via caveolae-mediated endocytosis and FN matrix is more stable when caveolae function is inhibited with caveolae-disrupting agents (118,119). Therefore, caveolae may play a major role in matrix stability/instability.

Transglutaminases

Functions and substrates

Transglutaminases (TGs) are a widely distributed enzyme family that comprises

of 9 members-TG1-7, Factor XIII-A (FXIII-A) and band 4.2 in mammals. TGs are capable of catalyzing transamidation of a protein-bound glutamine (Q) residue in a Ca^{2+}-ion dependent manner. All TG family members contain four main structural domains: an N-terminal β sandwich, an catalytic core, and two C-terminal-barrel domains. Upon activation, the TG enzyme undergoes a conformational change from a closed, compacted form to an extended, open one, which exposes the catalytic site of the enzyme (127). Ca^{2+}-ion promotes opening of the enzyme structure which activates the enzyme (128).

Transamidation is a two-step reaction that occurs between a protein-bound Q residue and a protein-bound lysine (K) residue or a primary amine/monoamine/ polyamine (serotonin, dopamine, putrescine, spermine, and spermidine) and results in the formation of a covalent ε-(γ-glutamyl) lysyl cross-link and the release of ammonia (128-130). The first option results in protein-protein crosslinking and formation of protein oligomers/polymers that are resistant to proteolytic degradation (130). The second reaction results in monoamination / polyamination of proteins. Both modifications have been shown to change the chemical properties of proteins as well as their biological functions (131-133). If reaction occurs in the absence of K donor residues and the presence of water, Q residue is deaminated into glutamic acid (**Figure 1**). Transamidation reactions always begin with the recognition of Q residues and thus a TG-reactive Q residue in a protein defines the substrate. K residues (and monoamines and polyamines) join the catalysis and react only after Q residue is bound to the enzyme (130). Non-catalytic functions for several TGs have also been

reported (128,130,134). For instance, band 4.2 is a structural protein in erythrocytes, and TG2 can act as an integrin-FN binding co-receptor or syndecan-4 interaction partner on the cell surface (135,136).

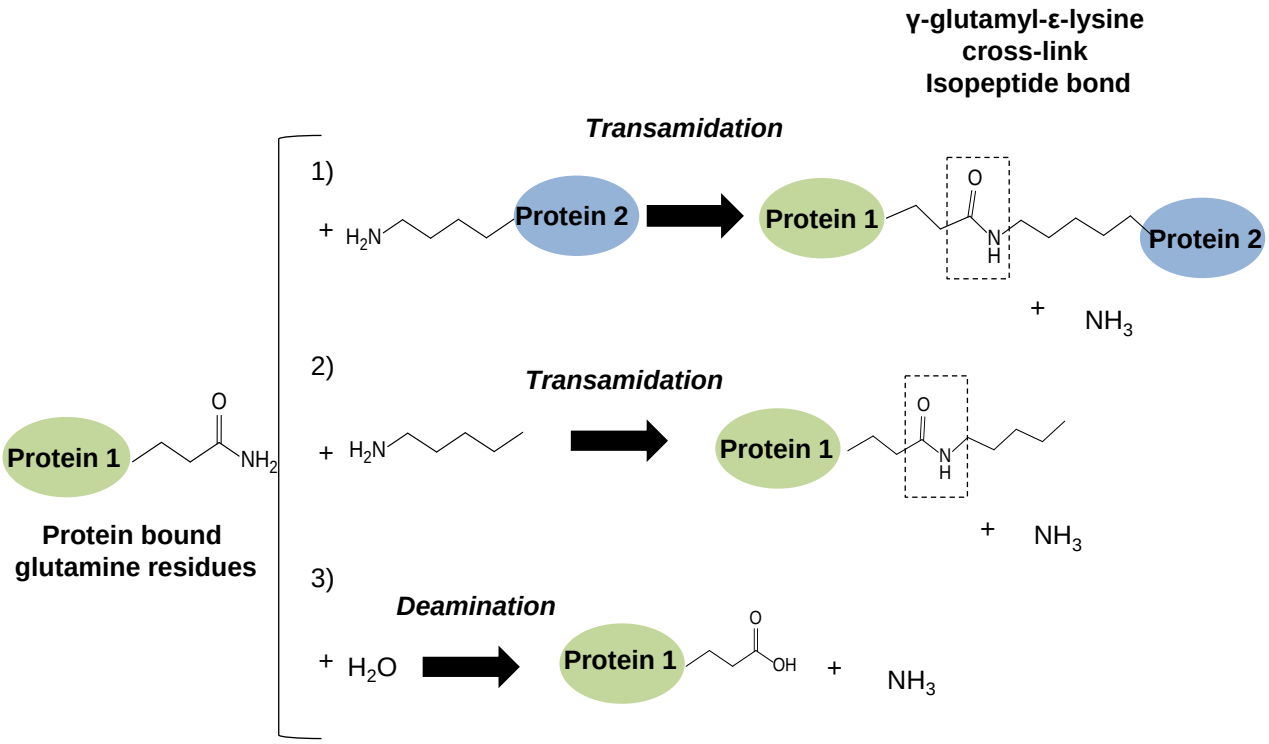

Figure 1. Catalytic reactions of TG enzymes. TGs can modify specific glutamine (Q) residues in proteins in three different ways. **1)** Q residues can be crosslinked to a lysine (K) residues to form γ-glutamyl-ε-lysine isopeptide crosslinks between the Q and K residues. This reaction creates protein polymers. **2)** Q residues can be also modified in the same reaction by primary amines or monoamines (here the primary amine, pentylamine/cadaverine). **3)** When reaction occurs in the absence of K donor residues in aqueous environment, Q residue is deaminated to glutamic acid (E).

TG enzymes are expressed in wide variety of tissues and found in different cellular compartments, where they participate in many biological functions ranging from tissue stabilization to cell signaling (130,137,138). They are also linked to some pathological conditions such as the celiac diseases and Huntington's disease, where TG2 enzyme is thought to play a role in the pathogenesis (139,140). TG-crosslinking activity has been primarily related to stabilization of proteins in both collagenous and non-collagenous ECM (141-143), as well as stabilization of cytoskeletal and intermediate filament components such as actin, β-tubulin, myosin and vimentin (44, 144,145). TGs can also crosslink cell surface receptors and crosslinking of β3-integrin to vascular endothelial growth factor receptor (VEGFR)-2 catalyzed by FXIII-A has been suggested to have a proangiogenic effect (146).

Although the relative positions of active site residues for TGs are conserved among the enzyme family, the differences of charge distributions exist among various isoenzymes (147) and this difference may be responsible for the different substrate specificities and biological functions of each isoenzyme. The TRANSDAB database lists more than 200 protein sequences that function as Q-donor TG substrates-several of them showing overlap between different TG enzymes. For example, there are at least four TG isoforms (TG1,TG2,TG3 and TG5) expressed in the mammalian epidermis and they play complementary roles in the formation of a specialized structure-the cornified cell envelope (148), and the enzymes show overlap in their substrate preferences, such as involucrin, loricrin, and small proline-rich protein (147, 149).

TG isoform-specific substrate peptides

The identification of TG isoenzyme specific substrates that have TG reactive Q residue(s) is essential to understanding TG enzyme function. Major advances have been made by Dr. Hitomi's group in identifying specific peptidic substrate sequences for all TG enzymes. The group used a phage display peptide library to identify the primary sequences around the reactive Q residue preferred by each TG. Random peptides are displayed on the surface of the phages and if phages displayed an efficient Q donor sequence; they were able to incorporate a biotin-labeled cadaverine into the sequence. The biotinylated phages could be purified by avidin-affinity chromatography and the peptide sequences form selected phages were then expressed as GST fusion proteins. Their abilities to act as Q donor substrates were evaluated using monodansyl cadaverine (a fluorescence labeled cadaverine) which incorporates into the Q residues of substrate proteins at the presence of TG activity. The highest affinity peptide for each was identified and peptides are listed in (**TABLE II**). Recently, by using highly reactive Q-donor substrate peptide F11 and T26 (specific to FXIII-A and TG2, respectively) and mass spectrometry, several possible TG substrate proteins were identified from MC3T3-E1 osteoblast cultures (150). These proteins were: glucose-regulated protein, Ras GTPase-activating-like protein, glutamate dehydrogenase 1, nATP synthase subunit α, α- and β-tubulin, vimentin, actin, hsp71, and hsp90. Using the information gathered from the active sites of different TGs and from those preferred substrate peptides, inhibitors that have greater specificity against the individual target TGs could be designed in the near future.

TABLE II: Known preferred substrate peptides for different transglutaminases. These peptide sequences have been used successfully in number of studies (151-155).

Enzymes	Substrate peptide sequences	References
TG1	K5: YE**Q**HKLPSSWPF	Sugimura Y et al. 2008
TG2	T26 : H**Q**SYVDPWMLDH	Sugimura Y et al. 2006
TG3	E51: PPPYSFY**Q**SRWV	Yamane A et al. 2010
TG6	Y25 : DDWDAMDE**Q**IWF	Fukui M et al. 2013
TG7	Z3S: YSL**Q**LPVWNDWA	Kuramoto K et al. 2013
FXIII-A	F11: D**Q**MMLPWPAVAL	Sugimura Y et al. 2006

TG Inhibitors

The development of TG isoenzyme specific inhibitors has been challenging due to the substrate overlap and lack of knowledge of how enzymes choose their substrates in the first place. The most ubiquitous mammalian transglutaminase is TG2 which has been the subject of many studies due to its association with a variety of disease states such as metastatic cancer, celiac disease, fibrosis and neurodegenerative disorders such as Huntington's (130,156). Therefore, development of selective inhibitors for TG2 activity may help to better understand the mechanisms behind those diseases and further apply inhibitors as novel therapeutic solutions. Inhibitors of general transamidation activity have been generally divided into three distinct subclasses based on their mechanism of inhibition, namely competitive amine inhibitors, reversible and irreversible inhibitors (157). The competitive amine inhibitors are probably the most commonly used, because of their commercial availability. They include putrescine, 5-(biotinamido) pentylamine (bPA), and cadaverine-derived inhibitors, like monodansyl cadaverine, and fluorescein cadaverine. These types of compounds inhibit protein-protein crosslinking by acting as competitive primary amine substrates that replace protein-bound K residues. These compounds can also be used as substrate identification probes as is done in this thesis.

Number of modern peptidic inhibitors has been developed and in this thesis work, we have used an irreversible TG inhibitor, NC9, to block TG crosslinking activity. NC9 has a commonly used substrate Cbz-Gln-Glyas the backbone (158) and it bears a

dansyl group that allows it to be tracked via antibody detection (**Figure 2**). The strategy behind the action of NC9 is that it mimics a TG substrate. However, instead of a Q residue, the inhibitor has a glutamine warhead moiety which attacks the thiol group of TG active site where it then covalently incorporates (159). NC9 is also known to change TG enzyme conformation by keeping it in an open form (160), while the reversible TG inhibitors do not. The reversible inhibitors interrupt enzyme activity by blocking substrate access to the active site without covalent incorporation, or simply through the competitive binding to the TG enzyme with Ca^{2+}, like the Zn^{2+} ion (157), since Ca^{2+} ion is needed for the activation of the enzyme. Other irreversible TG inhibitors, like 6-diazo-5-oxo-noerleucine (DON) containing peptides from the German company Zedira (Z-DON) can also act as TG enzyme preferred γ-glutamyl-containing substrates and attack the active site through covalent binding.

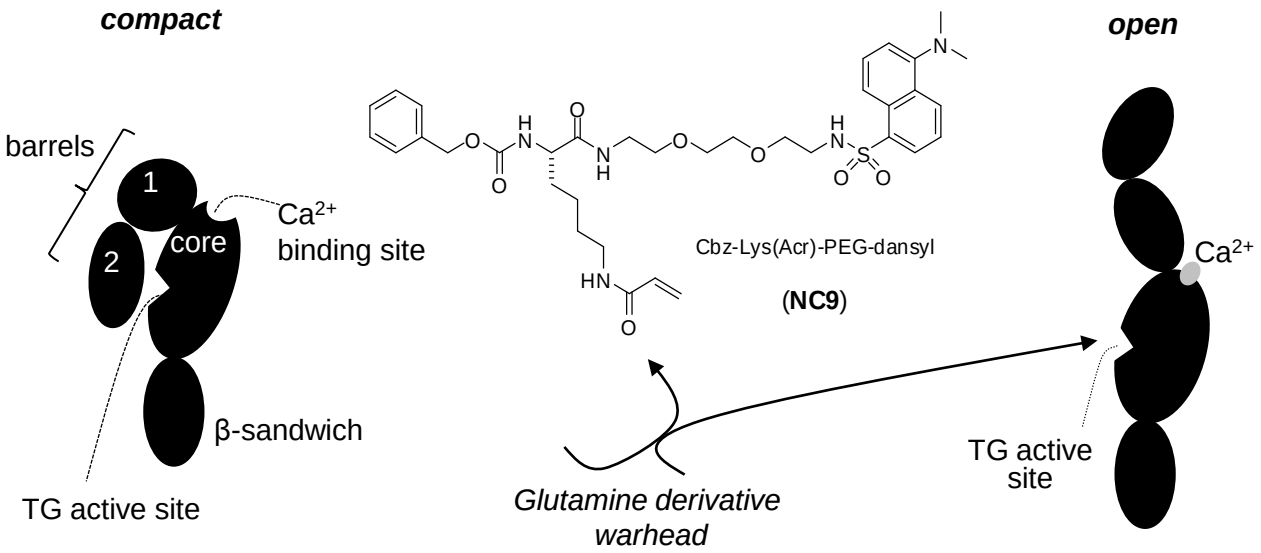

Figure 2. Structure of TG enzyme and the irreversible TG inhibitor NC9. The compact inactive TG enzyme has four domains: barrels 1 and 2, a catalytic core and β-sandwich. NC9 has the central peptidic scaffold and bears a Cbz-group, which confers affinity for the Q substrate binding site. NC9 also bears a dansyl group which is attached to the peptidic moiety through a short PEG (Poly(ethylene glycol)) spacer. The dansyl group allows tracking of NC9 in enzymes that it reacts with using antibodies (159). NC9 is also known to change TG enzyme conformation by keeping it in an open form (160).

Factor XIII-A

Factor XIII-A (FXIII-A) was first identified as a circulating plasma TG enzyme that is responsible for fibrin clot crosslinking in the final stage of the blood coagulation cascade (134,161). In circulation, FXIII-A is part of FXIII, a heterotetrameric coagulation factor that contains two catalytic A subunits (FXIII-A_2) and two noncatalytic B subunits (FXIII-B_2). FXIII-A consists of 732 amino acids with a molecular mass of 83 kDa (in humans). The B subunits serve as protective and inhibitory carriers for A subunits in plasma. For the circulating FXIII, the B subunits are synthesized by hepatocytes in liver and FXIII-A is made by cells of bone marrow origin (162,163). Besides the circulating form, FXIII-A can also be found in many cell types, ranging from monocytes, macrophages, megakaryocytes, chondrocytes, to osteoblasts and adipocytes (164-170). In those studies, FXIII-A was reported to be localized in various cellular and extracellular locations including in the cytosol, at the plasma membrane/cell surface, and in the extracellular matrix. We refer this pool of FXIII-A as the cellular FXIII-A (**Figure 3**).

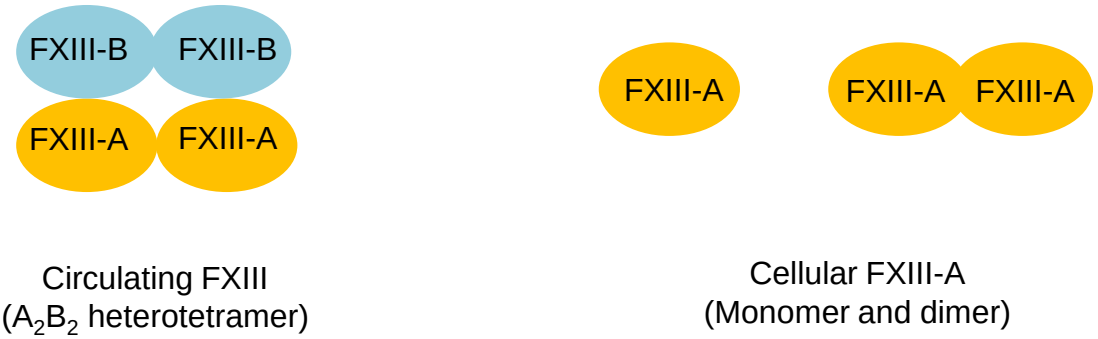

Figure 3. **Circulating and cellular FXIII-A.** Circulating FXIII-A exits as part of FXIII coagulation factor which is a heterotetramer that consists of two FXIII-A and two FXIII-B subunits. Cellular FXIII-A is known to exit as a dimer in the cytoplasm of platelets and monocytes/macrophages (134,171). In osteoblast cell cultures, FXIII-A has been reported to exit as monomers on the plasma membrane and dimers in the ECM (169).

In addition to its role in hemostasis, circulating plasma FXIII-A has also been associated in wound healing, tissue repair and angiogenesis (134,172). Meanwhile more and more attention has been paid to investigating the functions of cellular FXIII-A. It has been suggested that FXIII-A and its extracellular crosslinking activity can stimulate fibroblast proliferation adhesion, induce chondrocyte hypertrophy, regulate ECM deposition and stabilization, and modulate proliferation and differentiation of preadipocytes (167,169,170,173).

Factor XIII-A in bone and osteoblasts

TG2 was the first TG enzyme found in bone and cartilage with restricted distribution that correlated with the calcification of cartilage tissue (174). Shortly after it, the expression of FXIII-A was demonstrated in avian embryonic growth plate and then in mouse embryonic growth plate, where its expression correlated with matrix mineralization (175,176). Later on, TG2 and FXIII-A were also found expressed in the cartilage of avian epiphysis andin bone *in vivo*, where FXIII-A was particularly localized to osteoblasts, osteocytes, and found in the osteoid (142,166,177).

Our recent work has focused on understanding the role of cellular FXIII-A in osteoblasts during osteogenesis. For these cells, we have shown previously that our research model-MC3T3-E1 osteoblasts only express TG2 and FXIII-A TGs and FXIII-A appears to be the active enzyme during osteoblast differentiation (165). In cell culture, FXIII-A is found to be localized on the plasma membrane of osteoblast as rounded patches, and also externalized to the ECM as dimers where its function has been linked to the regulation of COL I secretion via modulating microtubule function

and dynamics, and stabilization of newly deposited ECM (159,169). Moreover, we have shown the FXIII-A TG activity is required for plasma FN assembly into bone matrix (143), which is crucial for bone formation *in vivo* (178).

In our previous work, we demonstrated that differentiating osteoblasts form high-molecular weight oligomeric Glu-tubulin whose formation was dependent on TG activity (157). We suggested that this form of Glu-tubulin was linked to stabilization of microtubule tracks to the plasma membrane during secretion of COL I. In *Chapter 3*, we further demonstrate that cellular FXIII-A can catalyze formation of a covalent high-molecular weight Glu-tubulin that is found on the plasma membrane of osteoblasts, and is thought to be linked to the secretion process owing to its increased stability. We learned from our previous studies that cellular FXIII-A is located on the plasma membrane of osteoblasts in rounded patches (157) and what these patches represent is not yet known. In the *Chapter 4*, we show a pool of cellular FXIII-A in the inner leaflet of osteoblast plasma membrane and it colocalizes in caveolae. The intracellular function of FXIII-A in osteoblast will be further discussed.

Rationale, hypothesis and aims

The following two specific hypotheses are investigated in this thesis:

Hypothesis 1—Detyrosinated (Glu) tubulin is a substrate for cellular FXIII-A transglutaminase and exists as a covalently stabilized form. This form is created to assist secretion of COL I.

Rationale—Microtubules are dynamic cytoskeletal components that grow and

shrink rapidly in a polymerization and depolymerization process. Microtubule components, α- and β-tubulin undergo numerous PTMs that modulate their dynamics and cellular functions. TG enzymes are expressed in wide variety of tissues and in different cellular compartments, where they participate in various biological functions ranging from tissue stabilization to cell signaling (130,137,138). TG-crosslinking activity has been primarily related to protein stabilization of collagenous and non-collagenous ECM (141-143), as well as cytoskeletal and intermediate filament components such as actin, β-tubulin, myosin and vimentin (44, 144,145). It has been recently reported that tubulin can be posttranslationally modified by TG enzymes and form covalent high-molecular weight oligomers during pollen tube growth (179, 180). It also has been suggested tubulin PTM may affect its association with membranes (181). In our previous work, we demonstrated that differentiating osteoblasts form high-molecular weight oligomeric Glu-tubulin whose formation was dependent on TG activity (159). We suggested that this form of Glu-tubulin was linked to the stabilization of microtubule tracks to the plasma membrane during secretion of COL I. In this thesis, we have further examined the location and function of the oligomeric Glu-tubulin in osteoblast cell culture and by *in vitro* TG activity assays. We hypothesized that the Glu-tubulin is a substrate for cell surface FXIII-A and the covalent crosslinking of TG activity creates high molecular weight Glu-tubulin on the plasma membrane with increased stability. We further hypothesized that the presence of high molecular weight Glu-tubulin on the osteoblast plasma membrane correlates with secretory activity of osteoblasts. Thus, the aim is to investigate if Glu-tubulin is a

substrate for cell surface FXIII-A in osteoblasts and if its crosslinking is linked to the secretion and deposition of COL I ECM.

Hypothesis 2—Intracellular FXIII-A localizes to caveolae in differentiating osteoblasts and regulates Cav-1 phosphorylation and its homo-oligomerization, as well as c-Src kinase activation.

Rationale—We learned from our previous studies that cellular FXIII-A is involved in regulating matrix secretion and deposition (159) and that cellular FXIII-A is located on the plasma membrane of osteoblasts in rounded patches. What these patches represent is not known. Caveolae are flask- or omega shaped plasma membrane invaginations, as well as in grape-like clusters or rosettes, and they have long been implicated in transport processes such as transcytosis, potocytosis and endocytosis, as well as in signal transduction (69,72). Cav-1, a caveolae marker and caveolae-associate protein, is expressed in osteoblast (88). It is known that Cav-1 is phosphorylated at Tyr14 by Src-kinases (94), which affect caveolae function and osteoblast maturation (103,108). Moreover, Cav-1 has been identified as a negative regulator for bone formation through skeletal phenotyping of Cav-1 deficient mice. These mice have increased bone formation and trabecular size (92). In this study, we hypothesize that patchy staining of FXIII-A on osteoblasts surface represents FXIIl-A associated with caveolae. We hypothesize that FXIII-A localized to caveolae regulates caveolae function in differentiating osteoblasts. Thus, the aim is to examine FXIII-A colocalization with Cav-1 by conventional and TIRF microscopy and by

preparation of caveolae-enriched membrane fractions. The aim is also to investigate the potential role of FXIII-A in caveolae and related signaling.

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Chapter 2–MC3T3-E1 cell line as a cell culture model to study caveolae and plasma membrane tubulin in osteoblasts

MC3T3-E1 cell line is a well characterized and studied pre-osteoblast cell line that is derived from newborn mouse calvaria (1). The MC3T3-E1 cells were isolated on the basis of expressing high TNAP which is an osteoblast differentiation marker. These TNAP expressing cells were then years later further subcloned based on their ability to express later markers of osteoblast differentiation, such as osteocalcin and bone sialoprotein (2,3). Among the MC3T3-E1 subclones that were identified, M14 subclone demonstrated high mineralizing activity and thus was used as a research model throughout the whole thesis. The MC3T3-E1/M14 cell differentiation and expression of TGs in these cells has been well described and characterized in our previous work (4). As an osteogenic cell line, the MC3T3-E1/M14 cells are excellent model to study various activities and functions of proteins involved in osteoblast differentiation (5).

