Maastricht University

Skin and Bones

Citation for published version (APA):

de Vos, I. J. H. M. (2018). *Skin and Bones: Studying the effects of MMP14 mutations*. [Doctoral Thesis, Maastricht University]. Datawyse / Universitaire Pers Maastricht. https://doi.org/10.26481/dis.20181219iv

Document status and date:
Published: 01/01/2018

DOI:
10.26481/dis.20181219iv

Document Version:
Publisher's PDF, also known as Version of record

Please check the document version of this publication:

• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
• The final author version and the galley proof are versions of the publication after peer review.
• The final published version features the final layout of the paper including the volume, issue and page numbers.

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy
If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Download date: 03 Nov. 2024

CHAPTER 8

Summary

Samenvatting

Summary

In 2007, we reported two brothers with a multisystem disorder encompassing progressive mitral valve insufficiency, osteopenia, thoracic kyphosis, craniofacial dysmorphism, dermal fibrosis, and severe nodulocystic acne [1]. In these patients, we identified a novel homozygous *MMP14* c.332G>A missense mutation. This gene encodes matrix metalloproteinase 14, a membrane-bound endopeptidase that primarily cleaves structural components of the extracellular matrix (ECM), which is expressed in various tissues including bone and skin [2-5]. Multiple lines of evidence suggested that this mutation could be pathogenic. As shown in **Chapter 1**, *in silico* analysis predicted that the resulting p.R111H substitution would be damaging. Secondly, a homozygous MMP14 p.T17R mutation has previously been reported in patients with a clinical diagnosis of Winchester syndrome (WS), a constellation of features similar to our patients' phenotype [6, 7]. Thirdly, the mouse models *Sabe* and *Cartoon* with homozygous MMP14 p.R92C and p.S466P missense mutation, respectively, share many skeletal features with our patients [8, 9]. Fourthly, there is significant phenotypical overlap between our patients and individuals with Frank-Ter Haar syndrome (FTHS) and multicentric osteolysis, nodulosis, and arthropathy (MONA), which are caused by homozygous loss-of-function mutations in *SH3PXD2B* respectively *MMP2* [10-16]. Importantly, the protein products of these three genes directly cooperate in the formation of podosomes, which are specialised membrane protrusions involved in ECM remodelling and invasive cell motility [17-20]. Together, this evidence led us to diagnose our patients with Wichester syndrome, pending confirmation of the p.R111H mutation's pathogenicity. In this thesis, we aimed to elucidate how mutation of *MMP14* results in the WS phenotype.

In **Chapter 2**, we first assessed how our novel p.R111H mutation, and previously reported human and murine mutations, affect MMP14. MMP14 is synthesised as a latent pre-proenzyme, whose activity is controlled by different processes, including proteolytic activation of the zymogen and exposure at the cell surface [2, 3, 21-24]. To reach the plasma membrane, the full-length zymogen contains an amino-terminal signal peptide (SP) for its insertion into the endoplasmatic reticulum (ER) membrane during translation [2, 3]. Next to the SP lies a prodomain that keeps the adjacent catalytic domain inactive [2, 3, 21, 25-27]. After ER insertion, pro-MMP14 is activated by sequential cleavage of its N-terminus by MMPs and proprotein convertases (PCs), and is subsequently trafficked to the plasma membrane [22, 28-32]. The abovementioned mutations are all present at sites that are thought to be crucial for normal activation and/or trafficking of MMP14: the SP (p.T17R), PC recognition motifs (p.R92C and p.R111H), and the hemopexin-like (Hx) domain (p.S466P) [2, 3, 7-9, 28-30, 33-35]. To assess the effect of these mutations on MMP14 processing, trafficking, and activity, we developed a novel *in vitro* model consisting of fibroblasts expressing either wild type