The MC3T3-E1/M14 pre-osteoblast subclone 14 as a main research model in this study was a generous gift from Dr. Renny T. Franceschi at the University of Michigan, School of Medicine. The cells were cultured in minimal essential medium (MEM) which was supplemented with 10% fetal Bovine Serum, 1% Penicillin-Streptomycin, 1% L-Glutamine, and 0.225 mM L-Aspartic acid. This medium is referred to as medium (M) only hereafter. Once the cells were plated (indicate as -1 day), they were allowed a 24 hours of proliferation to reach a confluence over 80%

before they were treated with 50 µg/ml ascorbic acid (AA) and β-glycerophosphate (β-GP) to induce MC3T3-E1/M14 pre-osteoblast differentiation, deposition of COL I and to allow matrix mineralization. This medium is referred to as differentiating medium (DM).

Preparation of caveolae enriched fractions from MC3T3-E1 osteoblasts

The presence of Cav-1 and Cav-2 in both human fetal osteoblasts and MC3T3-E1 cells was demonstrated by the group of Hauschka (6). Here we confirm that caveolae are present in differentiating MC3T3-E1/M14 (referred to as MC3T3-E1 osteoblasts here on). Cav-1 is not enriched by conventional protein extractions methods. Owing to the unique physiological properties that caveolae possess, they can be isolated via sucrose density gradient ultracentrifugation (more details about this method are described in manuscript two). As seen in **Figure 4A,** after ultracentrifugation of MC3T3-E1 osteoblast extracts, a faint light-scattering band can be visualized at the 35% sucrose-5% sucrose interface. This interface contains mainly caveolae material as well as lipid rafts form the integral plasma membrane. Treatment of the cells with 10 mM methyl-β-cyclodextrin (MβCD), which depletes cholesterol and disrupts caveolae, decreases this interface greatly. Examination of MC3T3-E1 osteoblast plasma membrane structures with transmission electron microscopy (TEM) shows different shapes of plasma membrane invaginations (**Figure 4B**) and clusters (**Figure 5C**). These represent caveolae in osteoblast.

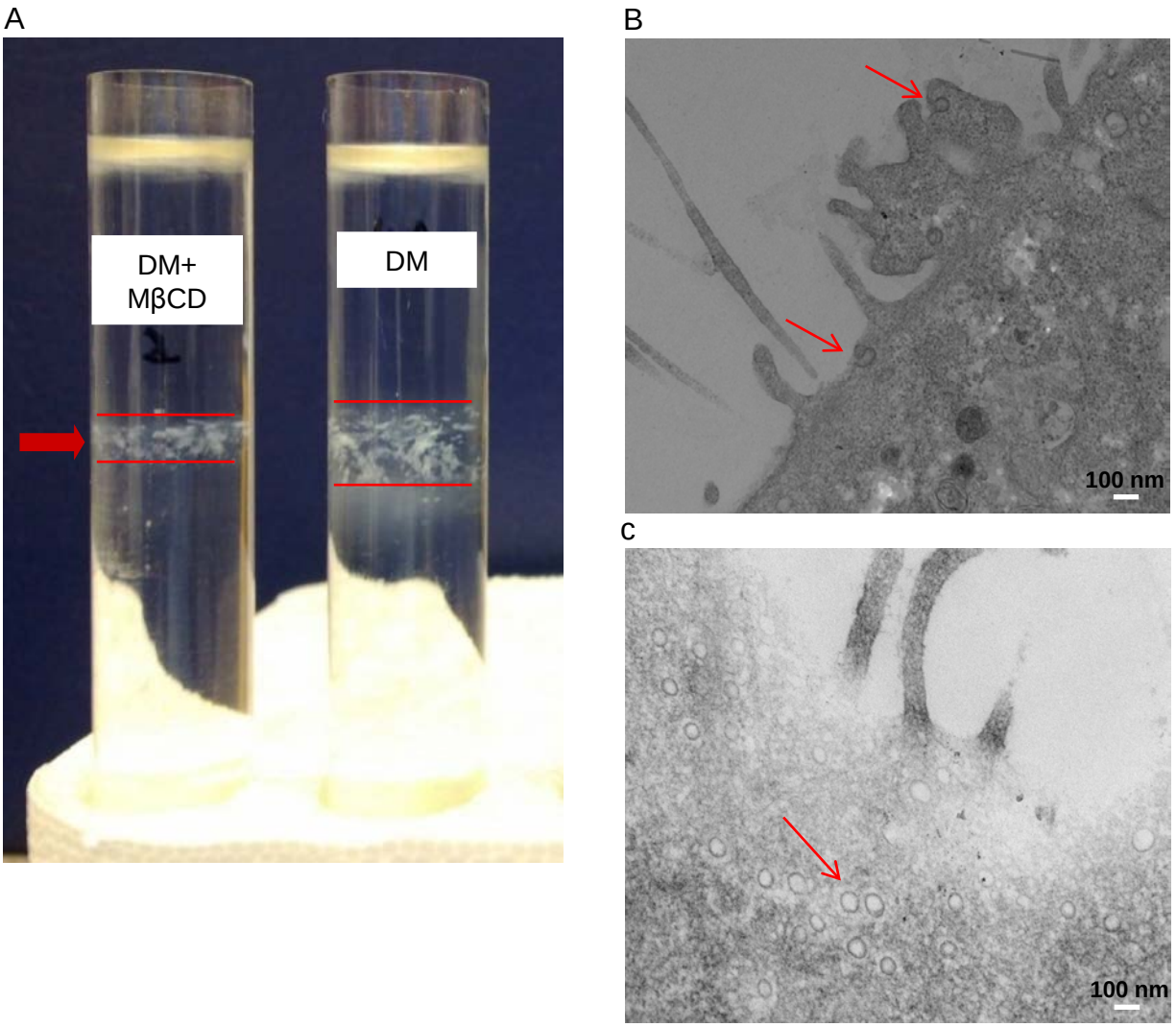

Figure 4. Caveolae in MC3T3-E1 osteoblasts. (A) After sucrose density gradient centrifugation, the so called caveolae enriched fraction (7) can be seen as an opaque band (arrow) between 35% and 5% sucrose layers. The caveolar layer is visibly thicker in differentiating osteoblasts (DM) than the MβCD treated cells (DM+MβCD). (B,C) Caveolae like structures (arrows) with different shapes can be visualized in differentiating MC3T3-E1 osteoblast at Day 5 by TEM. Cells are fixed by 4% paraformaldehyde/0.05% glutaraldehyde and processed with the standard embedding protocol for TEM. The bar equals 100 nm.

Plasma membrane tubulin in MC3T3-E1 osteoblasts

In our previous study, we reported that cellular FXIII-A was colocalized with membrane-associated tubulin and that activity arising from FXIII-A was linked to the promotion of COL I secretion process (8). With the material collected from sucrose density gradient ultracentrifugation, we further tested and demonstrate here the presence of plasma membrane tubulin in our cell culture system (**Figure 5**). The presence of cellular FXIII-A and its function is discussed in the *Chapter 4* of this thesis. In conclusion, the MC3T3-E1 osteoblast cell line is appropriate to study caveolae and plasma membrane tubulin, which we hypothesize to have interactions with the cellular FXIII-A in osteoblast.

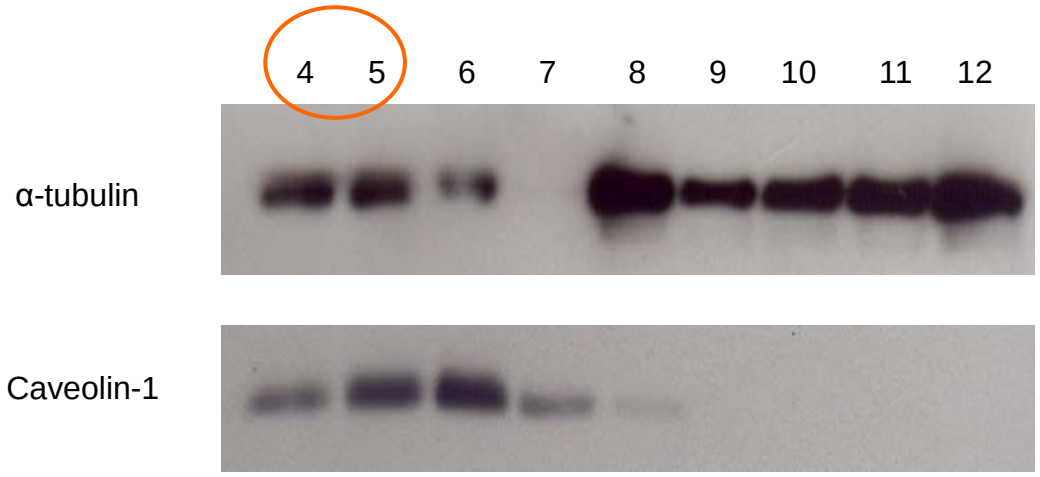

Figure 5. Western blotting of α-tubulin in the caveolae-enriched fractions of MC3T3-E1 osteoblasts. Western blot analysis of caveolae-enriched membrane fractions from sucrose density gradient centrifugation (12 in total). Fractions 1-3 contain negligible amount of protein and were not analyzed. Fractions 4 and 5 represent caveolae enriched fractions (7) and fractions 6-12 contain non-lipid raft membranes. Fractions 4 to 12 were analyzed by SDS-PAGE and Western blotting and detected for α-tubulin and Caveolin-1. α-tubulin co-fractionates with caveolin-1 in fractions 4 and 5 in differentiating osteoblasts on Day 5.

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Chapter 3–Detyrosinated Glu-tubulin is a substrate for cell surface Factor XIII-A in osteoblasts

From our previous work, we learned that FXIII-A enzyme and its crosslinking activity were co-localized with plasma membrane-associated α-tubulin in rounded patches. Inhibition of FXIII-A with NC9 destabilized microtubules as assessed by cellular Glu-tubulin levels. Furthermore, NC9 blocked modification of Glu-tubulin into 150 kDa high-molecular weight Glu-tubulin form which was specifically localized to the plasma membrane.

In this chapter, we provide new information that Glu-tubulin acts as a substrate of FXIII-A TG enzyme which creates an oligomeric Glu-tubulin with increased stability in differentiating osteoblasts. This high-molecular weight Glu-tubulin is membrane associated and found on the cell surface of osteoblasts where its presence is linked to COL I secretion and deposition during osteoblast differentiation and elaboration of ECM.

The study presented in this chapter was originally published in the *Journal of Amino Acids 2014 Volume 46, Issue 6, pp 1513-1526 © Springer-Verlag Wien*

Detyrosinated Glu-tubulin is a substrate for cellular Factor XIII-A transglutaminase in differentiating osteoblasts

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Abstract

Microtubule components α-and β-tubulin undergo a number of posttranslational modifications that modulate their dynamics and cellular functions. These modifications include polyamination and covalent crosslinking by transglutaminase enzymes. We have demonstrated previously that the less dynamic and more stable tubulin form - detyrosinated Glu-tubulin - is found in high-molecular weight, oligomeric complexes in bone-forming osteoblasts during differentiation and along with deposition of collagenous extracellular matrix. In this study, we report that oligomeric Glu-tubulin has high nocodazole tolerance, indicating further increased stability. We show that α-tubulin, which gives rise to Glu-tubulin, is a transglutaminase substrate in *in vitro* assays, and that it is crosslinked into oligomers (dimers, trimers and tetramers) by transglutaminase 2 and Factor XIII-A; β-tubulin was not crosslinked by transglutaminase activity. The oligomeric Glu-tubulin was specifically localized to the plasma membrane of osteoblasts as analyzed by subcellular fractionation, cell surface biotinylation experiments and total internal reflection fluorescence (TIRF) microscopy. Glu- and α-tubulin co-localized with cellular Factor XIII-A as analyzed by conventional and TIRF microscopy. The Factor XIII-A -specific substrate peptide bF11 co-localized with α-tubulin and acted as a competitive inhibitor to oligomerization of Glu-tubulin, attenuating its formation in cells. This was associated with significantly decreased type I collagen deposition and decreased secretory activity as measured by synaptotagmin VII levels on the osteoblast plasma membrane. Our results suggest that Glu-tubulin may exist as covalently stabilized form which may be linked to the

secretion and elaboration of collagenous extracellular matrix.

Key words:

- Glu-tubulin and detyrosinated tubulin
- Covalent crosslinking
- Factor XIII-A transglutaminase
- Type I collagen secretion

Abbreviations:

FXIII-A	Factor XIII-A
TG	Transglutaminase
MTs	Microtubules
bPA	5-(biotinamido)pentylamine
COL I	Collagen Type I
Syt VII	Synaptotagmin VII
pFN	Plasma FN
TIRF	Total Internal Reflection Fluorescence

Introduction

Microtubules (MTs) are dynamic cytoskeletal proteins that are essential for a number of cellular functions such as mitosis, motility and intracellular transport (Wloga and Gaertig 2010; Garnham and Roll-Mecak 2012). MTs are comprised of heterodimeric α-and β-tubulin units that polymerize and grow into molecular rods through a GTP-powered process. MT growth and shrinkage is highly dynamic and occurs rapidly by polymerization-depolymerization events. MTs are stabilized and blocked from depolymerization during various cellular functions including secretion and differentiation, and this occurs initially through posttranslational modifications that alter the ability of tubulin to bind other proteins (Garnham and Roll-Mecak 2012). Perhaps the best characterized and understood stabilizing modification is the enzymatic removal of the C-terminal tyrosine residue (detyrosination) on α-tubulin which creates a new C-terminal end having two glutamic acid residues (Liao and Gundersen 1998). This form is referred to as Glu-tubulin and is used as a marker for MT stability (Westermann and Weber 2003). Although detyrosination itself does not stabilize MTs, the process precedes further stabilization where Glu-tubulin has an improved ability to recruit stabilizing factors, such as MT-associated proteins (MAPs) and Tau, as well as cargo-carrying motor proteins such as kinesin-1 (Liao and Gundersen1998; Periset al. 2006; Periset al. 2009). Both α-and β-tubulin undergo a number of other posttranslational modifications that create subpopulations of tubulins with different properties that appear to fine-tune MT function in various cellular processes (Westermann and Weber 2003; Janke and Kneussel 2010; Janke and

Bulinski 2011; Song et al. 2013). In addition to detyrosination/tyrosination, α-tubulin undergoes acetylation and polyglutamylation which also increases the ability of these modified MTs to recruit motor proteins (Maas et al. 2009). Polyglycylation of α-tubulin and β-tubulin has been linked to regulation of cell growth (Xia et al. 2000). β-tubulin can be also glycosylated, glutamylated and phosphorylated at a serine and a tyrosine residue, of which the latter modification has been suggested to improve tubulin interaction with cellular membranes (Hargreaves et al. 1986; Garnham and Roll-Mecak 2012). The different posttranslational modifications of tubulin are reversible and can occur in various combinations (Eddé et al. 1991; Janke and Kneussel 2010; Garnham and Roll-Mecak 2012).

We and others have recently reported that tubulin can be also posttranslationally modified by transglutaminase (TG) enzymes and can form covalent high-molecular weight (HMW) oligomers (Del Ducaet al. 2009; Del Ducaet al.2013; Di Sandro et al. 2010; Al-Jallad et al. 2011; Song et al. 2013). TGs are a family of protein-crosslinking enzymes capable of creating an isopeptide bond between a glutamine (Q) residue and a lysine (K) residue or between a glutamine (Q) residue and primary amines such as putrescine, spermine, and spermidine (Williams-Ashman and Canellakis 1980; Lorand and Graham 2003; Iismaa et al. 2009). Formation of covalent isopeptide bonds between proteins leads to the formation of oligomers or polymers, whereas primary amine incorporation does not; however, both modifications have been shown to change the chemical properties of proteins as well as their biological functions (Truong et al. 2004; Lai et al. 2004; Lorand 2007). TG-crosslinking activity has been

associated with stabilization of protein networks in the extracellular matrix, and of intracellular protein systems because crosslinked protein complexes typically resistproteolytic degradation (Iismaa et al. 2009). The TG family contains currently 9 different enzymes-TG1-7, Factor XIII-A (FXIII-A) and band 4.2 (inactive enzyme). TG enzymes are expressed in wide variety of tissues and in different cellular compartments, sometimes working in combinations of two or more (Nurminskaya and Kaartinen 2006; Al-Jallad et al. 2011), where they participate in a variety of cellular functions ranging from tissue stabilization to cell signaling (Lorand 2007; Iismaa et al. 2009; Park et al. 2010). Although many TG substrates are extracellular proteins (Kaartinen et al. 2002; Kaartinen et al. 2005; Esposito and Caputo 2005; Beninati et al. 2009; Cui et al. 2014), they have been also shown to stabilize cytoskeletal and intermediate filament components such as actin, β-tubulin, myosin and vimentin (Maccioni and Seeds 1986; Safer et al. 1997; Nemes et al. 1997; Clement et al. 1998; Tseng et al. 2002; Esposito and Caputo 2005; Baumgartner and Weth 2007; Del Duca et al. 2009; Del Duca et al. 2013; Song et al. 2013). In recent studies, neuronal tubulin was shown to undergo TG-mediated polyamination, namely incorporation of putrecine, spermidine and spermine into its structure (Song et al. 2013); the modification was required for stabilization of MTs during neurite growth (Song et al. 2013). TG activity has also been reported to covalently modify actin and tubulin into HMW oligomers during pear pollen tube growth (Del Ducaet al. 2009; Del Ducaet al.2013; Di Sandro et al. 2010). In our previous work, we demonstrated that bone-forming osteoblasts also form HMW, oligomeric Glu-tubulin upon expression of

TG activity (Al-Jallad et al. 2011). This HMW form of Glu-tubulin was present specifically in membrane preparations of differentiating and secretory osteoblasts, and we suggested at that time that the Glu-tubulin oligomerization was linked to stabilization of MT tracks to the plasma membrane during secretion of type I collagen (COLI). In the present study, we have further examined the formation of oligomeric Glu-tubulin in osteoblasts *in vitro*. We report that the covalent crosslinking by TG creates plasma membrane-associated Glu-tubulin with increased stability. This form co-localizes with FXIII-A transglutaminase and its presence correlates with secretory activity of osteoblasts.

Materials and Methods

Peptides, proteins and antibodies

Biotinylated peptides (bF11: Biotin-DQMMLPWPAVAL, bF11QN: Biotin-DNMMLPWPAVAL) were synthesized by Biologica Co. (Nagoya, Japan) and by Biomatik Corp (Wilmington, DE, USA). Bovine tubulin protein (α/β-tubulin) was purchased from Cytoskeleton Inc. (Denver, CO, USA), pre-activated human FXIII-A enzyme was from Zedira (Darmstadt, Germany), paclitaxel and nocodazole were from Cell Signaling (Danvers, TX, USA). Mouse monoclonal anti-α-tubulin antibody, rabbit anti-Synaptotagmin VII were from Sigma (St. Louis, MO, USA) and anti-Glu-tubulin (rabbit polyclonal antibody) was from Millipore (Temecula, CA, USA). Anti-dansyl antibody was purchased from Molecular Probes (Eugene, OR, USA).

Polyclonal antibody against mouse FXIII-A was designed and generated by GenScript (Piscataway, NJ, USA) (Al-Jallad et al. 2011). Anti-biotin antibody (rabbit polyclonal) was from Rockland Immunochemicals (Gilbertsville PA, USA), anti-fibronectin from (Millipore, Temecula, CA, USA), mouse mAb 6-11B-1 specific for acetylated tubulin and rabbit pan-cadherin antibody from Abcam (Cambridge, MA, USA). Horseradish peroxidase linked anti-rabbit IgG and anti-mouse IgG were from Cell Signaling (Whitby, ON, Canada) and GE Healthcare Life Sciences (Baied'Urfe, QC, Canada), respectively. The AlexaFluor® secondary antibodies, DAPI, EZ-link sulfo-NHS-biotin and protein BCA assay kit were from Thermo Scientific (Waltham, MA, USA). All other reagents, if not specified below, were purchased from Sigma or Fisher Scientific.

Cell culture

Mouse calvarial pre-osteoblasts (MC3T3-E1) subclone 14 were (originally a generous gift from Dr. Renny T. Franceschi, University of Michigan, School of Dentistry, Ann Arbor, MI, USA) (Wang et al. 1999) were cultured in a humidified 37°C incubator at 5% CO_2 in modified alpha minimum essential medium (α-MEM) minus L-glutamine and L-aspartic acid (Gibco, Burlington, ON, Canada), supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin-streptomycin antibiotics, L-glutamine and L-aspartic acid. All treatments were initiated 24 hours after adhesion (Day 0), and cells were plated at 50,000 cells per cm^2 unless otherwise specified. Differentiation into mature osteoblasts was induced by supplementing the medium with 50 µg/ml ascorbic acid

(AA), and mineralization was induced by adding 50 µg/ml AA plus 10 mM β-glycerophosphate (βGP) as described previously (Al-Jallad et al. 2006). This medium was referred to as differentiating medium (DM). Control cells were treated with medium only (referred to as M). Media were changed every second day and TG activity was inhibited by using the irreversible inhibitor N-α-carbobenzyloxy-N-ε-acryloyl-L-lysine(2-(2-dansylaminoethoxy)ethoxy)ethanamie (NC9) (Keillor et al. 2008), which was used at 25 µM as before (Al-Jallad et al. 2011). The end point of all experiments was day 5. For immunofluorescence visualization of the bF11 andbF11QN inhibitors, a 1 µM concentration was used and peptides were administered to cells at day 4 and cells were fixed on day 5 for analysis. For inhibition of COL I deposition, bF11 was administered to cells at a 25-100 µM concentration from day 0 to day 6. The effect of bF11 and bF11QN peptides on cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay (Sigma). Peptides were not toxic to cells at any concentration (Online Resource 2). Plasma fibronectin-depleted serum was prepared as described previously (Cui et al .2014).

Transglutaminase-activity assays

TG-activity assays were performed according to our previously published method (Al-Jallad et al. 2006). Briefly, samples were incubated for 2 hours at 37°C with different concentrations of 5-(biotinamido) pentylamine (bPA) (Pierce /Thermo Scientific), 10 mM dithiothreitol (DTT) and 1µg α/β-tubulin mix and pre-activated

FXIII-A in a reaction buffer containing 3 mM $CaCl_2$ in Tris-buffered saline (TBS) at pH 8.0. After incubation, samples were processed for SDS-PAGE and Western blotting as below.

Preparation of protein extracts, subcellular fractions and cell-surface proteins

Total cellular protein preparations were obtained using RIPA buffer (150m MNaCl, 10 mM Tris-HCl (pH 7.2), 5 mM EDTA, 0.1% Sodium dodecyl sulphate (SDS), 1% Triton X-100, 1% Na-Deoxycholate). Cell extracts were homogenized by ultrasonication directly after extraction and centrifuged at 14000 × g for 15 minutes at 4°C. The supernatant was collected and hereafter is referred to as total protein extract. Subcellular protein fractions where prepared using Proteo Extract Subcellular Proteome Extraction Kit (S-PEK) from Calbiochem (Gibbstown, NJ, USA), following the manufacturer's instructions. Cytoplasmic (Cy), plasma membranous (Me) preparations were used for this study. Proteins presented on the cell surface were isolated using EZ-link sulfo-NHS-biotin (Thermo Scientific). Protein concentrations of all preparations were determined with a bichinchonic acid (BCA) protein assay kit (Thermo Scientific).

SDS-PAGE and Western blotting

Twenty five (25) µg of each sample was boiled in Laemmli buffer for 5 minutes, followed by electrophoretic separation of proteins by SDS-PAGE. Following electrophoresis, gels were transferred to PVDF membranes (Bio-Rad) and blocked

for 30 minutes in 5% non-fat dry milk in TBS-Tween (TBS-T), and then incubated at 4°C overnight in primary antibody diluted in TBS-T. Following incubation with primary antibody, membranes were rinsed in TBS-T, and then incubated with secondary antibody conjugated with horseradish peroxidase at room temperature. Secondary antibodies used were sheep anti-mouse (Invitrogen) and goat anti-rabbit (Cell Signaling). Bands were visualized using the ECL Plus kit (GE Healthcare) and chemiluminescence was detected using Kodak Biomax film.

Immunofluorescence microscopy

Cells were seeded on Nunc® Lab-Tek® chamber slides (Fisher) and cultured as described above. On day 5, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. Slides were washed three times with PBS and blocked with 2% bovine serum albumin (BSA) in PBS. This was followed by primary antibody incubation for 2h at room temperature, and a washing step with 0.1% BSA in PBS and incubation with AlexaFluor® secondary antibody conjugates. Nuclei were stained by DAPI (4', 6-diamidino-2-phenylindole). Samples were mounted with Prolong Gold Anti-Fade medium (Invitrogen) and dried overnight at room temperature. Cells were imaged using an Axioskop2 upright fluorescence microscope equipped with an AxioCam MRm camera and AxioVision4.8 imaging software from Zeiss. For supplemental experiments, some slides were permeabilized with a 10-minute incubation of 0.25% Triton-X100 in PBS, or fixed in methanol at -20 °C.

Total Internal Reflection Fluorescence Microscopy

MC3T3-E1 cells were plated and cultured (as above) on the FluoroDish^{TM} dishes (World Precision Instruments, Inc, Sarasota, FL, USA) coated with 1 µg/ml fibronectin (Sigma). On day 5, cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. Immunofluorescence staining of the cells was done by following the same protocol as for conventional immunofluorescence microscopy (as above). After the final wash of DAPI staining, samples were covered with PBS and kept inthe dark at 4°C prior to analysis. Samples were observed and visualized with an incident angle of 68 degrees using a Zeiss Axio Observer Z1 microscope in the multi-color TIRF illumination mode.

Collagen staining and quantification

Staining for COL I *in vitro* was performed by the Picrosirius method as described previously (Tullber-Reinert and Hundt, 1999). Briefly, cell layers were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), and then fixed in Bouin's Fluid (75% saturated aqueous picric acid, 3.7% formaldehyde, 5% glacial acetic acid) for one hour at room temperature on an orbital shaker. Following fixation, cell layers were washed for 15 minutes, then twice for one minute, in distilled water. COL I was stained by incubating in Sirius Red staining solution (100 mg/ml Sirius Red (Sigma) in saturated aqueous picric acid) for one hour at room temperature with gentle shaking. Unbound dye was removed by washing twice for one minute with 0.01 M HCl. Stain was then dissolved in 0.1 N NaOH,

diluted in PBS, and quantified by spectrophotometry at 562 nm in triplicates. Serial dilutions of COL I from calf skin was applied to 96-well plates, dried overnight, stained as described above, and used to generate standard curves.

Statistical analysis

Error bars represent standard error of the mean (SEM) from three separate samples or experiments. Statistical significance was determined by the Student's *t*-test.

Results

TG activity in osteoblasts creates a membrane-associated, 150 kDa Glu-tubulin with increased stability

To investigate TG-mediated oligomerization of tubulin, we first examined the formation of 150 kDa Glu-tubulin in differentiating MC3T3-E1 osteoblast cultures. As evident from Western blotting conducted after SDS-PAGE under reducing conditions, Glu-tubulin is detected in the cells in two forms, as a 50 kDa monomer and as a 150 kDa HMW form which was specifically found in the membrane preparations (Me) of osteoblasts (Fig. 1a). Inhibition of TG activity in osteoblasts using the irreversible TG inhibitor NC9 (Keillor et al., 2008; Al-Jallad et al., 2011), decreased levels of the 150 kDa Glu-tubulin showing that its formation is dependent on TG activity (Fig. 1a) similarly as we have shown before (Al-Jallad et al. 2011). This covalent modification did not occur in acetylated tubulin (Ac-tub), another posttranslationally stabilized form of α-tubulin (Garnham and Roll-Mecak 2012) which was found only in its monomeric 50 kDa form in osteoblasts (Fig. 1b). To examine whether 150 kDa Glu-tubulin responds to chemical modulators of MT dynamics, MC3T3-E1 osteoblasts were treated with different concentrations of nocodazole (destabilizer) or paclitaxel (stabilizer) during their differentiation. As seen in Figure1c, nocodazole treatment of the cells rapidly and dose-dependently destabilized all of the 50 kDa Glu-tubulin. Nocodazole also affected levels of 150 kDa Glu-tubulin dose dependently, however, the oligomeric form was considerably more resistant to destabilization. Conversely, paclitaxel dose-dependently increased formation of 50 kDa Glu-tubulin, but had no

effect on the formation of 150 kDa Glu-tubulin, strongly indicating that there is an additional rate-limiting step in the formation of 150 kDa Glu-tubulin. To investigate whether the formation of 150 kDa Glu-tubulin might involve a plasma membrane TG activity, we detected the presence of the activity in the membrane fractions of non-differentiating and differentiating osteoblasts inhibited byNC9 which incorporates covalently and irreversibly into active TG enzymes and can be detected via its dansyl group. As seen in Figure 1d, detection of the dansyl group of NC9 in cytoskeletal and membrane fractions of osteoblasts by Western blotting showed that the inhibitor was specifically in the membrane fraction of both non-differentiating (medium only treated, M) cells and differentiating (differentiation medium, DM) cells and bound to a68 kDa enzyme. This molecular weight (MW) corresponds to the MW of cellular FXIII-A in the mouse osteoblast culture system (Al-Jallad et al. 2011). In summary, Glu-tubulin appears to undergo a specific TG-mediated stabilization step in osteoblasts that results in the formation of membrane-associated, oligomeric 150 kDa Glu-tubulin that has increased stability compared to 50 kDa Glu-tubulin.

α-Tubulin is a specific TG substrate and can be crosslinked into HMW forms of 100, 150 and 250 kDa (dimers, trimers and polymers) *in vitro*

As demonstrated, differentiating MC3T3-E1 osteoblasts have oligomeric Glu-tubulin whose formation is dependent on TG activity. We next examined if tubulin serves as a crosslinking substrate *in vitro* and investigated whether this modification is specific to α-tubulin, which is known to give rise to Glu-tubulin (β-tubulin has a

different C-terminus and is not detyrosinated). This was tested by an established TG substrate assay involving protein incubation with a TG enzyme and 5-(biotinamido) pentylamine (bPA) (Al-Jallad et al. 2006). In this reaction bPA replaces lysine residues in crosslink formation and incorporates into TG-reactive glutamine residues of a substrate protein in the presence of a TG enzyme (Iismaa et al. 2009). We have previously shown that osteoblasts express two TGs - TG2 and FXIII-A - the latter being the main crosslinking enzyme in osteoblasts (Al-Jallad et al. 2006; Al-Jallad et al. 2011; Cui et al. 2014); thus, FXIII-A was the chosen enzyme for this assay. Incubation of an α/β-tubulin mix with a pre-activated human FXIII-A and increasing amounts of bPA resulted in a dose-dependent, strong labeling as well as formation of covalent (SDS-resistant) oligomeric tubulin forms as analyzed by Western blotting and detection by biotin antibody for bPA label (Fig. 2a). α/β-tubulin mix formed dimers, trimers, tetramers and pentamers all of which were also labeled with bPA, this being indicative of the existence of several TG-reactive Q-residues (Fig. 2a). A negative control without α/β-tubulin was used to confirm that the biotin-labeled bands were from tubulin and do not result from bPA incorporation into FXIII-A enzyme. We further incubated the α/β-tubulin mix with FXIII-A or TG2 enzyme alone without bPA, and detected the samples with specific α-tubulin and β-tubulin antibodies to investigate whether the modification was specific to FXIII-A and crosslinking of α-tubulin. As evident from Figure 2b, the modification was not specific to FXIII-A; the TG2 enzyme was capable of creating α-tubulin oligomers. Crosslinking of the α/β-tubulin mix with FXIII-A and detection with both α-tubulin and β-tubulin antibodies (Fig.2c) showed

strong and specific polymerization of the α-tubulin, while β-tubulin did not form any oligomers. Furthermore, the observation that the pure α/β-tubulin preparations contained also oligomerized α-tubulin, but no oligomers of β-tubulin (Fig. 2c), supported the finding and indicated that the α-tubulin is more prone to forming oligomers.

α-tubulin and Glu-tubulin are found on the plasma membrane and co-localize with FXIII-A

The observation that 150 kDa Glu-tubulin specifically associated with membrane fractions in MC3T3-E1 osteoblast cultures suggests that it may be associated with the plasma membrane of the cells and possibly also found on the cell surface. Although it is well established that tubulin links to the inner leaflet of the plasma membrane via linker molecules (microtubule plus-end tracking proteins (TIPs)) such as EB1 (Ligon et al. 2003; Dixit et al. 2009), it has been suggested, although not yet fully established that tubulin alone can associate with the plasma membrane and even penetrate the plasma membrane to extrude to the cell surface (Zisapel and Littauer 1979; Rubin et al. 1982; Quillen et al. 1985; Wolff 2009). It has been suggested that modifications such as phosphorylation may promote tubulin insertion into membranes (Hargreaves et al. 1986; Atashi et al. 1992). The concept that tubulin is on the outer leaflet of the osteoblast plasma membrane is central to the concept that it undergoes crosslinking by FXIII-A since FXIII-A is generally considered only to catalyze the crosslinking reaction within the high Ca^{2+} environment outside the cell, and because we have

demonstrated in our previous work that FXIII-A (and TG2) are not active inside osteoblasts, but are found on the cell surface (Al-Jallad et al. 2011). Therefore, we examined whether α-and Glu-tubulin were associated with the osteoblast plasma membrane and whether they are found on the cell surface, and if they co-localize there with FXIII-A. To do this, we used Total Internal Reflection Fluorescence (TIRF) microscopy which is a specialized technique to study cell-surface and plasma membrane-associated molecules (Axelrod 2001; Jaiswal and Simon 2007; Mattheyses et al. 2010; Simons and Gerl 2010). TIRF microscope only excites fluorophores in a restricted region of the specimen immediately adjacent to the bottom of the microscope slide upon which cells are examined using this method, which allows the study of molecules on the plasma membrane (Mattheyses et al. 2010). As seen in Figure 3a, TIRF microscopy using a 68-degree angle and penetration depth of less than 80 nm into the plasma membrane shows clear α-tubulin staining where it appears close to the plasma membrane in~ 5-7μm patches. Analysis of α-tubulin, Glu-tubulin and FXIII-A with TIRF microscopy shows co-localization of both forms of tubulins with FXIII-A in these structures (Fig. 3b). We have previously published co-localization of FXIII-A and tubulin in similar structures using a conventional IF microscope where specimens were prepared by only fixing with formaldehyde (not permeabilized with Triton-X100) (Al-Jallad et al. 2011). In these experiments, we assigned the patchy staining pattern to tubulin at the periphery of the osteoblasts. Here the α-tubulin visualization with TIRF microscopy resembles these images obtained with conventional IF microscopy. Cells fixed with only

formaldehyde showing filamentous α-tubulin patches (Online Resource 1a). This suggests that the formaldehyde-fixation method may be also used to visualize tubulin at the cell periphery. As seen in Online Resource 1a-c, formaldehyde fixing blocks visualization of the intracellular tubulin network which can be seen only after Triton-X100 permeabilization or after methanol fixation at -20 °C. Conversely, the two methods do not show patchy tubulin staining suggesting that the detergent treatment interferes with detection of tubulin patches, possibly by abolishing critical lipid components from the cell membrane (Online Resource 1).

Since the plasma membrane is on average 7 nm thick, visualizing through a depth of 80 nm with TIRF microscopy is not a *bone fide* demonstration of the presence of a protein on the outer leaflet of the plasma membrane. Thus, we further examined the possibility of the Glu-tubulin being on the outer leaflet of the osteoblast membrane by cell-surface biotinylation experiments. For this, cell surface-specific biotinylation and subsequent pull-down experiments were done using cell-impermeable sulfo-NHS-biotin. Western blotting of α- and Glu-tubulin from this cell-surface biotinylated material showed clearly that both Glu-tubulin and α-tubulin are found on the surface of the osteoblast, but that the presence of Glu-tubulin (both 50 kDa and 150 kDa forms) on the cell surface is specific to the differentiated and COL I-depositing cells producing extracellular matrix (Fig. 3c). The presence of oligomeric Glu-tubulin on the cell surface is dependent on TG activity since inhibitor NC9 abolishes the 150 kDa Glu-tubulin while the 50 kDa form appears largely unaffected. This suggests that Glu-tubulin first localizes to the cell surface and is

subsequently crosslinked upon co-localizing with FXIII-A. Immunodetection of cell-surface β1-integrin was used as a positive control for Western blotting using the same biotinylated material (Fig. 3c). We conclude that Glu-tubulin (α-tubulin) can be found on the outer leaflet of the plasma membrane (on the cell surface) where it appears to acts as a substrate for FXIII-A.

The specific FXIII-A substrate peptidebF11co-localizes with α-tubulin in osteoblasts and attenuates α-tubulin crosslinking *in vitro*

To further confirm that the α-tubulin (and thus Glu-tubulin) is crosslinked by FXIII-A in osteoblast cultures, we used the FXIII-A-specific substrate peptidebF11 to examine whether it co-localizes with α-tubulin. This isozyme-specific Q-donor substrate probe was developed from a phage-display random peptide library (Sugimura et al. 2006; Watanabe K et al. 2013) and it specifically incorporates into substrates of FXIII-A. The peptide bears a biotin label in the N-terminus for antibody detection. Immunofluorescence staining of nonpermeabilized osteoblasts and their analysis by conventional IF microscopy shows co-localization of the bF11 peptide with α-tubulin in rounded patches (Fig. 4a). Use of control bF11QN peptide where a Q residue has been changed to an N residue (which eliminates its reactivity to FXIII-A) showed no staining. TIRF microscopy confirmed the co-localization of bF11 and α-tubulin on the osteoblast plasma membrane (Fig. 4b). We next tested the ability of the bF11 peptide to covalently integrate into α-tubulin using an *in vitro* TG-activity assay. In this assay, bF11 incorporated into α-tubulin as did bPA. It also acted as a

competitive inhibitor to decrease the oligomerization of α-tubulin *in vitro* when applied at a high concentration (100μM) (Figure 4C, last lane). bPA-labeled α-tubulin is detected at 50 kDa and 150 kDa. The 75 kDa band here arises from labeling of the preactivated human FXIII-A added in the mixtures. These results demonstrate that α-tubulin has both TG-reactive Q residues and TG-reactive K residues that are capable of covalently reacting and incorporating bPA and bF11, respectively.

bF11 peptide inhibits Glu-tubulin crosslinking and COL I deposition in osteoblast cultures

We have previously linked FXIII-A activity in osteoblast cultures to COL I extracellular matrix deposition via mechanisms that involve *i*) promotion of COL I-containing secretory vesicle trafficking to the plasma membrane via microtubule tracks and Glu-tubulin crosslinking to stabilize the secretory process, and *ii*) the stabilization of a fibronectin matrix scaffold for COL I deposition into the extracellular space. To examine if the bF11 peptide was able to also block COL I secretion and deposition, we used bF11 in osteoblast cultures at a high dose where it would be expected to act as a competitive inhibitor and attenuate the formation of oligomeric 150 kDa Glu-tubulin. Firstly, as seen in Figure 5a, 100 μM bF11 decreased the formation of 150 kDa Glu-tubulin in differentiating osteoblast cultures (DM) similarly as NC9 as evident from Western blots of cytosolic and membrane fractions of differentiating osteoblasts. Examination of the effect of bF11 on COL I matrix accumulation in osteoblast cultures showed that it inhibited the process in a

concentration-dependent manner when the inhibitory peptide was applied to the cell cultures for 6 days (25-100 μM concentrations were tested). Quantification of Picrosirius Red staining (which stains extracellular fibrillar COL I) in the cultures showed significant (-39% and -63%) decreases in COL I levels at 50 and 100 μM bF11 concentration, respectively (Fig. 5b). An unexplained slight but significant increase in COL I deposition was observed when using bF11 at the 25 μM concentration and bF11QN at the 100 μM concentration. bF11 treatments did not affect cell viability (Online Resource 2).

To examine whether the secretory process was defective in bF11-treated cells, we analyzed synaptotagmin VII (Syt VII) levels in the membrane fractions. It has been shown that Syt VII becomes part of the cell membrane during successful exocytosis making Syt VII detectable at the cell surface of secretory cells (Chapman 2002). As seen also in Figure 5c, Syt VII levels were decreased (-29%) in membrane fractions of bF11-treated cells, supporting the notion that secretory vesicle transport to the cell membrane is impaired following this inhibition. Of importance here is also the observation that Syt VII is found on the plasma membrane of osteoblasts only in differentiating (COL I-synthesizing) osteoblasts. Nondifferentiating medium-treated osteoblasts retained Syt VII in the cytosolic compartment (Fig. 5c).

As outlined above, we have shown before that inhibition of FXIII-A with bF11 in osteoblast cultures interferes with plasma FN (pFN) assembly (Cui et al. 2014) as also seen in Online Resource 2a. Thus, as a control experiment, we examined whether the lack of a pFN network affects oligomerization of Glu-tubulin. This was

done by culturing osteoblasts in pFN-depleted serum. As seen in Online Resource 2b, pFN depletion from serum did not affect the formation of oligomeric 150 kDa Glu-tubulin. This demonstrates that Glu-tubulin crosslinking and pFN matrix stabilization are two separate functions of FXIII-A. In summary, formation of 150 kDa Glu-tubulin is associated with FXIII-A activity on the cell surface and COL I secretion in osteoblasts.

Discussion

Both α- and β-tubulin undergo a number of posttranslational modifications which influence MT function and dynamics in many cell types. In this study, we describe details of a FXIII-A-mediated covalent oligomerization of Glu-tubulin in osteoblasts. We report that this oligomerization, which creates a 150 kDa Glu-tubulin form, is specific to α- and Glu-tubulin. This HMW Glu-tubulin appears associated with the plasma membrane and found on the outer leaflet of osteoblast cells where it co-localizes with FXIII-A. Oligomerization appears to create a more stable form of Glu-tubulin whose function is likely linked to highly specialized COL I trafficking in osteoblasts to produce an extracellular matrix. While our studies are the first to link HMW Glu-tubulin formation to COL I secretion, the role of detyrosination and the formation of Glu-tubulin has been linked to the exocytotic secretory process in other studies. It has been speculated that Glu-tubulin binds kinesin motor protein(s) with high affinity during cell polarization, differentiation and secretory vesicle trafficking (Liao and Gundersen 1998). It has also been shown that Glu-tubulin slows down the velocity of the kinesin protein while it moves along MT tracks. This change in velocity has been suggested to allow more ‘consistent delivery of cargo’ or ‘asymmetric’ secretion in the cell (Garnham and Roll-Mecak 2012). Indeed, COL I secretory activity in osteoblasts is consistent with such a concept. COL I is the most abundant protein in vertebrates and constitutes approximately 30% of the weight of bone (Feng and McDonald 2011). Its biosynthesis is a highly complex process involving important extracellular processing steps where it is carefully assembled into long fibrils and then

fibers (Kadler et al. 1996). In bone, osteoblasts are thus a cell type responsible for a high level of continuous, polarized secretory activity from the apical cell membrane that occurs over weeks while the cell resides at the bone tissue surface (Long 2012). Thus, an extra step to stabilize MT tracks radiating to the cell membrane involving covalent oligomerization of tubulin may be required to achieve this extensive and stable secretion.

In the present study, we describe that the oligomeric 150 kDa Glu-tubulin form is plasma membrane-associated since it is found only in membrane fractions as assessed by subcellular protein analysis. While the mechanism by which this oligomerization might promote Glu-tubulin association with the plasma membrane remains unknown, it is possible that the crosslinking results in complexation with another protein that is capable of penetrating the phospholipid layer. Another intriguing possibility is that oligomerization of Glu-tubulin changes tubulin solubility to lipids which may promote its association with membranes in a manner similar to that which has been described for phosphorylated β-tubulin and its association with vesicle lipid bilayers (Hargreaves et al. 1986). It is known that solubility changes are fairly common upon covalent crosslinking by TGs (Lai et al. 2004; Shin et al. 2008).

We also report that both 50kDa and 150 kDa Glu-tubulin/α-tubulin are found on the cell surface where we believe the covalent crosslinking and oligomerization occurs. While the presence of tubulin on the cell surface remains a concept that needs further evidence, it is not a completely novel idea. The existence of cell surface tubulin was first shown in 1976 with an approach involving labelling of the surface of

pigeon erythrocytes (known to contain tubulin, unlike human erythrocytes) with colchicine/Sepharose beadswhich bind to β- and α-tubulin (Zenner and Pfeuffer 1976). The authors of this study concluded that since colchicine extends at most 11–12Å into the membrane, that it binds to material on the cell surface. Further evidence of the existence of cell-surface tubulin comes from studies of malignant cells where it has been reported for neuroblastoma cells (Zisapel and Littauer 1979), for transformed lymphocytes (Bachvaroff et al. 1980) and for a lymphoblast line (Quillen et al. 1985). In addition to that occurring in lymphocytes, tubulin penetration to the outer leaflet of the plasma membrane has been shown via surface labeling of intact cells in a human cultured monocyte-like cell line, U937 (Por et al. 1991). In this study, the authors used an indirect staining procedure based on a biotinylated anti-mouse IgG antibody, and flow cytometry using antibodies to actin and tubulin which detected both these proteins on the cell surface in U937 cells. The role of cell-surface tubulin is not known in any of the above cell types; however, the authors of these studies suggested that it may be linked with malignancy and leukemic transformation. Our work provides further evidence of the potential existence of cell-surface tubulin in another, nonmalignant cell type (osteoblasts), and suggests that it may represent a form of tubulin that can be modified and further stabilized by TGs on the cell surface.

Evidence that tubulin is a substrate for TG in mammalian cells was first introduced in 1986 by Maccioni and Seeds using clonal mouse neuroblastoma N18 cells (Maccioni and Seeds 1986). The authors of this study suggested that this

modification could mediate covalent crosslinking of microtubules to other cellular components. To date, most studies regarding tubulin suggest that TGs stabilize this MT protein, or regulate the interactions between tubulin and membrane-associated or cytoskeletal proteins, to facilitate biological events such as cell secretion, placental development, and axonal growth and maintenance (Robinson et al. 2007; Del Duca et al. 2009; Al-Jallad et al. 2011; Song et al. 2013). The latest report on neural cells has shown that tubulin can be polyaminated by TG2 in the intracellular space in a process that regulates axon growth (Song et al. 2013). TGs require a fairly high Ca^{2+}-concentration for activation which induces a significant conformational change in the enzymes that expose their active site (Pinkas et al.2007). This knowledge has been at the center of a debate on whether TGs can act as crosslinking enzymes in the intracellular compartment where Ca^{2+}-concentration is relatively low. With regard to this, strong evidence exists showing that monoamination (serotonylation, histaminylation, dopaminylation of Q residues) (Vowinckel et al. 2012) and polyamination (incorporation of spermine, spermidine, putrescine into Q residues) occurs in the intracellular compartment (Seiler and Deckardt 1976; Cooper et al. 1997; Jeon J-H et al. 2003; Song et al. 2013). It thus seems reasonable to consider that TGs may be activated inside the cell via Ca^{2+} oscillations, or that binding of other small proteins to intracellular TGs could induce conformational change and activation. In our system, the active TG enzyme appears to be FXIII-A (Al-Jallad et al. 2011, Cui et al. 2014) which has not heretofore been reported to catalyze crosslinking reactions inside cells. Although FXIII-A is found inside osteoblasts, where it may have functions

in cell signaling, its crosslinking activity is only found on the plasma membrane outer leaflet of the cells as well as in the extracellular matrix. Thus, for FXIII-A-mediated oligomerization of tubulin to occur, it would be required to be outside the cells. Oneintriguing possibility related to this discussion is that tubulin may be monoaminated and/or polyaminated by TG2 inside certain cells, and oligomerized by FXIII-A outside the cells, thus providing two modes of stabilization depending on the cell type and function of MTs related to the respective cell biology of different cell types.

In summary, in differentiating osteoblasts, Glu-tubulin can act as a substrate for FXIII-A transglutaminase which creates oligomeric Glu-tubulin with increased stability. This form of Glu-tubulin is membrane-associated where its presence is linked with COL I secretion and deposition during osteoblast differentiation and the associated elaboration of a collagenous extracellular matrix.

Acknowledgements

We would like to thank Mrs. Aisha Mousa for assistance in all phases of this study, and Dr. Erika Wee of the Advanced BioImaging Facility (ABIF) of McGill University's Life Science Complex for excellent assistance in using TIRF microscopy. This study was supported by grants to MTK from the Canadian Institutes of Health Research (CIHR) (MOP-119403) the CIHR Institute for Musculoskeletal Health and Arthritis (IMHA) (IMH-89827). KH was supported by a Grant-in-Aid for Scientific Research (B) (No. 23380200) from JSPS, Japan (KH). SW received studentship stipends from the China Scholarship Council (CSC) and Faculty of Dentistry, McGill University. CC received a studentship stipend from China the Scholarship Council (CSC).

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical standards

The manuscript does not contain clinical studies or patient data.

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Figure 1

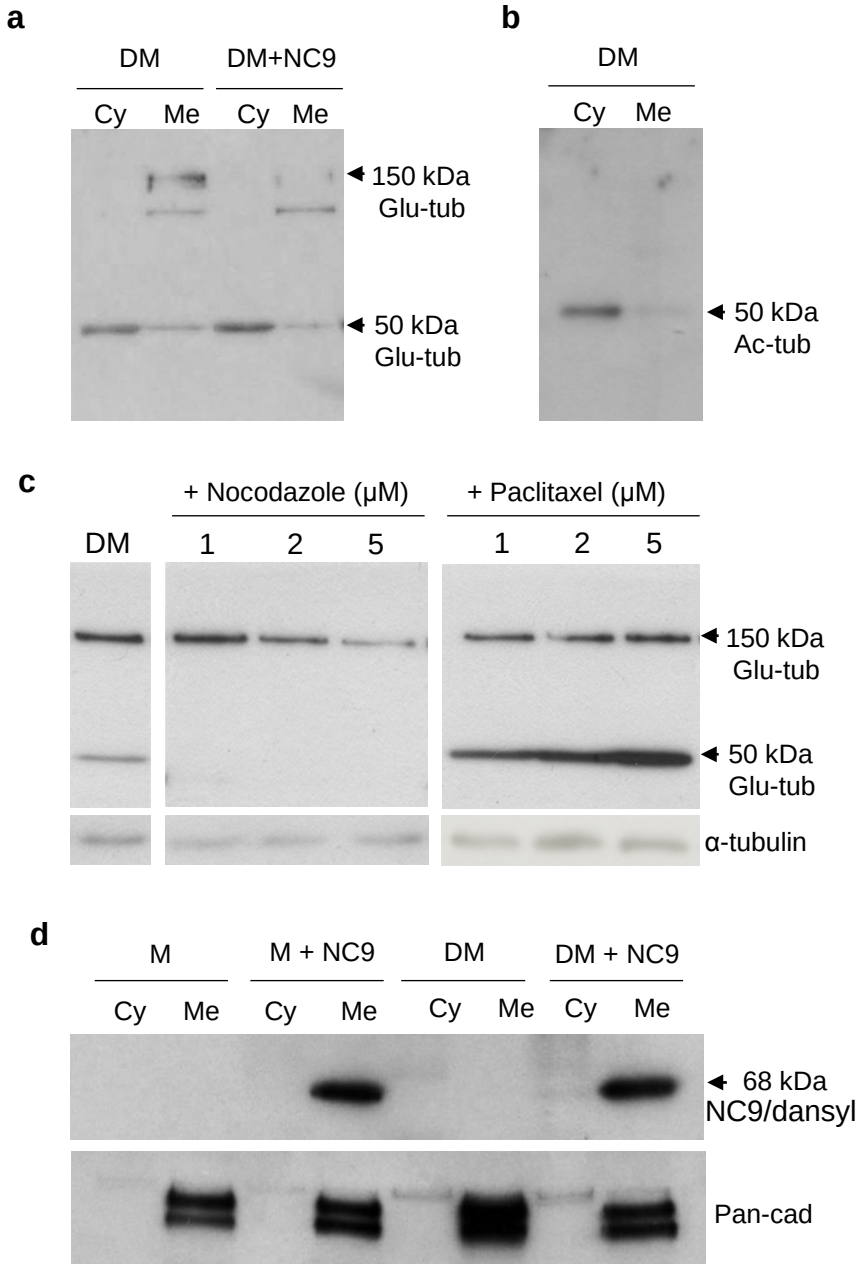


Fig. 1. TG activity on the osteoblast cell membrane creates a high-molecular weight, membrane-associated, 150 kDa Glu-tubulin with increased resistance to destabilization. (a) Western blot analysis of Glu-tubulin (Glu-tub) in cytosolic (Cy) and membrane (Me) fractions of differentiating MC3T3-E1 osteoblasts (DM). Glu-tubulin is found in two forms, as a 50 kDa monomer in the cytosolic fraction and as a 150 kDa high-molecular weight form in the membrane fraction. The TG inhibitor, NC9, decreases the levels of 150k Da Glu-tubulin. (b) Acetylated tubulin (Ac-tub) is not found as a high-molecular weight form as analyzed by Western blotting. (c) The 150 kDa Glu-tubulin has increased stability, since nocodazole treatment of differentiating osteoblasts causes a destabilization and disappearance of the 50 kDaGlu-tubulin, whereas the 150 kDa Glu-tubulin shows increased resistance to this destabilization. Paclitaxel, a microtubule stabilizer, causes a dose-dependent increase in the levels of 50 kDa Glu-tubulin, whereas the 150 kDa Glu-tubulin does not respond to paclitaxel treatment. α-Tubulin was used as a loading control. (d) Western blot analysis of the dansyl group of NC9 (a TG inhibitor) shows its specific incorporation into the membrane fraction of osteoblasts indicating the presence of the enzyme on the plasma membrane. Pan-cadherin (Pan-cad) was used as loading control. NC9 labeled a TG enzyme with a MW of 68kDa.

Figure 2

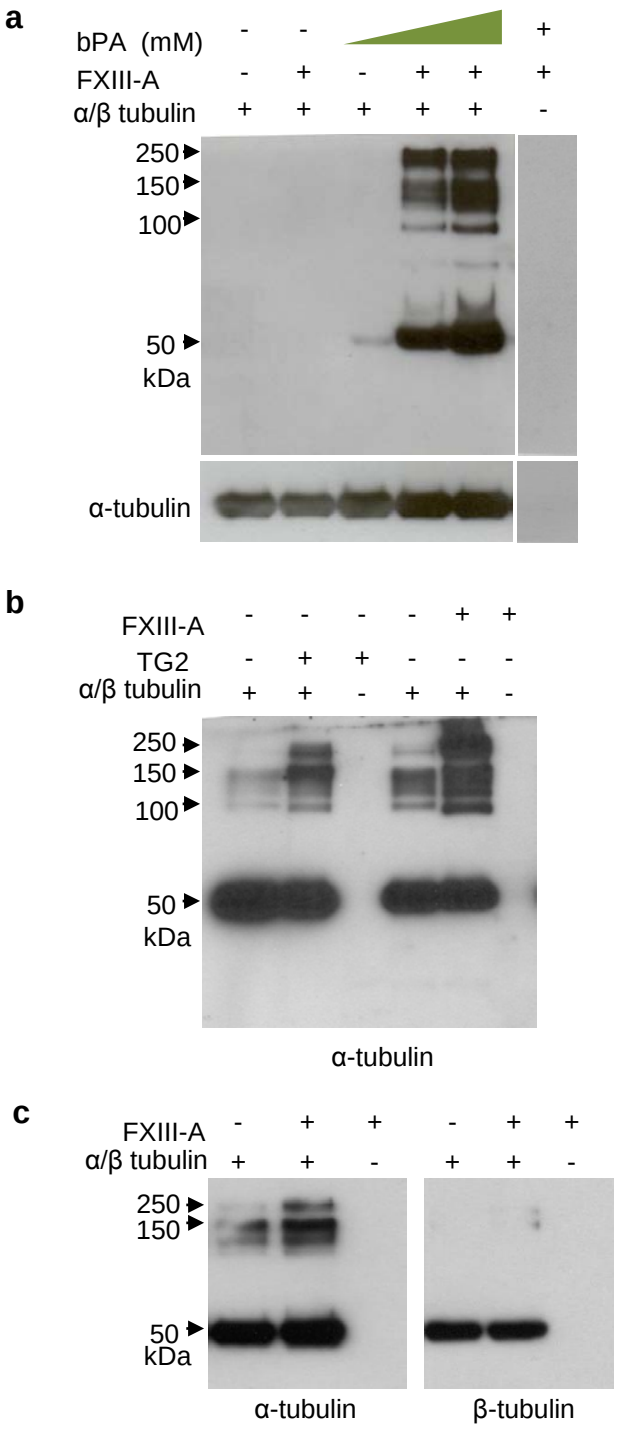


Fig. 2. **α-Tubulin but not β-tubulin, is a TG substrate and can be crosslinked into high-molecular weight forms of 100, 150 and 250 kDa (dimers, trimers and pentamers).** (a) TG-substrate assay of α/β-tubulin mix was performed by using biotin-pentyl amine (bPA) which incorporates into Q residues of TG substrates in the presence of TG enzyme(s). Western blot analysis using biotin-antibody shows that bPA label incorporates into 50 kDa tubulin as well as into complexes of tubulin. Dimers, trimers and other-sized polymers are readily detected. (b) Tubulin can act as substrate for both TG2 and Factor XIII-A as demonstrated by the formation of high-molecular weight forms of α-tubulin upon incubation with both enzymes. Western blot detection was done for α-tubulin. (c) α-Tubulin is a specific substrate for FXIIIA as demonstrated by the formation of high-molecular weight complexes in the presence of the enzyme. β-Tubulin does not form complexes and is thus not a FXIII-A substrate.

Figure 3

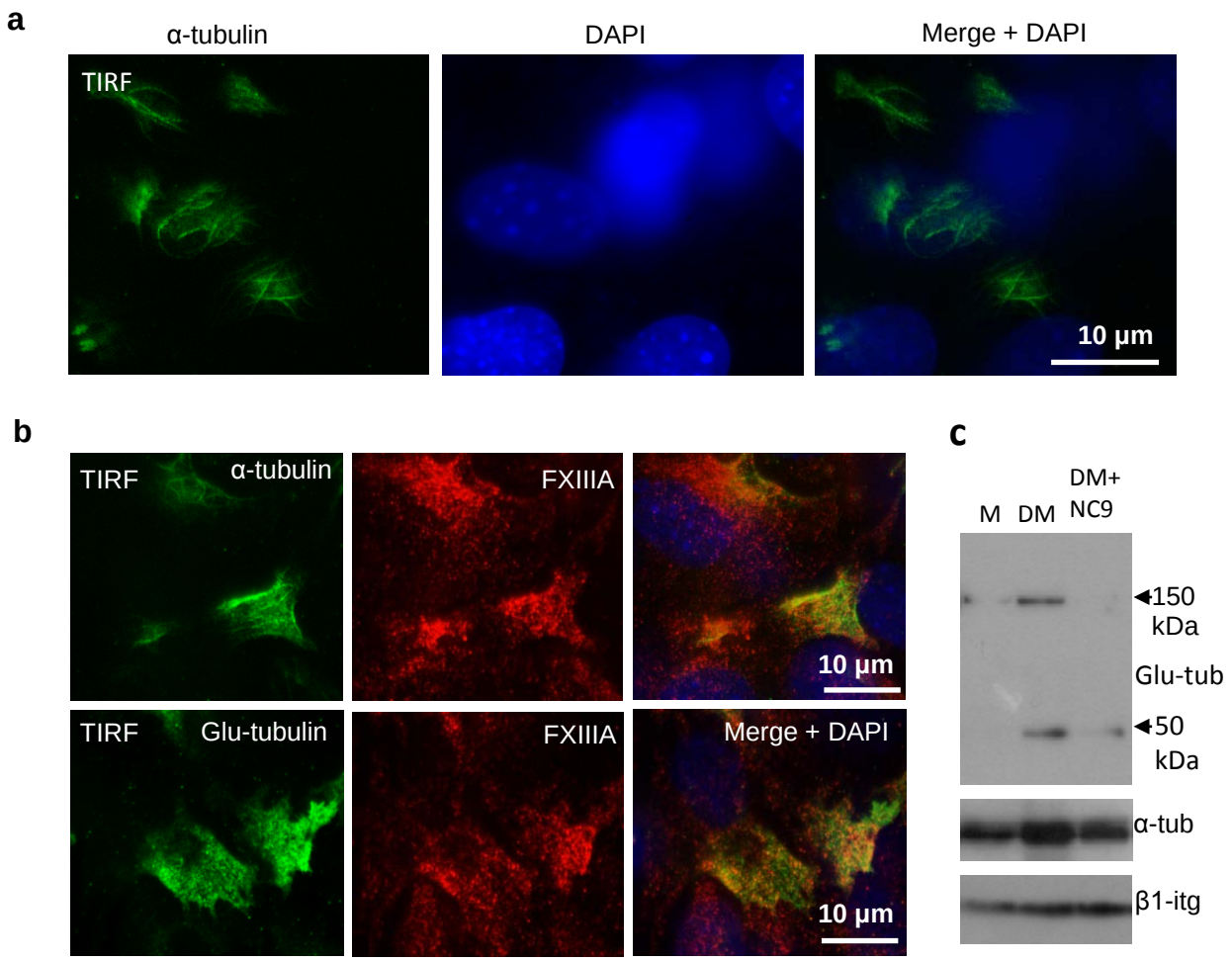


Fig. 3. **α- and Glu-tubulin are found on the plasma membrane and cell surface of osteoblasts where they co-localize with Factor XIII-A.** (a) TIRF microscopy of α-tubulin (green, Alexa Fluor® 488) and DAPI (blue) (visualized in EPI mode) in formaldehyde-fixed and nonpermeabilized osteoblasts on day 5. Analysis shows α-tubulin in 5-7 µm patches at the periphery of the cells. Visualization was done with an incident angle of 68 degrees reaching to a depth of 80 nm. (b) TIRF microscopy co-localization (yellow) of FXIII-A (red, Alexa Fluor® 568) and α-tubulin and Glu-tubulin (green, Alexa Fluor® 488) in differentiating osteoblasts. (c) Both α- and Glu-tubulin are detected on the cell surface as determined by Western blotting of cell-surface biotinylated protein extracts. A non-cell permeable biotinylation reagent was used for preparing and purifying the cell-surface material. Differentiating osteoblasts (DM) have more high-molecular weight Glu-tubulin on the cell surface compared to nondifferentiated osteoblasts treated with medium only (M). Inhibition of TG activity with NC9 results in a decrease of the high-molecular weight Glu-tubulin on the cell surface. β1-integrin (β1-itg) was used as the loading control for cell-surface material.

Figure 4

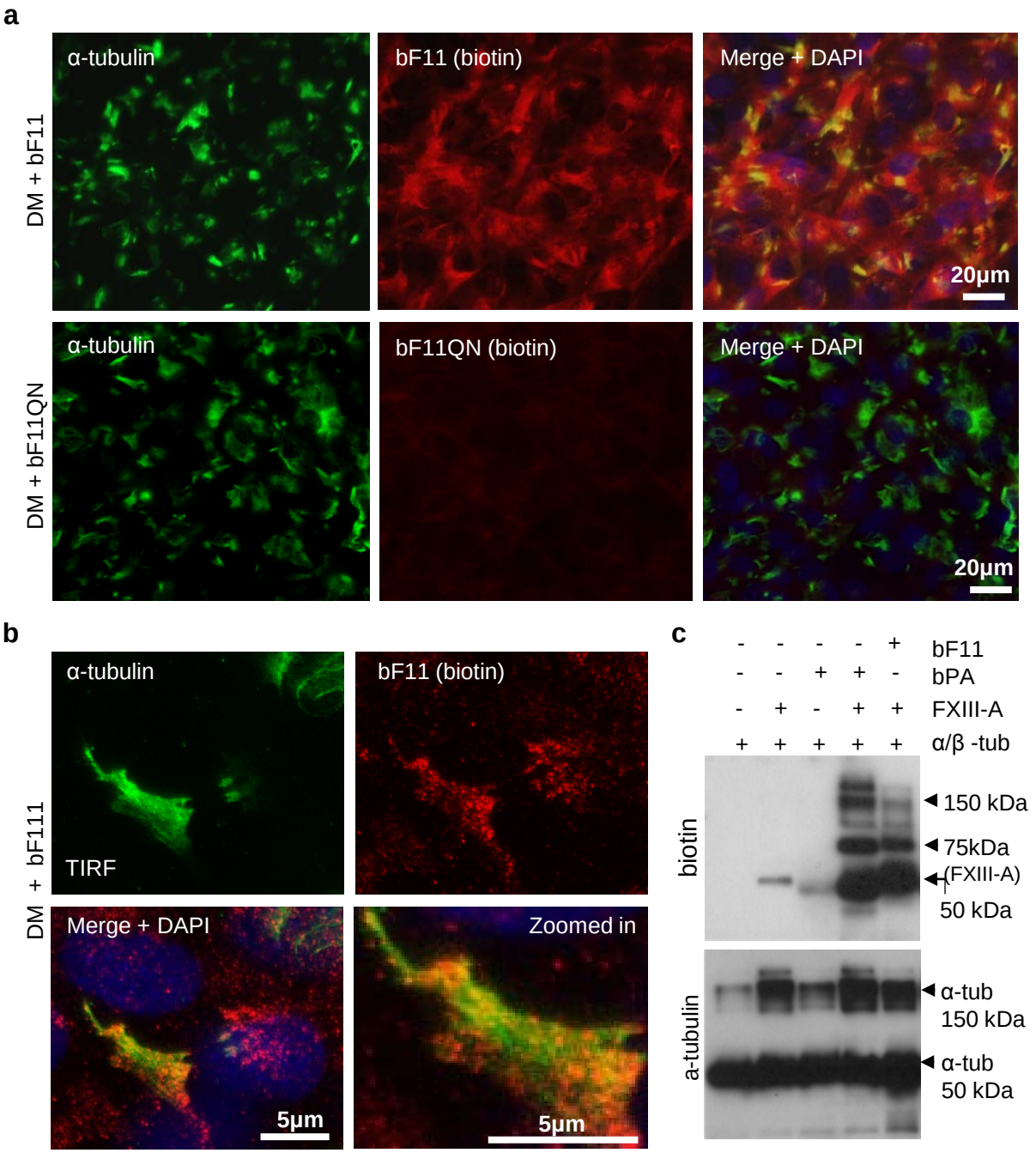


Fig. 4. The Factor XIII-A-specific substrate peptide bF11 co-localizes with α-tubulin and inhibits α-tubulin crosslinking *in vitro*. (a) Immunofluorescence microscopy of bF11 incorporation into osteoblast cell cultures and co-localization with α-tubulin. bF11 was detected with anti-biotin antibody (red, Alexa Fluor® 568) co-localized (yellow) with α-tubulin (green, Alexa Fluor® 488). bF11QN control peptide does not incorporate into osteoblast cultures and no signal is detected. (b) TIRF microscopy co-localization (yellow) of bF11 (biotin) (red, Alexa Fluor® 568) with α-tubulin and Glu-tubulin (green, Alexa Fluor® 488) in differentiating osteoblasts (DM) on day 5 informaldehyde-fixed, nonpermeabilized cells. (c) Western blot analysis of bF11 peptide incorporation into α-tubulin in the presence of FXIII-A (last lane). bF11 incorporates into α-tubulin in a manner similar to biotinylated pentylamine (bPA), but is more efficient in blocking the formation of high-molecular weight complexes of α-tubulin. The 75 kDa band arises from bPA-labeled FXIII-A. Since bF11 incorporates into the Kresidues of TG substrates and bPA into the Q residues, these results show that α-tubulin can provide both K and Q residues for crosslinking.

Figure 5

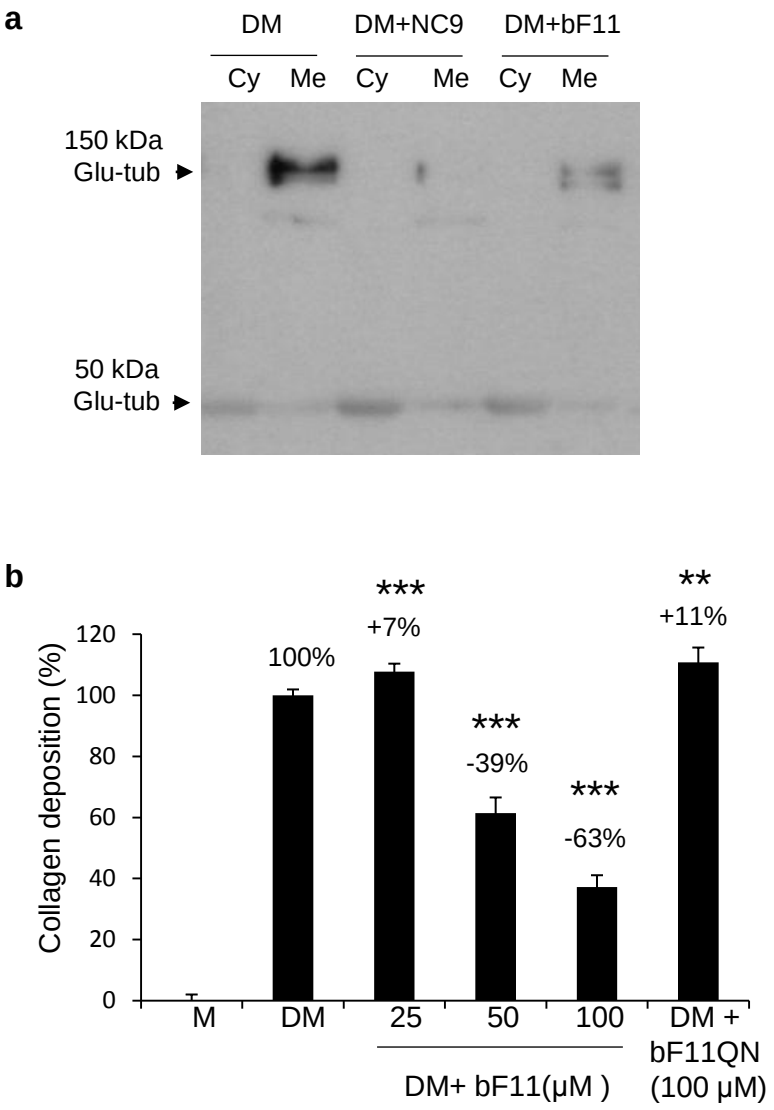
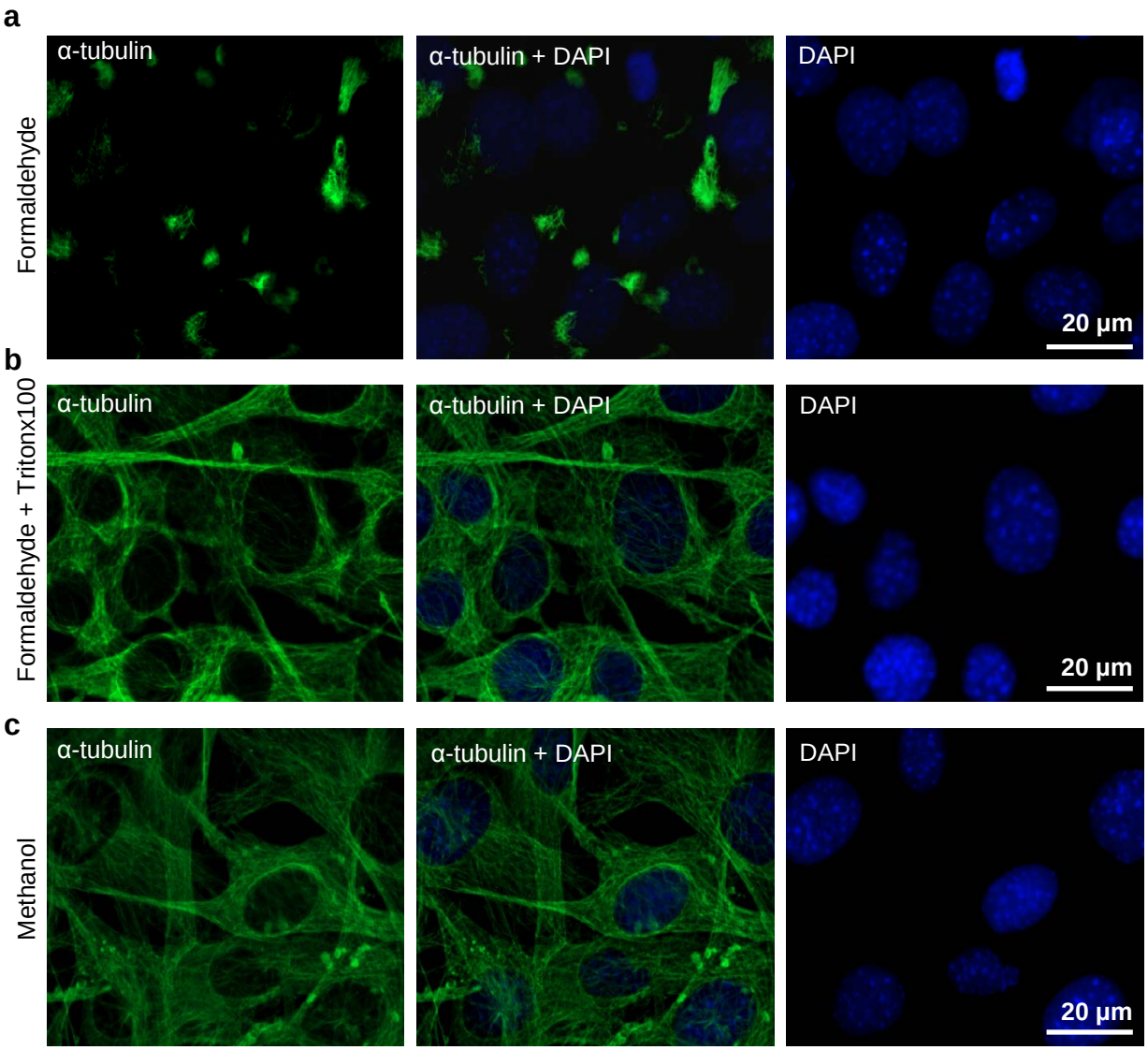

a
DM
DM+NC9
DM+bF11
Cy
Me
Cy
Me
Cy
Me
150 kDa
Glu-tub
50 kDa
Glu-tub
b
Collagen deposition (%)
120
100
80
60
40
20
0
100%
***
+7%
***
-39%
***
-63%
**
+11%
M
DM
25
50
100
DM +
bF11QN
(100 µM)
DM+ bF11(µM)
c
M
DM
DM+bF11
Cy
Me
Cy
Me
Cy
Me
Syt VII
Pan-cad

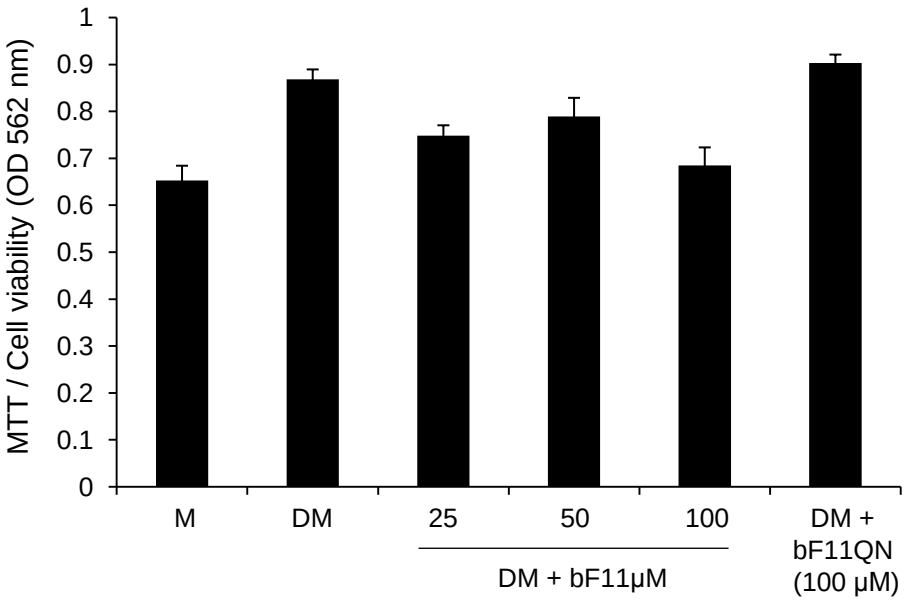
Fig. 5. The Factor XIII-A-specific substrate peptide bF11 attenuates levels of the 150 kDa Glu-tubulin in osteoblasts and inhibits COL I extracellular matrix accumulation. (a) Western blot analysis of 150 kDa Glu-tubulinin cytosolic (Cy) and membrane (Me) fractions of differentiating osteoblasts upon bF11 treatment. NC9 treatment was used here as a positive control to demonstrate successful inhibition. bF11 (100 µM) treatment results in a decrease in 150 kDa HMW Glu-tubulin levels in membrane fractions similar to that seen after treatment with NC9. **(b)** COL I deposition in osteoblast cultures upon bF11 treatment (25-100 µM). COL I levels were quantified by Picrosirus red staining. The COL I levels of nondifferentiating (and thus non-COL I depositing) osteoblasts was subtracted as background (0%). COL I levels in differentiating osteoblasts (DM) were set at 100%. bF11 treatment results in a dose-dependent and significant decrease of COL I deposition at concentrations of 50 and 100 µM (-39% and -63%, respectively). A slight, but significant increase in COL I deposition is observed at 25 µM of bF11 (+7%) and at 100µMof bF11QN (+11%). Data are presented as mean values SEM. ** $p \leq 0.01$.*** $p \leq 0.001$.Student's *t*-test relative to control (DM). **(c)** Western blot analysis of synaptotagmin VII (Syt VII) levels in cytosolic (Cy) and membrane (Me) fractions of differentiating osteoblasts upon bF11 treatment. Differentiating treatment (DM) results in an appearance of Syt VII in the membrane fraction as an indicator of successful exocytosis of proteins related to osteoblast differentiation and the elaboration of a collagenous extracellular matrix. bF11 treatment decreases Syt VII levels in the membrane fraction by 29%, indicating that the secretory process was impaired. Pan-cadherin was used as the loading control.

Online Resource 1



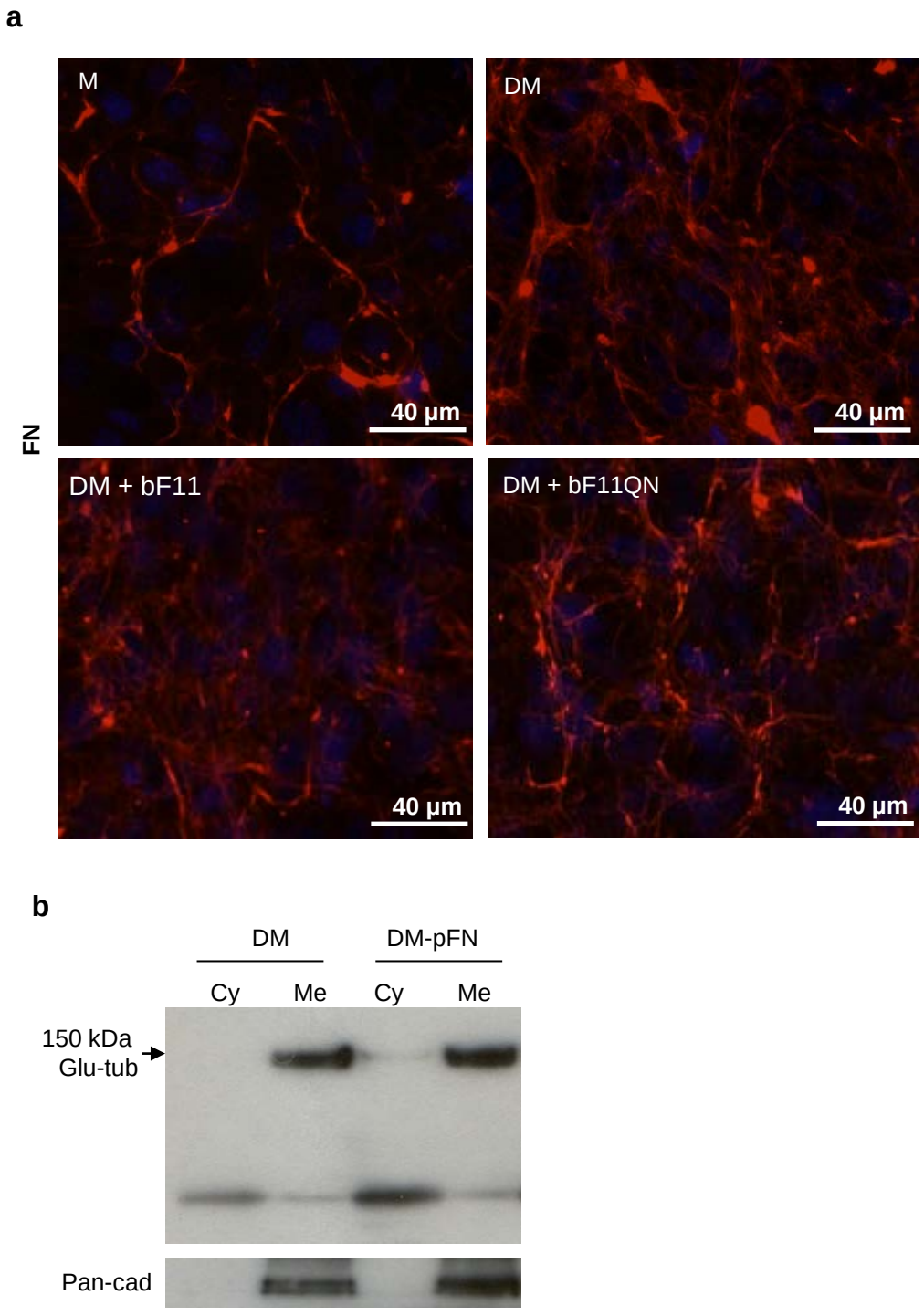
Online Resource 1: Immunofluorescence microscopy of α-tubulin in differentiating osteoblasts prepared for analysis by different methods. (a) Osteoblasts fixed with 3.7% formaldehyde (no permeabilization) and stained for α-tubulin (green, Alexa Fluor® 488) and DAPI (blue). Visualization shows α-tubulin in 5-7 µm patches similar to what was visualized by TIRF microscopy. **(b)** Osteoblasts fixed with 3.7% formaldehyde and permeabilized with Triton-X100 and stained for α-tubulin and DAPI. Only the intracellular tubulin network is visible; no α-tubulin patches are observed. **(c)** Osteoblasts fixed with methanol at -20 C and stained for α-tubulin and DAPI. The intracellular α-tubulin network is visible, and no patches are detected.

Online Resource 2



Online Resource 2: Cell viability assay (MTT assay) of osteoblasts treated with different concentrations (25-100 μM) of bF11 and bF11QN control peptide (100 μM). Peptides were not toxic to cells as cell viability remained between that of medium only treated (M) and differentiating cells (DM).

Online Resource 3



Online Resource 3: The absence of a pFN matrix network in osteoblast cultures does not affect 150 kDa Glu-tubulin formation in osteoblast cultures. Differentiating osteoblasts (DM) were cultured in plasma fibronectin (pFN)-depleted serum (DM-pFN) and control medium (DM). **(a)** pFN matrix assembly was analyzed by immunofluorescence microscopy (red, Alexa Fluor® 568) showing a slight decrease in pFN assembly into matrix after bF11 treatment. bF11QN had no effect on the formation of the pFN matrix network. **(b)** Western blot analysis of Glu-tubulin in cytoskeletal (Cy) and membrane (Me) fractions of osteoblasts cultured in plasma fibronectin (pFN)-depleted serum (DM-pFN) and control medium (DM). No change is observed upon removal of pFN /pFN matrix.

Chapter 4–Cellular Factor XIIIA transglutaminase localizes in caveolae and regulates caveolin-1 phosphorylation, homo-oligomerization and c-Src signaling in osteoblasts

We have shown previously that TG inhibitor, NC9, colocalized with FXIII-A on the plasma membrane of osteoblast in a patch-type staining pattern and the inhibition of TG activity affect osteoblast differentiation. Caveolae are flask- or omega shaped plasma membrane invaginations, as well as in grape-like clusters or rosettes, which have been implicated in vesicular transportation and signal transduction. It is possible that those FXIII-A patches could associate with caveloae membrane. Moreover, caveolin-1, which is necessary for both the structure and function of caveolae, has been proposed to be a negative regulator for bone formation through skeletal phenotyping of Cav-1 null mice. FXIII-A and caveolin-1 both have important role in bone formation.

In this chapter, we investigated the nature of the FXIII-A patchy staining on the plasma membrane of osteoblasts. We hypothesized that FXIII-A is localized in specialized membrane compartments - caveolae. And FXIII-A localized in caveolae regulates caveolae phosphorylation, homo- oligomerization and related signaling in differentiating osteoblasts.

The study presented in this chapter was submitted to Journal of Histochemistry & Cytochemistry.

Cellular Factor XIIIA transglutaminase localizes in caveolae and regulates caveolin-1 phosphorylation, homo-oligomerization and c-Src signaling in osteoblasts

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Abbreviated Short Title: Factor XIII-A is found in caveolae

Abstract

Transglutaminases (TGs) are a family of widely distributed enzymes that catalyze protein crosslinking by forming a covalent isopeptide bond between the substrate proteins. We have shown that MC3T3-E1 osteoblasts express Factor XIII-A (FXIII-A) and the extracellular crosslinking activity of FXIII-A is involved in regulating matrix secretion and deposition. In this study, we have investigated the localization and potential role of the intracellular FXIII-A. Conventional immunofluorescence microscopy and TIRF microscopy analysis showed that FXIII-A co-localizes with caveolin-1in specialized membrane structures, caveolae,in differentiating osteoblasts. Caveolae-disrupting agent methyl-β-cyclodextrin abolished FXIII-A staining and co-localization with caveolin-1 from the osteoblast plasma membrane. The presence of FXIIIA in caveolae was confirmed by preparing caveolae-enriched cellular fractions using sucrose density gradient ultracentrifugation followed by Western blotting. Despite this association of FXIII-A with caveolae, there was no detectable transglutaminase activity in caveolae as measured by monodansylcadaverine incorporation.TG inhibitor NC9 - which can alter TG enzyme conformation - localized to caveolae and displaced FXIII-A from these structures when added to the osteoblast cultures. The decreased FXIII-A levels in caveolae after NC9 treatment increased c-Src activation which resulted in caveolin-1 phosphorylation, homo-oligomerization and Akt phosphorylation, suggesting cellular FXIII-A has a role on regulating c-Src signaling in osteoblasts.

Key words: Factor XIII-A transglutaminase, Caveolae, Caveolin-1, c-Src kinase, Osteoblast differentiation

Abbreviations: FXIII-A, Factor XIII-A; TG, Transglutaminase; Cav-1, Caveolin-1; MDC, Monodansylcadavarine; TIRF, Total Internal Reflection Fluorescence

Introduction

Transglutaminases (TGs) are a family of widely distributed, Ca^{2+}-dependent enzymes with diverse functions ranging from intracellular signaling to extracellular matrix stabilization. The TG family includes nine enzymes - TG1-7, Factor XIII-A (FXIII-A) and band 4.2. TG enzymes can catalyze several, two-step reactions that result in posttranslational modifications of glutamine residues - however, several TGs have been reported to also have noncatalytic functions (Lorand and Graham 2003; Beninati et al. 2009; Iismaa et al. 2009, Muszbek et al. 2011, Eckert et al. 2014). The best known and most studied catalytic function of TG enzymes is the protein transamidation reaction that occurs between a protein-bound glutamine residue and a protein-bound lysine or primary amines resulting in the formation of a γ-glutamyl-ε-lysyl crosslink, also referred to as an isopeptide bond. This crosslinking in certain extracellular matrix substrate proteins has been reported to stabilize both collagenous and noncollagenous extracellular matrices (Hohenadl et al. 1995; Akagi et al. 2002; Kaartinen et al. 2002; Esposito C and Caputo I 2005; Al-Jallad et al. 2006; Cui et al. 2014). The crosslinking is also essential for cornified cell envelope formation in terminal keratinocyte differentiation where covalent crosslinking occurs in proteins such as involucrin, loricrin, and small proline-rich protein (Eckert et al. 2005; Hitomi 2005). Crosslinking by TGs can also be involved in integrin-growth factor coupling where FXIII-A-catalyzed extracellular crosslinking of β3-integrin to VEGFR-2 results in VEGFR-2 activation and promotion of angiogenesis (Dardik et al. 2005). In addition to protein crosslinking, several noncatalytic function for TGs have been reported, and

these include the function of TG2 as a fibronectin-binding co-receptor for β1-integrin in human fibroblasts and erythroleukemia cells (Akimov et al. 2000), and the cytoskeletal scaffolding function of band 4.2 which maintains membrane integrity in erythrocytes (Das et al. 1994; Satchwell et al. 2009).

FXIII-A is best known as a circulating, plasma TG enzyme responsible for fibrin crosslinking that occurs as the last step of the blood coagulation cascade (Ariëns et al. 2000; Muszbek et al. 2011), but circulating FXIII-A also contributes to wound healing and tissue repair (Verderio et al. 2004; Iismaa et al. 2009; Muszbek et al. 2011). FXIII-A is part of a heterotetrameric FXIII complex which consists of two FXIII-A units and two inhibitory FXIII-B units. The B units are released from the complex via thrombin cleavage of FXIII-A which induces the TG activity of FXIII-A. The circulating FXIII-A is produced by cells of bone marrow origin (Wölpl et al. 1987; Poon et al. 1989). FXIII-A (which is not bound to FXIII-B) is also found in many tissues and is expressed by monocytes, macrophages, megakaryocytes, chondrocytes, osteoblasts and adipocytes – in these cell types FXIII-A is found in various cellular and extracellular locations including in the cytosol, at the plasma membrane/cell surface, and secreted into the extracellular matrix (Muszbek et al. 1985; Nurminskaya and Kaartinen 2006; Nakano et al. 2007; Johnson et al. 2008; Piercy-Kotb, 2011; Malara et al. 2011; Myneni et al. 2014). A number of reports show that FXIII-A and its crosslinking activity outside the cell (being either cell surface-bound or soluble), can regulate fibroblast adhesion, induce chondrocyte hypertrophy, regulate extracellular matrix secretion and stabilization, and promote angiogenesis (Ueki et al. 1996; Dardik

et al. 2005; Al-Jallad et al. 2006; Nurminskaya and Kaartinen 2006; Johnson et al. 2008; Al-Jallad et al. 2006; Cui et al. 2014). FXIII-A is also found inside cells, but whether it has a function the intracellular compartment, is not known.

Our recent work has focused on understanding the role of FXIII-A in osteoblasts during osteogenesis. For these cells, we have shown previously that FXIII-A is produced by osteoblasts and is externalized to the extracellular matrix where its function has been linked to stabilization of plasma fibronectin matrix (Al-Jallad et al. 2006, 2011; Piercy-Kotb et al. 2011; Cui et al., 2014). Cell-associated FXIII-A resides on the extracellular side of the osteoblast plasma membrane where it appears to facilitate the formation of high-molecular weight detyrosinated tubulin which is linked to the secretion and elaboration of collagenous extracellular matrix in these cells (Wang et al., 2014). Both these extracellular functions involve transamidation activity. We also previously reported by immunofluorescence microscopy that FXIII-A in osteoblasts appears as small patches at the plasma membrane that did not always co-localize with Glu-tubulin (Al-Jallad et al. 2011; Wang et al. 2014). In the present study, we hypothesized that those patches represent FXIII-A which is associated with specialized lipid structures - caveolae. Caveolae are specialized, omega-shaped lipid raft plasma membrane invaginations that are involved in vesicular transport processes such as transcytosis, endocytosis and potocytosis, as well as in signal transduction (Razani et al. 2002; Cohen et al. 2004; Baker and Tuan 2013). The aim of this study was to examine if intracellular FXIII-A is associated with caveolae and if it has an intracellular function in osteoblasts. We report here that intracellular FXIII-A

does indeed localize to caveolae in differentiating osteoblasts, where it regulates caveolin-1 phosphorylation and its homo-oligomerization, and c-Src kinase activation in a manner that does not appear to involve its transamidation activity, thus suggesting that FXIII-A also has a noncatalytic function as has been similarly reported for other TG enzymes.

Materials & Methods

Antibodies and reagents

Methyl-β-cyclodextrin, monodansylcadaverine and 2-(N-morpholino)ethanesulfonic acid (MES) were from Sigma(St. Louis, MO, USA). Src family kinase inhibitor PP2, c-Src and p-c-Src (Tyr424) (antibody 9A6) antibodies were purchased from Santa Cruz (Dallas,TX, USA). Anti-dansyl antibody was purchased from Molecular Probes (Eugene,OR, USA). Polyclonal antibody against mouse FXIII-A was designed and generated by GenScript (Piscataway, NJ, USA) (Al-Jallad et al. 2011). Mouse anti-p-caveolin-1(Tyr14) was from BD Science(San Jose, CA, USA). Rabbit anti-caveolin-1 antibody was from Abcam (Cambridge, MA, USA). Anti-p-Akt (Ser473), anti-pan-Akt, anti-β1-integrin, horseradish peroxidase linked anti-rabbit IgG and anti-mouse IgG were from Cell Signaling (Whitby,ON, Canada) and GE Healthcare Life Sciences (Baied'Urfe, QC, Canada), respectively. TG2 mouse monoclonal antibody (CUB7402/TG100), the AlexaFluor® secondary antibodies, DAPI, EZ-link Sulfo-NHS-SS-biotin, High-Capacity NeutrAvidin Agarose and protein BCA assay kit were from Thermo Scientific (Waltham, MA, USA).All other reagents, if not otherwise

specified, were purchased from Sigma or Fisher Scientific.

Cell culture and treatments

Mouse calvarial preosteoblasts (MC3T3-E1) subclone 14 were originally a generous gift from Dr. Renny T. Franceschi, University of Michigan, School of Dentistry, Ann Arbor, MI, USA (Wang et al. 1999). Cells were cultured in a humidified 37°C incubator at a 5% CO2 level in modified alpha minimum essential medium (α-MEM) minus L-glutamine and L-aspartic acid (Gibco, Burlington, ON, Canada), supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin-streptomycin antibiotics, L-glutamine and L-aspartic acid. All treatments were initiated 24 hours after adhesion (Day 0), and cells were plated at a density of50,000 cells per cm2 unless otherwise specified. Differentiation into mature osteoblasts was induced by supplementing the medium with 50 µg/ml ascorbic acid (AA) and 10 mM β-glycerophosphate as described previously (Al-Jallad et al., 2006). This medium with additives is referred to as differentiation medium (DM). Control cells were treated with medium only (without AA and BGP, referred to as M). Media was changed every second day. TG activity was inhibited by using the irreversible inhibitor N-α-carbobenzyloxy-N-ε-acryloyl-L-lysine (2-(2-dansylaminoethoxy)ethoxy)ethanamid(NC9) (Keillor et al., 2008), which was used at a concentration of 25 µM as described previously (Al-Jallad et al. 2011). Src kinase inhibitor PP2 was added from day 0 to day 2. For FXIII-A and caveolin-1 co-fractionation and immunofluorescence microscopy experiments, day 5 cells were used. For TG activity labeling experiments, monodansylcadaverine (MDC) (100 µM)

was added to cell culture media at day 4 for 24 hours only, and this was followed by immunofluorescence microscopy staining or by protein extraction at day 5. To study the effect of MDC on caveolin-1 homo-oligomerization, cells were treated with MDC from day 0 to day 5 with two concentrations (50 μM and 100μM) of MDC. All experiments were repeated at least three times.

Preparation of protein extracts

Total cellular protein preparations were obtained using buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.2), 5 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 1% Na-deoxycholate. Cell extracts were homogenized by ultrasonication directly after extraction and were centrifuged at 14000 × g for 15 minutes at 4°C. The supernatant was collected and hereafter is referred to as total protein extract. Orthovanadate and sodium fluoride were added into the above buffer for studying protein phosphorylation.

Sucrose density gradient ultracentrifugation

MC3T3-E1 cells were cultured on 150-mm plates and cells were detached from the plates by gentle scraping in the presence of 1% Triton-X 100/MES-buffered saline (MBS). Cell preparations were transferred to a pre-chilled Dounce (glass-glass) homogenizer and incubated on ice for 20 minutes. Cells were then homogenized on ice and mixed with 90% sucrose/MBS and transferred into an ultracentrifuge tube. Thirty five (35) % sucrose in MBS/Triton X-100 was applied on top of the

sample/90%sucrose/MBS layer. A visible interface remained evident between the two density layers. Finally, 5% sucrose in MBS/Triton X-100 was layered on top of the 35% sucrose layer. The preparation was centrifuged for 16–20 hours at 39,000 rpm (4°C) in a SW41Ti rotor (Beckman), this providing a maximum force equivalent (bottom of tube) of approximately 260,000 g and an average force (middle of the tube) of approximately 188,000 g. At completion of the centrifugation, a faint light-scattering band, which consisted of the buoyant lipid raft/caveolar material, became visible at the 35% sucrose-5% sucrose interface. Samples were collected from the gradient from the top down as 1 ml fractions. Fractions 4 and 5 are defined as the bona fide caveolae-enriched fractions (Parolini et al., 1999). Fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

SDS-PAGE and Western blotting

Twenty five (25) µg of each sample was boiled in SDS-sample buffer for 5 minutes, which was followed by electrophoretic separation of proteins by SDS-PAGE. For visualization of caveolin-1 oligomers, samples were not boiled before loading. Following electrophoresis, proteins from the gels were transferred to PVDF membranes (Bio-Rad) and blocked for 30 minutes in 5% nonfat dry milk in TBS-Tween (TBS-T), and then incubated at 4°C overnight in primary antibody diluted in TBS-T. Following incubation with primary antibody, membranes were rinsed in TBS-T, and then incubated with secondary antibody conjugated with horseradish

peroxidase at room temperature. Secondary antibodies used were sheep anti-mouse (Invitrogen) and goat anti-rabbit (Cell Signaling). Bands were visualized using the ECL Plus kit (GE Healthcare) and chemiluminescence was detected using Kodak Biomax film.

Immunofluorescence microscopy

Cells were seeded on Nunc® Lab-Tek®chamber slides (Fisher) and cultured as described above. On day 5, cells were fixed with 3.7% formaldehyde in PBS for 10 minutes at room temperature. Slides were washed three times with PBS and blocked with 2% bovine serum albumin (BSA) in PBS. This was followed by primary antibody incubation for 2 h at room temperature, and a washing step with 0.1% BSA in PBS and incubation with AlexaFluor® secondary antibody conjugates. Nuclei were stained by DAPI (4', 6-diamidino-2-phenylindole). Samples were mounted with Prolong Gold Anti-Fade medium (Invitrogen) and dried overnight at room temperature. Cells were imaged using an Axioskop2 upright fluorescence microscope equipped with an AxioCam MRm camera and AxioVision v4.8 imaging software from Zeiss.

Total Internal Reflection Fluorescence Microscopy

TIRF microscopy was done as described by us previously (Wang et al., 2014). Briefly, MC3T3-E1 cells were seeded on FluoroDish^{TM} plates (World Precision Instruments, Inc, Sarasota, FL, USA) that were coated with 1 µg/ml fibronectin (Sigma) and cultured as above. On day 5, cells were fixed with 3.7% formaldehyde in PBS for 10

minutes at room temperature. Immunofluorescence staining of the cells was done by following the same protocol as for conventional immunofluorescence microscopy as above. After the final wash of DAPI staining, samples were covered with PBS and kept in the dark at 4C prior to analysis. Samples were observed and visualized with an incident angle of 68 degrees using a ZeissAxio ObserverZ1 microscope in the multi-color TIRF illumination mode. Antibody controls for TIRF analysis are presented in Supplemental Figure 1.

Results

FXIII-A associates with caveolae in differentiating osteoblasts

To test our hypothesis that intracellular FXIII-A is associated with caveolae, we examined FXIII-A co-localization with caveolin-1 (Cav-1) in nondifferentiating (preosteoblasts) and differentiating osteoblasts. Cav-1 is a marker for caveolae, and a necessary protein for both the structure and function of caveolae (Razani et al. 2002). Conventional immunofluorescence microscopy showed only very weak Cav-1 and FXIII-A staining in medium only treated preosteoblasts (M), while differentiating osteoblasts (treated with differentiating media, DM) showed strong staining as well as clear co-localization of FXIII-A and Cav-1 in small patches (Fig. 1A). Color correlation distribution analysis that reflects extent of co-localization is presented in Figure 1B – data shows that not all stainable FXIII-A is found co-localized with Cav-1. The induction of FXIII-A upon the differentiation treatment (DM media) was consistent with our previous studies (Al-Jallad et al. 2011; Piercy-Kotb et al., 2012; Wang et al. 2014).

To further investigate whether the presence of FXIII-A in the patches is dependent on the integrity of caveolae, we used a caveolae-disrupting agent - methyl-β-cyclodextrin (MβCD) - which depletes cellular cholesterol and thus eliminates caveolae (Razani et al. 2002). As expected, a 30-minute treatment of the cells with MβCD abolished Cav-1 staining and simultaneously eliminated FXIII-A staining in the patches (Fig. 1). To further confirm the association of FXIII-A with Cav-1, we used Total Internal Reflection Fluorescence (TIRF) microscopy, which is a high-resolution, specialized technique to study cell-surface and plasma membrane-associated molecules (Axelrod 2001; Jaiswal and Simon 2007; Mattheyses et al. 2010; Simons and Gerl 2010). As seen in Figure 2, FXIII-A co-localized with Cav-1 in differentiating osteoblasts at day 5 as analyzed by TIRF microscopy. The higher magnification image revealed punctate and patchy caveolae staining and the clear presence of FXIII-A in these specialized, small membranous structures. Although secondary antibodies give slight dotted background in TIRF microscopy (Supplemental Figure 1), the rounded patchy staining is only observed with primary antibody present (Supplemental Figure 1B). Also, to confirm that the fixation and permeabilization method used allows staining for an integral membrane protein; we stained the cells for insulin receptor β-subunit (IR-β) which is located in the inner leaflet of the plasma membrane and known to associate with caveolae. As seen in Supplemental Figure 2, IR-β is stained and is found in similar patches as Cav-1.

To further confirm that FXIII-A is found in caveolae, we prepared caveolae-enriched cell fractions using sucrose density gradient centrifugation of osteoblast

homogenates. Caveolae membranes are highly enriched in glycol-sphingolipids and cholesterol, making them extremely light; therefore, they have a low buoyant density in sucrose (Razani et al. 2002; Cohen et al. 2004). They are also resistant to solubilization by nonionic detergents (Triton X-100, NP-40). Triton X-100 solubilizes most of the cellular proteins, which remain in the 45% sucrose layer. This leaves the caveolae and lipid rafts in the in soluble interface between 35 - 5% sucrose. Further separation of the sucrose gradient into 12 analyzable 1 ml fractions shows that caveolae are found in fractions 4 and 5 as analyzed by Cav-1 detection by Western blotting (Fig. 3). These two fractions 4 and 5 have been identified as the *bone fide* caveolae fractions (Parolini et al., 1999). The rest of the material includes non-lipid raft associated proteins and Cav-1 was also found in this material. As demonstrated in Figure 3A and B, FXIII-A, with a molecular weight between 50-75 kDa (as we have published before for mouse tissues) (Al-Jallad et al., 2011; Myneni et al., 2014) co-purified with caveolae-enriched fractions 4 and 5 in differentiating osteoblasts. FXIII-A cannot be detected in fractions 4 and 5 of preosteoblasts, which is consistent with our immunofluorescence data (Fig. 3A). Disruption of caveolae with MβCD reduced Cav-1 levels in fractions 4 and 5 (Fig. 3C), but did not completely eliminate the FXIII-A detection. It is possible that even the low Cav-1 levels in caveolae maintain FXIII-A association. The fairly weak detection of FXIII-A in Western blots merely reflects the quality of the antibody in this application and does not likely reflect the actual quantity of FXIII-A in these structures. The strong detection of FXIII-A in caveolae by immunocytochemistry with the same antibody strongly supports our

conclusion and its presence in these structures. In summary, intracellular FXIII-A in osteoblasts associates with specialized membrane structures - caveolae - which are increased in differentiating osteoblasts. The co-localization of FXIII-A with Cav-1 (which resides on the inner membrane leaflet) is dependent on the integrity of the caveolae.

NC9 decreases FXIII-A levels in caveolae which increases phosphorylation and homo-oligomerization of Cav-1 in caveolae-enriched fractions and c-Src activity

To continue to understand the function of FXIII-A in caveolae, we hypothesized that NC9 – an irreversible TG inhibitor – may be able to interfere/alter with its function as it has been show to associate noncovalently with enzyme inside the cells (Al-Jallad et al., 2011) and it has been reported to change the conformation of TG enzymes from the closed to open form (Colaket al. 2011; Caron et al. 2012). As seen in Figure 4A, detection of the dansyl group of NC9 by immunofluorescence microscopy showed co-localization with Cav-1, this suggesting that NC9 interacts with caveolae inside the cells. We then examined if NC9 had any effects on the levels of FXIII-A in caveolae fractions 4 and 5 on days 2, 4 and 6 of cell differentiation. Western blotting showed that NC9 decreased the presence of FXIII-A in caveolae (Fig. 4B). NC9 did not affect the total FXIII-A levels in the cells as seen by immunoblotting of total cell extracts (Fig. 4B) nor did it change Cav-1 levels in caveolae (Fig. 4C). This strongly suggests that the association of NC9 with FXIII-A promotes the dissociation of FXIII-A from

caveolae. To examine whether NC9 has effects on Cav-1 levels and/or function in caveolae, we considered that Cav-1 can be phosphorylated at Tyr14 by Src-kinases (Aoki et al. 1999), which promotes Cav-1 self-association to form noncovalent, high-molecular weight homo-oligomers that contain 14 to 16 individual Cav-1 molecules. These Cav-1 oligomers represent the functional assembly units of caveolae (Parolini et al. 1999; Razani et al. 2002; Cohen eta l. 2004). Thus, we examined Cav-1 phosphorylation and homo-oligomerization in the caveolae fractions at the same time points. The analysis showed that the decreased FXIII-A levels in caveolae were associated with an increase in both homo-oligomerization and phosphorylation of Cav-1 (Fig. 4B). The Cav-1 levels in fraction 4 and 5, and in total cell extract, were not affected by NC9 treatment (Fig. 5Band C). TG2, which is also expressed by osteoblasts and co-localizes with FXIII-A (Al-Jallad et al., 2011) was not detected in the caveolae fractions (Fig. 4B), although it was clearly expressed by the cells and found in total cell extracts (Fig. 4B).TG2 also did not co-localize in with Cav-1 (Supplemental Figure 3). Taken together, these data show that noncovalent interaction of NC9 with FXIII-A interferes with its ability to associate with caveolae, and that the decreased levels of FXIII-A in caveolae affect Cav-1 phosphorylation and homo-oligomerization.

It is known that Cav-1 is phosphorylated at Tyr14 by Src kinases (Aoki et al. 1999), which affects caveolae function and regulates osteoblast maturation (del Pozo et al. 2005; Peruzzi et al. 2012). It has also been shown that the internalization of certain integrin-regulated membrane domains requires the same phosphorylation of Cav-1

on Tyr14 (Del Pozo et al. 2005). Cav-1 phosphorylation is generally orchestrated by c-Src kinases that are activated by autophosphorylation at Tyr424 (in mouse) (Roskoski 2005) which in turn is promoted by c-Src association with Cav-1. To examine whether c-Src kinase activity and signaling was increased by NC9 treatment, we analyzed c-Src activation/phosphorylation at Tyr424. The data presented in Figure 5A show a clear increase in p-c-Src in total cell extracts upon NC9 treatment levels, this implying activation. To examine if c-Src was responsible for the observed NC9-induced increased phosphorylation and homo-oligomerization of Cav-1, we applied a Src kinase inhibitor PP2 to the cells and examined its effects on the NC9-mediated increase of p-Cav-1 and Cav-1 oligomers on day 2 of cell differentiation. As seen in Figure 5B, PP2 treatment markedly decreased the NC9-inducedphosphorylation and homo-oligomerization of Cav-1 down to the levels of control cultures, without affecting total Cav-1 levels. Analysis of c-Src and p-c-Src levels in the caveolae-enriched fractions on days 2, 4 and 6 of cell differentiation showed that only nonphosphorylated c-Src was associated with caveolae and that NC9 increased this association (Fig. 5C). This supports the concept that FXIII-A acts as a blocking molecule between the Cav-1 and c-Src interaction. The observation that no p-c-Src was seen in caveolae fractions also suggests that once autophosphorylated, p-c-Src dissociates from caveolae and may continue its cytosolic signaling function (Fig. 5C). Indeed, the increased cytosolic p-c-Src induced by NC9 was associated with increased phosphorylation of a c-Src downstream target – p-Akt (Ser473). The p-Akt levels were decreased with c-Src inhibitor (PP2)

treatment (Fig. 5D). These data strongly suggest that NC9-mediated displacement of FXIII-A from caveolae stimulates c-Src activation and induction of related signaling pathway. c-Src was found as two bands on day 6. This may be linked to its fragmentation and perhaps inactivation when preosteoblasts mature into osteoblasts around that time point (Park et al. 2004; Miura et al. 2004).

Caveolae show no transamidase activity

Finally, we examined whether FXIII-A has transamidase activity in caveolae. For this we used monodansylcadaverine (MDC) - a TG activity probe that is capable of penetrating the cell membrane and incorporating into substrate proteins in the presence of an active enzyme. As seen in Figure 6A, the dansyl group of MDC does not co-localize with Cav-1 as assessed by immunofluorescence microscopy. MDC also did not get incorporated covalently into any substrates in caveolae-enriched fractions 4 and 5 as seen by the lack of dansyl-detection in Western blots of these fractions while total cell extracts showed strong labeling (Fig. 6B). The isolation of caveolae does not involve harsher extraction conditions that would render all labeling undetectable. Furthermore, cells treated with MDC throughout the differentiation show no changes of Cav-1 homo-oligomerization compared with NC9 treated cells (Fig. 6C), suggesting that transamidation activity is not required to regulate Cav-1 homo-oligomerization. This suggests that FXIII-A in caveolae is likely inactive as a transglutaminase.

Discussion

Osteoblasts express FXIII-A transglutaminase which is found both in the cells and secreted into the extracellular matrix. In our previous work, we showed that the secreted, extracellular soluble FXIII-A promotes plasma fibronectin stabilization, crosslinking and fibrillogenesis into matrix, whereas the cell-associated FXIII-A is located on the outer plasma membrane of osteoblasts where its crosslinking action is linked to regulation of microtubule stabilization that in turn associates with secretion of type I collagen (Al-Jallad et al. 2006, 2011; Piercy-Kotb et al. 2011, Wang et al., 2014). Both these functions involve its crosslinking activity. In this study, we show that FXIII-A also co-localizes inside the cells with a major caveolae protein, Cav-1, which is known to associate with the inner leaflet of plasma membrane caveolae. Caveolae are specialized membrane structures that regulate endocytosis and signal transduction via clustering receptors and many cell-signaling molecules into a confined space. FXIII-A is associated with caveolae specifically in differentiated MC3T3-E1 osteoblasts, and its presence there is dependent on the integrity of caveolae as demonstrated by their co-reduction from the plasma membrane after treatment with the caveolae-disrupting agent MβCD. Caveolae-associated FXIII-A does not appear to have transamidating activity;however, the TG inhibitor NC9 interacts with the enzyme causing what appears to be displacement of FXIII-A from the caveolae. This displacement may be mediated by NC9-induced conformational change and the opening of FXIII-A structure, or by NC9 attacking the exposed FXIII-A active site that may also be used for the interaction with Cav-1. We further showed

that the decreased FXIII-A levels in NC9-treated caveolae was associated with increased phosphorylation and subsequent homo-oligomerization of Cav-1 which was mediated by increased activity of c-Src. It has been suggested Cav-1 binds and maintains Src family kinases in an inactive configuration, and that Cav-1 homo-oligomer formation modifies the interaction of Cav-1 with Src kinases in a manner that may lead to the release of these kinases from caveolae (Li et al. 1996; Wei et al. 1999). Furthermore, it has also been shown that Src kinase-mediated phosphorylation of Cav-1 increases Cav-1 homo-oligomerization suggesting that Cav-1 and Src kinases amplify each other's activation (Li et al. 1996; Wei et al. 1999; Del Pozo et al. 2005). Our results above imply that the presence of cellular FXIII-A in caveolae attenuates both Cav-1 homo-oligomerization and c-Src kinase activity which indicates that FXIII-A may act as a suppressor (and a switch) of their functions. The precise mechanism of how FXIII-A regulates this remains unknown; however, it may involve a direct interaction between FXIII-A and Cav-1 or interaction via a mediator protein. We hypothesize that this FXIII-A: i) inhibits cytosolic c-Src from associating with Cav-1, ii) inhibits Cav-1-mediated activation (autophosphorylation) of c-Src, and iii) inhibits p-c-Src-mediated Cav-1 phosphorylation. This may be viewed as FXIII-A in caveolae acting as a blocking molecule that inhibits c-Src and Cav-1 interaction which maintains c-Src in an inactive state. The action of FXIII-A in this process does not seem to require its TG activity.

Our previous studies have focused on the role of extracellular FXIII-A (both secreted and plasma membrane-associated) in matrix deposition and stabilization in

osteoblast cultures. The present study extends the possible roles for FXIII-A in bone biology, describing for the first time in osteoblasts a potential function for intracellular FXIII-A. The observation that FXIII-A regulates c-Src activity is well-aligned with the results that we have obtained previously using NC9 as an inhibitor of osteoblast culture maturation which results in an arrest of matrix deposition and osteoblast maturation. c-Src belongs to the SRC family of nonreceptor tyrosine kinases, which includes at least ten members (Lyn, Fyn, Lck, Hck, Fgr, Blk, Yrk, Gfr, Yes and c-Src) all sharing high homology in their domain structure (Brown and Cooper 1996). It has been reported that c-Src is involved in the regulation of osteoblast differentiation, where decreased c-Src expression enhances osteoblast differentiation and bone formation, and c-Src kinase activity is essential for osteoclast function and bone resorption (Marzia et al. 2000; Miyazaki et al. 2004) which makes c-Src a major negative regulator of bone mass. c-Src deficient osteoblasts exhibit a cell-autonomous alteration that leads to increased osteoblast numbers and increased bone formation in vivo, as well as accelerated osteoblast differentiation and associated increased matrix production (Marzia et al. 2000). Here, consistent with these previous findings, we have shown that Akt phosphorylation is increased as part of c-Src activation. p-Akt plays a critical role in controlling the survival and apoptosis of cells, as well as in insulin signaling, which integrates bone remodeling and energy metabolism in osteoblasts (Hermann C et al. 2000; Ferron et al. 2010; Tonks KT et al. 2013). Interestingly, cell-surface TG2 has also been reported to inhibit c-Src activity via its role in promoting integrin clustering on the cell surface (Janiak et al. 2006;

Condello et al., 2013). We have shown previously that TG2 and FXIII-A co-localize on the osteoblast surface (Al-Jallad et al., 2011) and a number of studies have shown tight functional links and synergy between the roles of TG2 and FXIII-A in bone and cartilage, as well as compensation in the case where one is absent (Nurminskaya and Kaartinen 2006; Johnson et al. 2008; Tarantino et al. 2009; Deasey et al. 2012). Thus, it is tempting to speculate that the potential synergistic role of the two enzymes in osteoblasts could be linked to the regulation of c-Src activity and subsequent signaling in a manner where TG2 would modulate its activity on the outer leaflet of the cell membrane, and FXIII-A would modulate its activity on the inner leaflet of the cell membrane.

Caveolae have been linked to cellular events that can both promote or inhibit osteoblast differentiation (Li et al. 1996; Yamamoto et al., 1998; Marzia et al. 2000; Fulzele et al. 2010). The role of caveolae and Cav-1 as a negative regulation of bone formation is based on solid animal studies (Rubin et al. 2007). Skeletal phenotype of Cav-1-knockout mice showed increased trabecular number and thickness. The authors speculated that Cav-1 deficiency leads to increased osteoblast differentiation suggesting that Cav-1 helps to maintain osteoblast progenitors in a less-differentiated state. Links to positive regulation of osteoblast function are based on the observations that caveolae sequester BMP-2 and vitamin D receptors (Huhtakangas et al. 2004; Chen et al. 2012; Bonor et al. 2012; Baker and Tuan 2013) on the osteoblast plasma membrane and regulate insulin signaling at least in mesenchymal stem cells (Baker and Tuan 2013). Due to the potential dual role of caveolae in both

promotion and inhibition of osteoblast differentiation, it is plausible that additional factors, such as FXIII-A, are required to modulate these specific functions. In future studies, we will continue to address and refine the role of FXIII-A in regulation of other caveolae-related functions.

In summary, our study suggests that intracellular FXIII-A is associated with caveolae in osteoblasts where its presence inhibits c-Src activation and c-Src-mediated Cav-1 phosphorylation, homo-oligomerization and function. This function for FXIII-A is novel, and appears not to involve its enzymatic transamidation activity adding to the known noncatalytic function of TG enzymes in other cell types. The data from this paper, together with our previous work, adds a new modality on how intracellular FXIII-A maybe contribute to the regulation of bone formation.

Acknowledgements

We would like to thank Dr. Erika Wee ofthe Advanced BioImaging Facility (ABIF) of McGill University's Life Science Complex for excellent assistance in using TIRF microscopy, and Dr. Simon Tran (McGill University) for the use of his immunofluorescence microscope. This study was supported by a grant to MTK from the Canadian Institutes of Health Research (CIHR). SW received studentship stipends from the China Scholarship Council (CSC) and Faculty of Dentistry of McGill University.

Competing Interest Statement

The authors declare that they have no competing interests.

Ethical standards

The manuscript does not contain clinical studies or patient data.

Author contribution statement

SW designed the study and performed immunofluorescence microscopy, TIRF microscopy, Western blotting, carried out all data analysis and wrote the manuscript. MTK designed the study and edited the manuscript. Both authors have read and approved the final manuscript.

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Figure 1

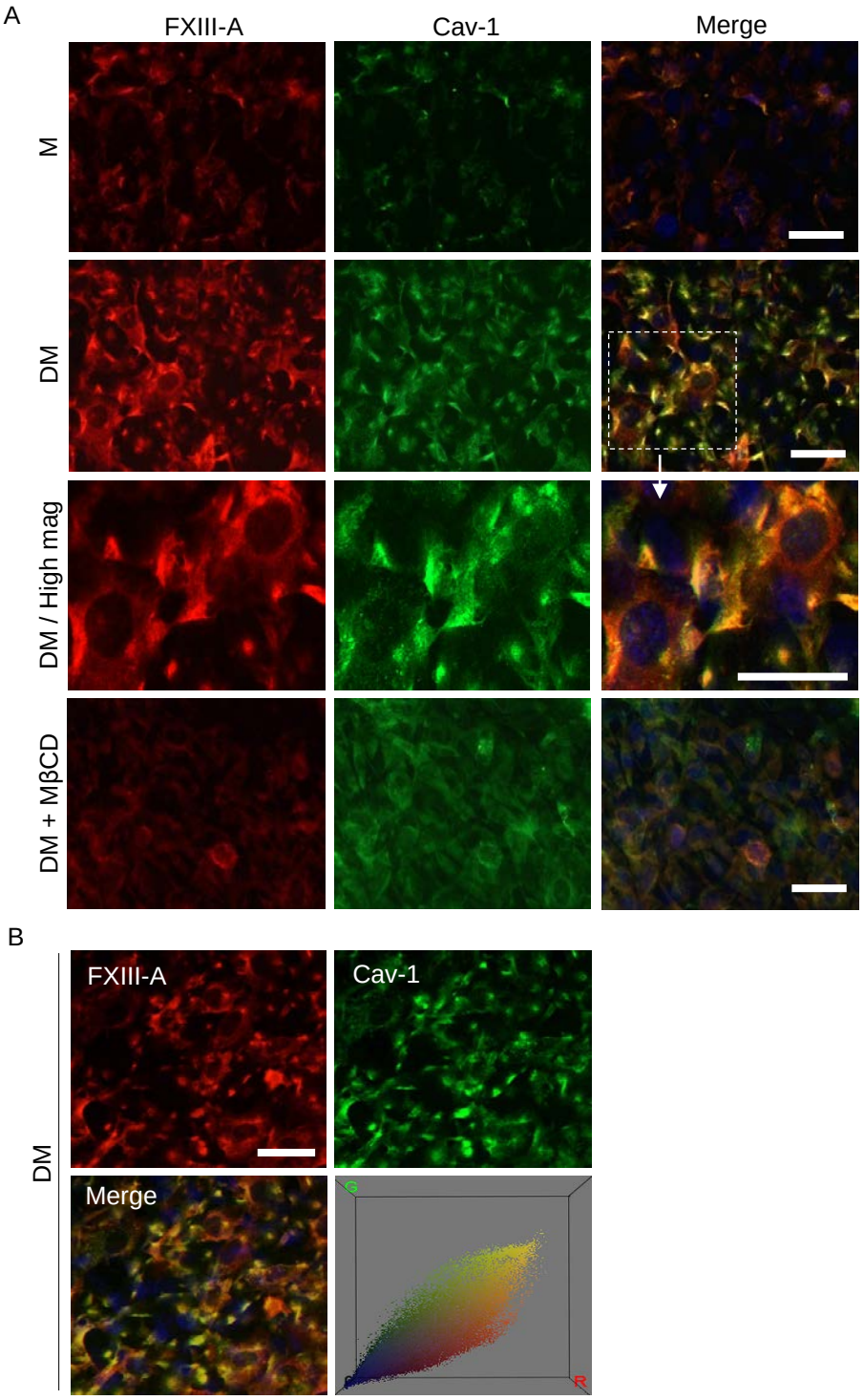


Fig. 1. Cellular FXIII-A co-localizes with caveolin-1in differentiating osteoblasts. (A) Immunofluorescence microscopy of FXIII-A (red) and caveolin-1(Cav-1) (green) in osteoblasts on day 5. Medium-only treated, nondifferentiating preosteoblasts (M) showed very weak FXIII-A and Cav-1 staining, while differentiating osteoblasts (DM) showed strong staining and co-localization (yellow) of the two in a patchy pattern (DM / high mag). Incubation of the cells with a caveolae-disrupting agent methyl-β-cyclodextrin (MβCD) for 30 min at 37°C abolished both FXIII-A and Cav-1 staining. Bars equal 20μm. (B) Color correlation distribution constructed from co-localization images using the Color Inspector 3D plug-in of Image J.

Figure 2

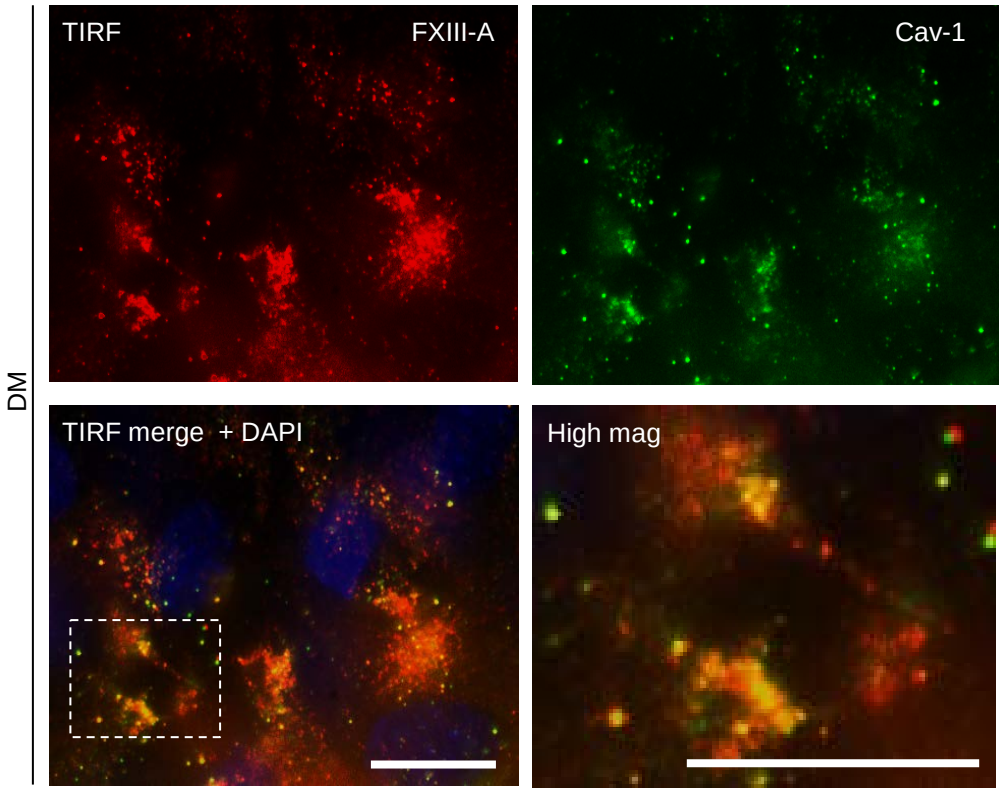


Fig. 2. TIRF microscopy of FXIII-A and caveolin-1 co-localization. TIRF (Total Internal Reflection Fluorescence) microscopy analysis of FXIII-A (red) and caveolin-1 (Cav-1) (green) in differentiating osteoblasts on day 5. The analysis confirms the co-localization and the higher magnification image (lower panel on right) reveals punctate caveolae staining and shows the clear presence of FXIII-A in these specialized membrane structures. Bars equal 20μm.

Figure 3

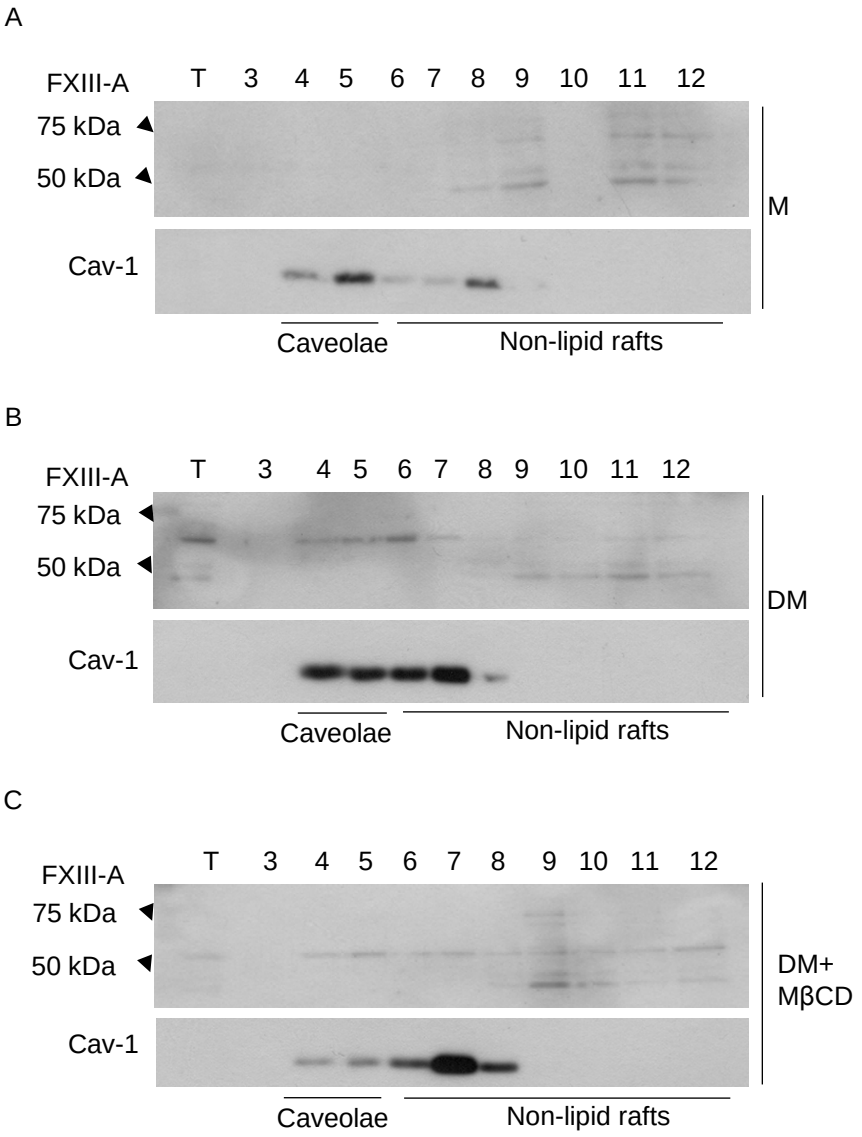


Fig. 3. Western blotting of FXIII-A in the caveolae-enriched membrane fractions of osteoblasts. Caveolae-enriched fractions were prepared using sucrose density gradient centrifugation of osteoblast homogenates which were fractionated into 1 ml fractions of which 3-12 were analyzed by Western blotting. Fractions 4 and 5 are *bone fide* caveolae-containing fractions, and fractions 6-12 contain non-lipid raft membranes. Upper blots were detected with anti-mouse FXIII-A antibody. Arrowheads point to molecular weight markers. (A) Nondifferentiating (medium only treated, M) preosteoblasts show no FXIII-A detection in fractions 4 and 5. (B) FXIII-A is detected in caveolin-enriched fractions 4and 5 in differentiating osteoblasts (differentiating media, DM). (C) Treatment of differentiating osteoblast cultures with the caveolae-disrupting agent methyl-β-cyclodextrin (MβCD) decreased Cav-1 levels in fractions 4 and 5, and also resulted in a reduction in FXIII-A; however, the treatment did not abolish either completely.

Figure 4

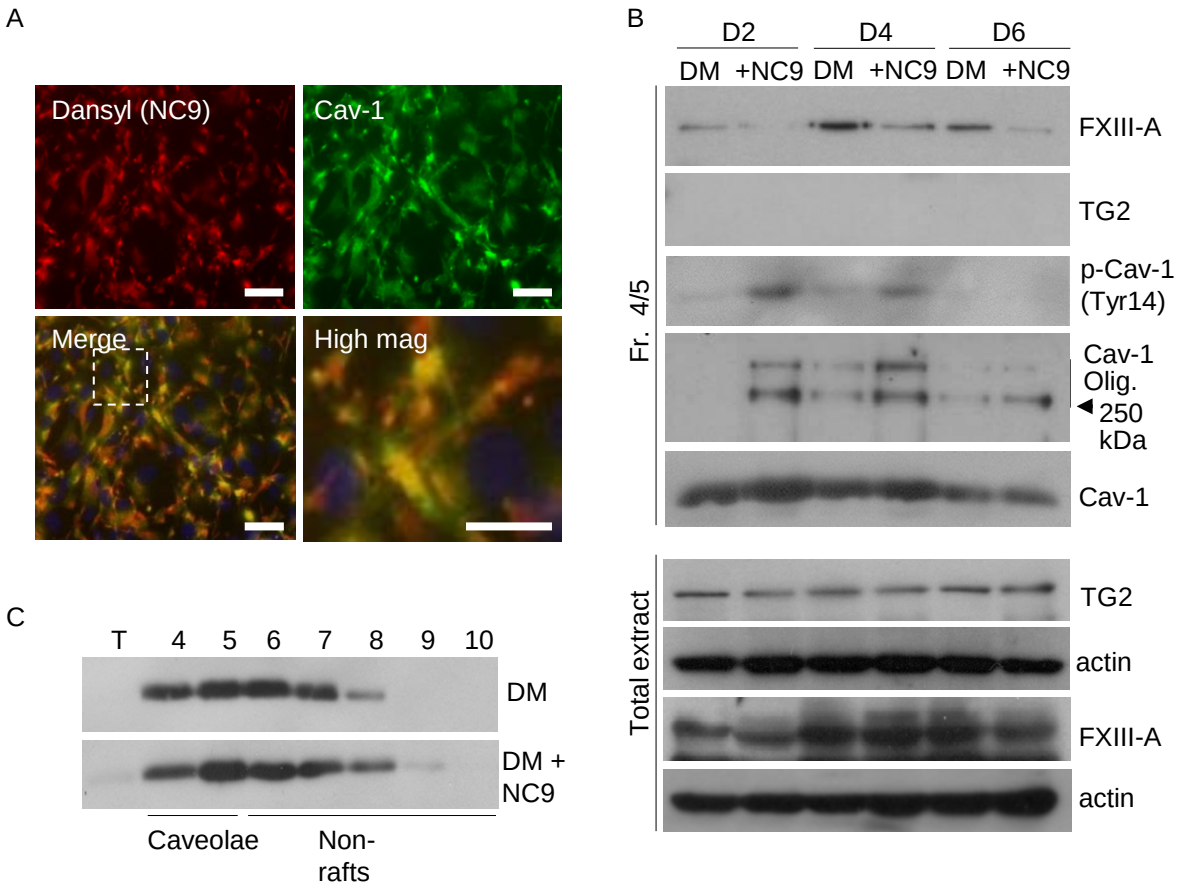


Fig. 4. NC9 displaces FXIII-A from caveolae and affects the homo-oligomerization and phosphorylation status of Cav-1 in caveolae-enriched fractions. (A) Immunofluorescence microscopy of NC9 in differentiating osteoblasts. NC9 (dansyl, red), when administered to the osteoblast cultures associates with caveolae (Cav-1, green) as seen by their co-localization (yellow). Bar equals 20µm. (B) Western blotting of FXIII-A levels and Cav-1 status in caveolin-enriched fractions (4/5) and total cell extracts of osteoblasts.NC9-treated cells have decreased FXIII-A levels while total extracts show no change in FXIII-A protein levels. The decreased FXIII-A levels in caveolae is associated with increased Cav-1 phosphorylation (p-Cav-1(Tyr14)) and increased homo-oligomerization. Total Cav-1 levels remain similar in NC9-treated and control cultures.TG2 was not detected in the caveolae fractions, but was abundant on all total cellular fractions. (C) Western blot analysis of Cav-1 from all fractions from the membrane component isolation confirmed that with, and without NC9 treatment, total levels and the distribution of Cav-1 remains the same.

Figure 5

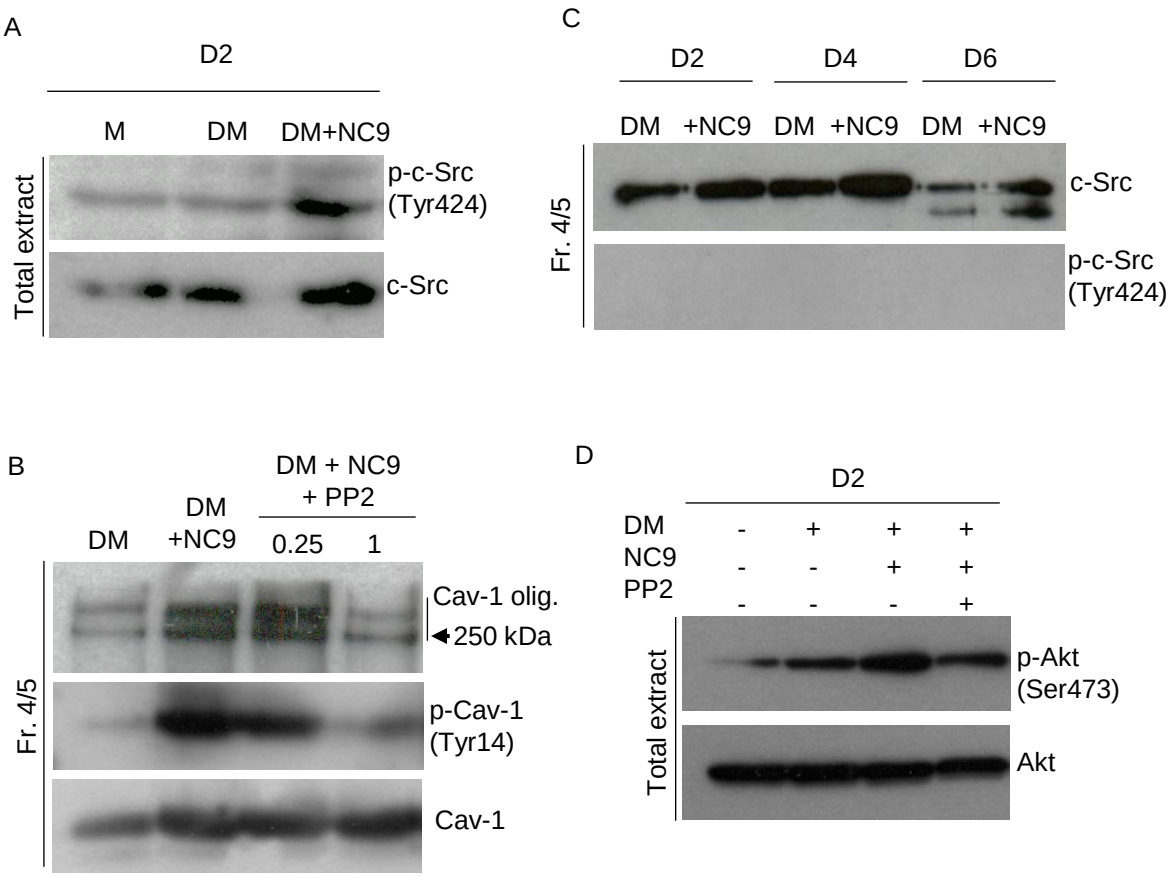


Fig. 5. NC9 promotes Src-kinase phosphorylation and activation. (A) Analysis of phosphorylation levels of c-Src (p-c-Src(Tyr424)) in total extracts of differentiating (DM) osteoblasts show increased phosphorylation in NC9-treated cells compared to controls. (B) Inhibition of Src kinase activity in NC9-treated cultures with PP2 markedly decreased both phosphorylation and homo-oligomerization of Cav-1 in caveolae-enriched fractions (Fr. 4/5) to the levels of control cultures, without affecting total Cav-1 levels, thus showing the involvement of c-Src in these modifications of Cav-1. (C) c-Src is associated with caveolae-enriched fractions (Fr. 4/5) and its presence there is increased in NC9-treated, differentiated osteoblasts. p-c-Src (Tyr424) does not remain associated with caveolae and is mostly found in the cytosol as seen in the above analysis of total osteoblast extracts. (D) Analysis of phosphorylation of a c-Src downstream signaling target, Akt in differentiating osteoblasts (DM) treated with NC9 show an increase in p-Akt compared to controls. Src kinase inhibitor PP2 reduces the NC9-induced increase in Akt phosphorylation.

Figure 6

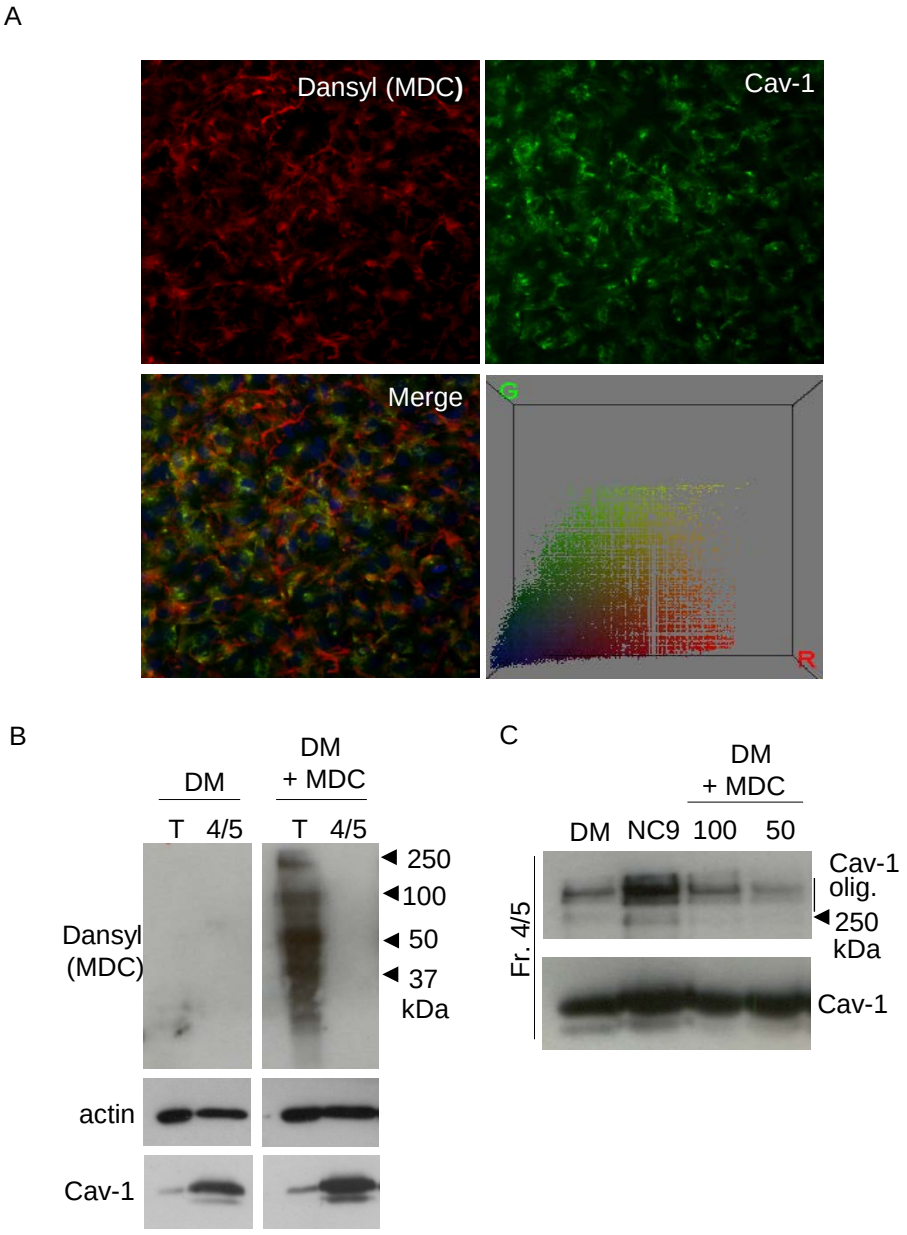
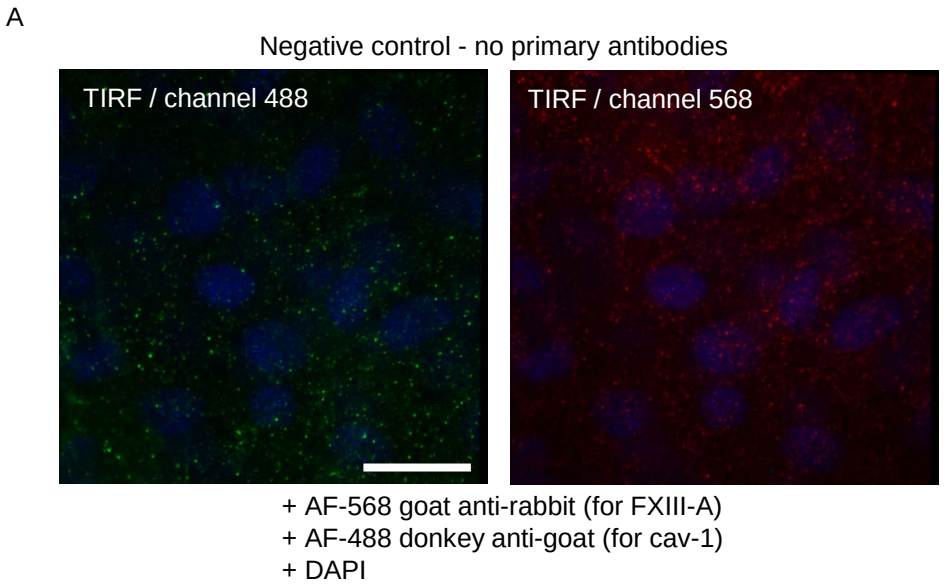
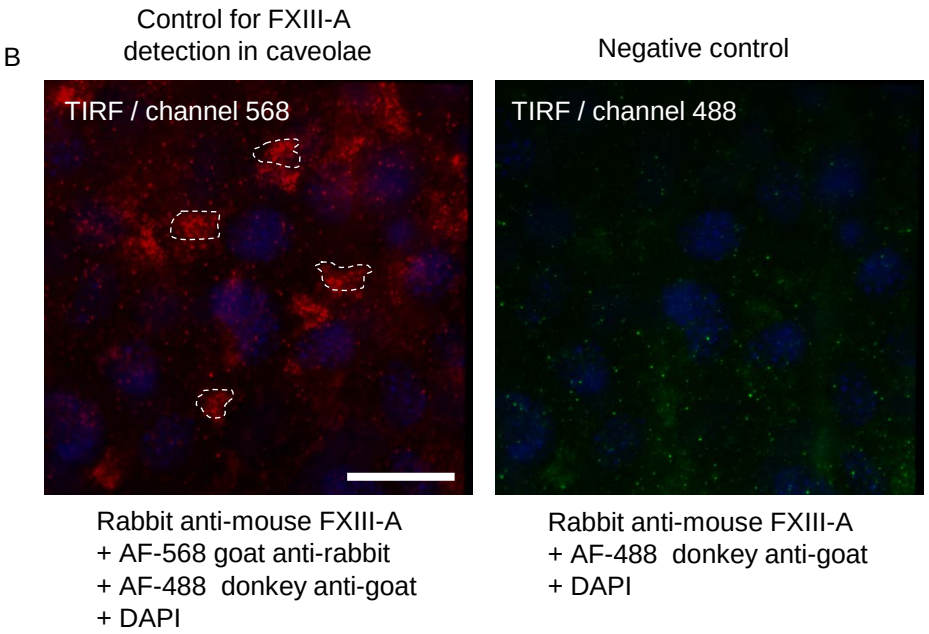


Fig. 6. No transamidase activity is detected in caveolae. (A) *In situ* TG activity was analyzed in differentiating osteoblast cultures by growing the cells in the presence of monodansyl-cadaverine (MDC) as described in Materials & Methods. Covalent incorporation of MDC into TG substrates to reveal enzyme activity was visualized by detecting the dansyl-group of MDC by immunofluorescence microscopy. No co-localization of the MDC/dansyl-group (red) with caveolin-1 (Cav-1) (green) was observed although MDC has incorporated into cell layers, mostly to extracellular matrix fibrils. Color correlation distribution was constructed using the Color Inspector 3D plug-in of Image J and shows no correlation. Bar equals 20µm. (B) Western blotting of MDC in total osteoblast extracts (T) versus caveolae fractions (4/5) of differentiating osteoblasts (DM) showing no detection of covalently linked MDC in caveolae although it was clearly detected in the total extracts. Actin and Cav-1 were used as loading controls for total cell extracts and caveolae enriched fractions, respectively. (C) Cells treated with MDC from day 0 to day 5 with two concentrations (50 µM and 100µM) show no changes of Cav-1 homo-oligomerization compared with NC9 treatment that increased the formation of homo-oligomers.

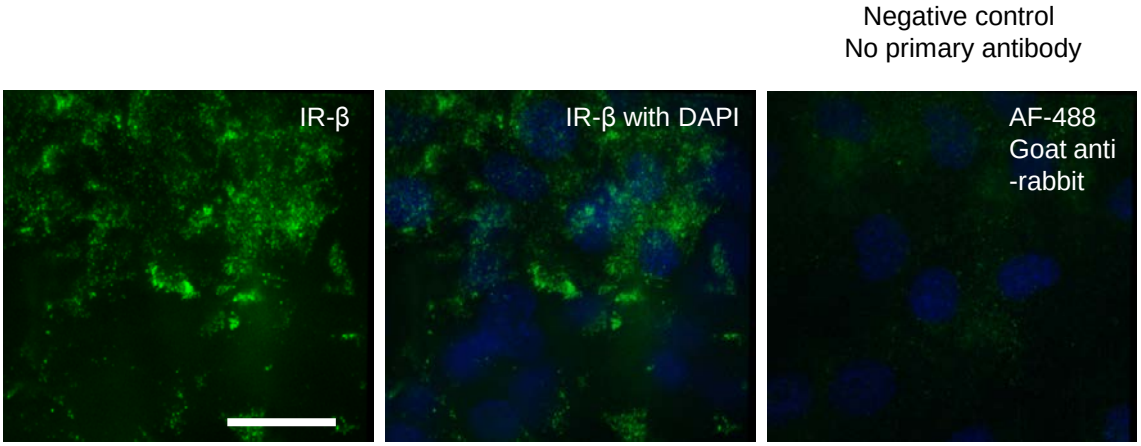
Supplementary Figure 1





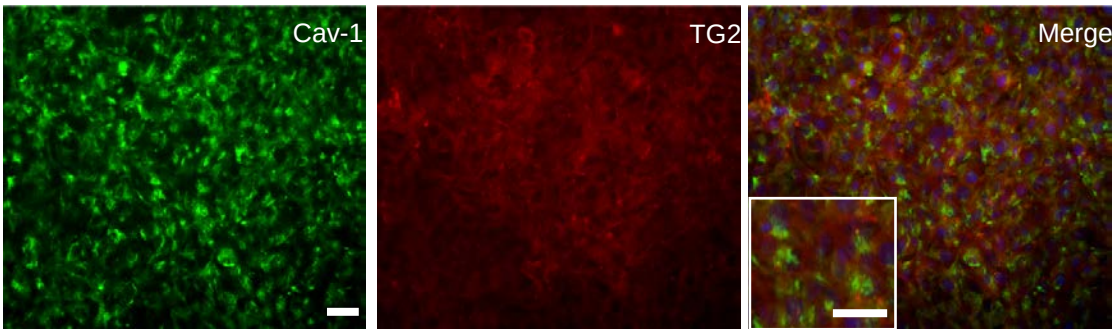
Supplementary Figure 1. Antibody controls for TIRF microscopy. (A) Primary antibody omission control. Fixed cells were incubated with two secondary antibodies, donkey anti-goat Alexa Fluor® (AF) 488 (green) and goat anti-rabbit Alexa Fluor® 568 (red), that were used in Cav-1 and FXIII-A co-localization study. While small dots are detected, no patchy clusters are seen. (B) Rabbit anti-mouse FXIII-A primary antibody with two secondary antibodies (goat anti-rabbit Alexa Fluor® 568 and donkey anti-goat Alexa Fluor® 488) as well as with donkey anti-goat Alexa Fluor® 488 (used for Cav-1). While small dots are detected, patchy clusters (dashed lines) are seen only with rabbit anti-mouse FXIII-A and its correct secondary. Bar equals 20μm.

Supplementary Figure 2



Supplementary Figure 2. TIRF microscopy analysis of an integral membrane protein - insulin receptor β (IR-β) in osteoblasts fixed with 3.5% formaldehyde and without permeabilization. IR-β (green) staining is seen as clusters which is distinguished from the staining with secondary antibody (goat anti-rabbit Alexa Fluor® 488) alone. Bar equals 20μm.

Supplementary Figure 3



Supplementary Figure 3. TG2 also does not co-localize in with Caveolin-1. Immunofluorescence microscopy of caveolin-1(Cav-1) (green) and TG2 (red) in osteoblasts on day 5. Bars equal 40 μm.

Conclusion& Future Directions

Conclusions

FXIII-A is a member of the TG enzyme family that has the ability to modify Q residues via its transamidation activity that in most circumstances involves the formation of crosslinked protein polymers. TGs also have functions that do not involve transamidation. These transamidation-independent functions have been identified at least for TG2 and band 4.2 (erythrocyte TG). Based our previous work, TGs appear to be tightly linked to osteoblast differentiation and bone formation and in our cell culture system, FXIII-A appears to be the active TG enzyme responsible for transamidation/crosslinking in the ECM, however FXIII-A is also present in osteoblast cells. In this thesis, we further investigated the functions of this cellular FXIII-A in differentiating MC3T3-E1 osteoblast. Our results suggest that cellular FXIII-A has both crosslinking and non-catalytic/non-crosslinking functions in osteoblast.

In *Chapter 3*, we show that FXIII-A activity in osteoblasts targets the stabilized, detyrosinated tubulin, also known as Glu-tubulin. FXIII-A forms a high 150 kDa molecular weight membrane associated Glu-tubulin from the monomeric form. *In vitro* TG activity assay suggests that the covalent modification of tubulin occurs to α-tubulin, which is known to give rise to Glu-tubulin, while β-tubulin does not react to TG activity. By subcellular fractionation, cell surface biotinylation experiments and TIRF microscopy, we first identify a pool of α-tubulin which is found on the plasma membrane of osteoblasts as well as on the cell surface. The importance of TG activity to the formation of Glu-tubulin and COL I accumulation was confirmed by using a

FXIII-A specific peptide probe and competitive inhibitor, F11, which is biotinylated (bF11). This study serves as a follow-up study to better understand how tubulin crosslinking or lack thereof affects matrix secretion and deposition from our previous work. Our work demonstrates that tubulin can be modified by TG activity. While TG mediated polyamination was demonstrated before as outlined in Chapter 1, our work is the first to demonstrate clearly that Glu-tubulin and tubulin can be also crosslinked into high molecular weight polymers in a cellular system. Furthermore, the observation that the high molecular weight Glu-tubulin is specifically membrane-associated brings evidence to the concepts that tubulin associates with membranes and PTMs can alter tubulin properties. How this crosslinking alters tubulin and promotes its membrane-association remains unknown, however, it is possible that crosslinking promotes its hydrophobicity as many crosslinked proteins generally show altered solubility.

We also learned from our previous studies that cellular FXIII-A is located on the plasma membrane of osteoblasts in rounded patches. In *Chapter 4*, we further demonstrated that those patches represent FXIII-A in caveolae. FXIII-A co-localizes with Cav-1 in the inner leaflet of the plasma membrane in differentiating osteoblasts. Despite the presence of FXIII-A, caveolae had no detectable TG activity as measured by monodansylcadaverine incorporation. NC9, which is capable of altering TG enzyme conformation, was found to colocalize in caveolae and to displace FXIII-A from these structures. The decreased FXIII-A levels in caveolae associated with increased Cav-1 phosphorylation and homo-oligomerization that result from c-Src

activation. Since c-Src has long been implicated to be a negative signaling factor for bone formation, the function of FXIII-A regulating c-Src activity presents a new potential angle to understanding the importance of cellular FXIII-A during osteoblast differentiation.

To summarize, we propose that cellular FXIII-A in osteoblasts have both crosslinking and non-catalytic/non-crosslinking functions on outer and inner leaflet of the osteoblast plasma membrane, respectively. First, cellular FXIII-A promotes secretion and elaboration of COL I ECM via facilitating microtubule association with plasma membrane. This process is linked to the presence of Glu-tubulin on the plasma membrane. This function of FXIII-A requires its crosslinking activity (**Figure 6**). Meanwhile, FXIII-A is also found on the inner leaflet of the plasma membrane where it functions in a transamidation-independent manner. It appears that FXIII-A keeps c-Src inactive with regard to Cav-1 phosphorylation, resulting in a favorable condition (signaling) for osteoblast differentiation (**Figure 7**).

The findings presented in this thesis represent new information on functions of cellular FXIII-A in osteoblast. The original findings are presented in the following section.

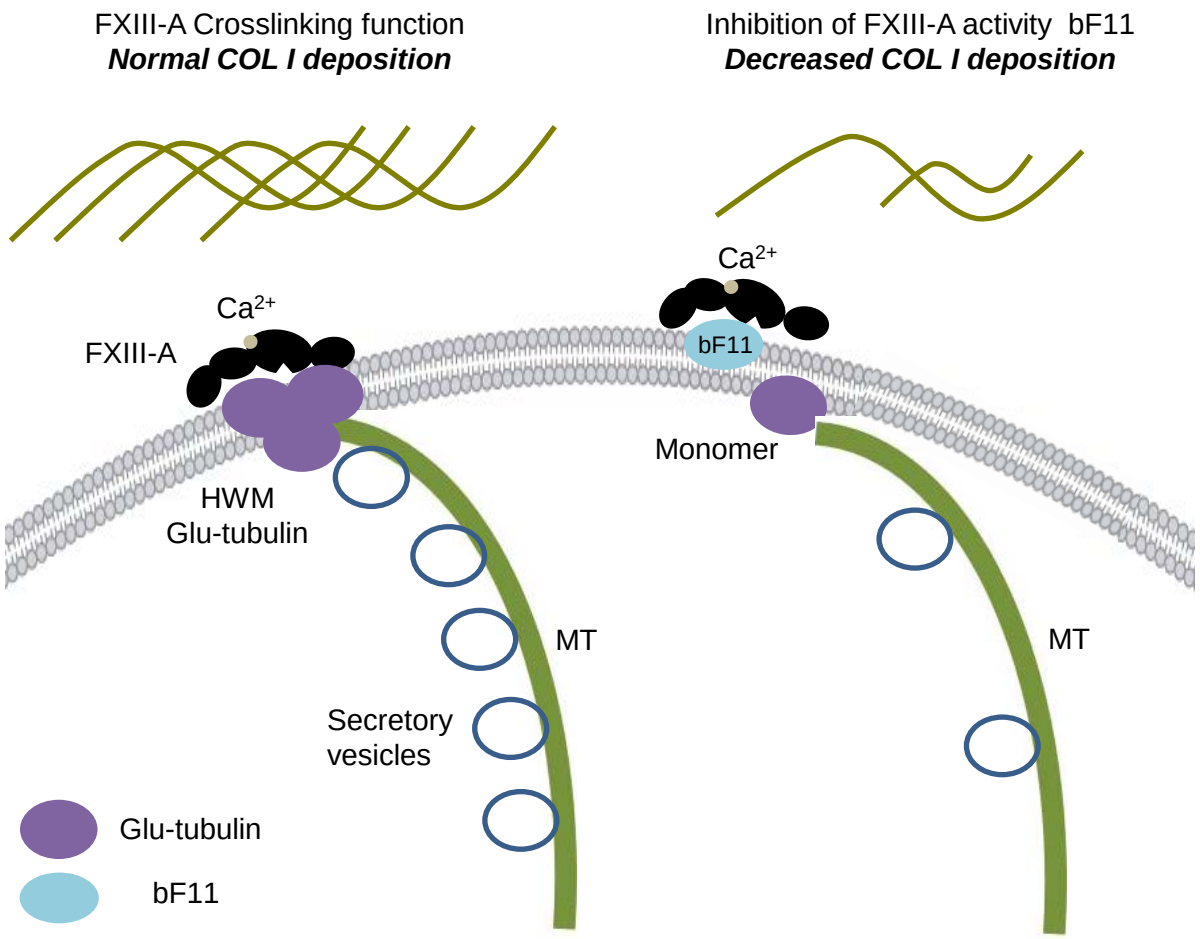


Figure 6. Proposed model for FXIII-A on the plasma membrane of osteoblasts. The oligomeric Glu-tubulin is specifically localized to the plasma membrane of osteoblast. Inhibition of FXIII-A activity (by specific substrate peptide bF11) attenuates its formation in cells. This is associated with significantly decreased COL I deposition and decreased secretory activity as measured by Synaptotagmin VII (secretory vesicle marker) levels on the osteoblast plasma membrane. Collectively, this means that membrane-associated high molecular weight Glu-tubulin is linked to COL I secretion in osteoblasts.

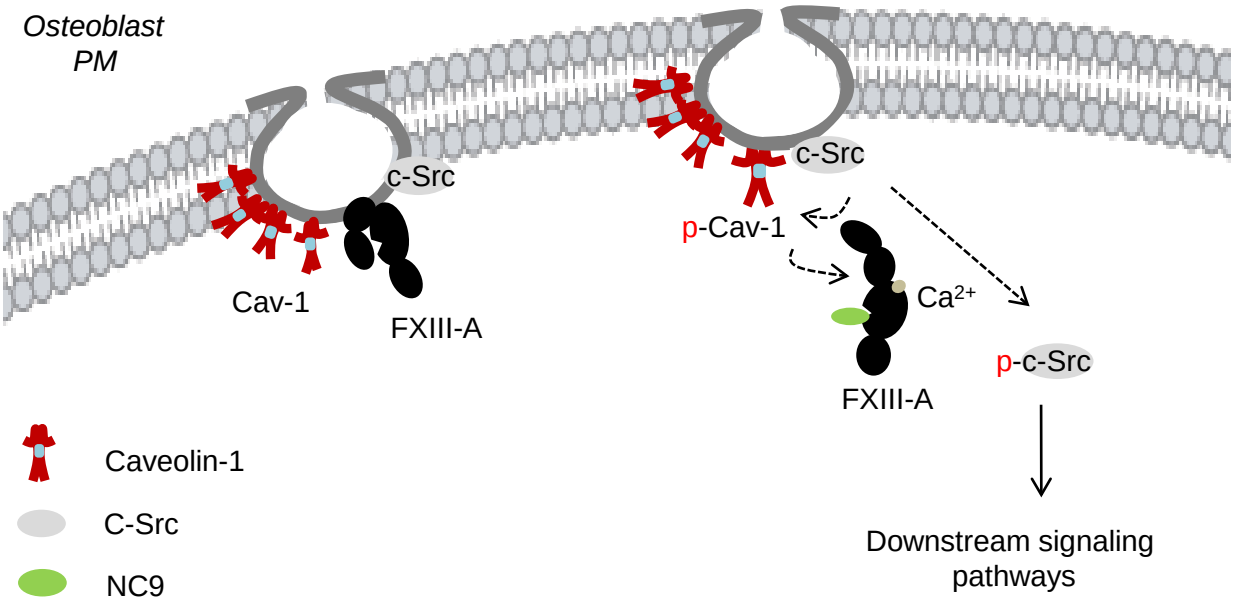


Figure 7. Proposed model for FXIII-A on the inner leaflet of osteoblast membrane. FXIII-A is found in co-localized with Cav-1, a caveolae marker. We propose that the presence of FXIII-A keep it inaccessible to c-Src. NC9 – a TG inhibitor which is capable of interacting with TG enzyme catalytic site as well as altering enzyme conformation from the closed to the open form – displaces FXIII-A from caveolae which allows c-Src to associate with Cav-1 to phosphorylate it. This interaction also promotes phosphorylation and activation of c-Src. p-c-Src does not remain bound to caveolae, and relocates to the cytosol where it continues its downstream signaling activities (such as phosphorylation of Akt).

Original contributions

- 150 kDa Glu-tubulin is found in the membrane preparations of osteoblasts and responds to nocodazole (destabilizer) or paclitaxel (stabilizer) treatments, and showed increased resistance to destabilization.
- α-Tubulin, but not β-tubulin, is a TG substrate and can be crosslinked into high-molecular weight forms of 100, 150 and 250 kDa (dimers, trimers and pentamers) *in vitro*.
- α- and Glu-tubulin are found on the plasma membrane of osteoblasts where they co-localize with FXIII-A.
- The specific FXIII-A substrate peptide bF11 co-localizes with α-tubulin in osteoblasts and attenuates α-tubulin crosslinking *in vitro*.
- A FXIII-A specific substrate peptide F11 inhibits COL I ECM accumulation and attenuates levels of the 150 kDa Glu-tubulin in osteoblasts.
- Cellular FXIII-A is co-localized with Cav-1 in differentiating osteoblasts.
- FXIII-A co-fractionates with Cav-1 in differentiating osteoblasts.
- Caveolae do not have transamidase activity.
- FXIII-A displacement from caveolae affects the homo-oligomerization and phosphorylation status of Cav-1.
- NC9 promotes c-Src-kinase phosphorylation and its downstream signaling activation.

Future directions

Despite advances made in the studies presented in this thesis, several questions remain. To gain further insight into the cellular functions of FXIII-A it would be important to:

- Perform GFP-FXIII-A transfections to visualize FXIII-A in caveolae and the outer leaflet of the plasma membrane via confocal microscopy and z-stacking.
- Examine microtubule dynamics (polymerization and depolymerization rates via microscopy) with α-tubulin-GFP transfected differentiating FXIII-A knockout and WT osteoblasts.
- Confirm above studies with primary cell cultures from FXIII-A knockouts.
- Immunoprecipitate or otherwise purify the high molecular weight, 150 kDa Glu-tubulin from membrane fractions of differentiating MC3T3-E1 cells to further characterize its biochemical properties compared to monomeric tubulin(s).
- Investigate other potential substrates for FXIII-A in osteoblasts using biotinylated F11 substrate peptide labeling approach followed by immunopurification of the labeled proteins and their identification by proteomics.
- Analyze other caveolae-mediated cellular functions, such as ECM endocytosis changes in FXIII-A inhibited or null cells.
- Examine of roles of FXIII-A are linked, i.e., assessment of interactions between microtubules and caveolae with-without TG inhibition. The association between microtubules and caveolae has been proposed by Insel group and Bloom group.