(WT) or mutant MMP14 with two different tags at their amino respectively carboxyl terminus. As expected, the full-length WT fusion protein was sequentially processed along the biosynthetic-exocytotic pathway and finally localised at the cell surface as a functionally active enzyme. We provided the first direct evidence for MMP14 SP removal in the ER *in vitro*. The p.T17R mutation impaired ER insertion and subsequent processing and trafficking of MMP14. In contrast, MMP14 R111H was processed and trafficked seemingly normally. However, the p.R111H mutation partially impaired MMP14's catalytic activity and its stimulatory effect on cell migration *in vitro*, suggesting R^{111} is a crucial residue for pro-MMP14 activation. It is possible that the impaired activity is caused by a subtle aberration in pro-domain cleavage that we were unable to detect by Western blot. Compared to the patients originally reported by Winchester et al., our patients had a relatively mild phenotype, correlating best with mutant MMP14's ability to activate pro-MMP2 *in vitro* [6]. Our results thus confirmed the pathogenicity of the novel hypomorphic *MMP14* allele, causing a mitigated form of WS. Compared to WS patients, the *Mmp14* mutant mice have a more severe phenotype. In line with this, the p.R92C mutation dramatically impaired MMP14's intracellular trafficking and activity *in vitro*. This implies a more important role for the R^{89}-R-P-R-C^{93} PC recognition motif in MMP14 activation and subsequent trafficking than previously thought [23, 31, 36]. Although the *Sabe* and *Cartoon* phenotype are virtually indistinguishable and the respective mutations impaired MMP14's trafficking and pro-MMP2 cleavage, MMP14 S466P retained its ability to digest gelatin and stimulate cell motility [8, 9]. A possible explanation for this discrepancy is that in contrast to MMP14 R92C, some MMP14 S466P still makes it to the plasma membrane, leaving its functions that do not require homodimerisation intact [37-40]. Homodimerisation might be impaired by a dose effect, or by a direct effect of the p.S466P mutation on the Hx domain [41]. In addition, the p.S466P mutation might alter MMP14's substrate specificity, enhancing gelatin digestion, but not pro-MMP2 cleavage. Taken together, we demonstrated that these four mutations each impair MMP14 functional activity in a unique manner, resulting in a similar clinical phenotype. We subsequently used 3D *in vitro* and *in vivo* models to assess how the loss of MMP14 function resulted in specific aspects of the WS phenotype.

One aspect of the WS and FTHS phenotype of our particular interest is acne [1, 6, 10, 11, 42]. Although it is well known that acne is characterised by cystic sebaceous glands i.e. comedones, it is unknown why such cystic changes occur. The sebaceous gland is, in essence, a hollow, branched epithelial structure that is separated from the type I collagen-rich dermis by a basement membrane [43]. Morphogenesis of such structures generally relies on *de novo* lumen formation and branching morphogenesis [44]. Planar cell polarity (PCP) and ECM remodelling play a crucial role in these two processes and when disturbed, can result in cyst formation [45-48]. Interestingly, multiple cell types

depend on MMP14's catalytic activity for branching morphogenesis, and on cell surface localisation of MMP14 for normal PCP [49-52]. Therefore, we hypothesised that disrupted luminogenesis and branching morphogenesis of the sebaceous gland due to impaired function of MMP14 or, given their direct functional link outlined above, SH3PXD2B, could underly comedogenesis. As the acne in our patients was successfully treated with 13-is retinoic acid, we hypothesised that this drug could correct the putative defective branching morphogenesis [1]. In **Chapter 3**, we conducted a pilot study to assess the effects of *Mmp14* or *Sh3pxd2b* knockdown (KD) and retinoid treatment on luminogenesis and branching morphogenesis of epithelial cells *in vitro* [53, 54]. We demonstrated that KD of either of these genes impaired lumen formation in reconstituted basement membrane (Matrigel™) and reduced branching morphogenesis in type I collagen gel, whereas retinoid treatment directly stimulated both processes. The disturbed luminogenesis upon KD of *Mmp14* or *Sh3pxd2b* is suggestive of impaired PCP [52]. In addition, the observed shortened tubule length in crosslinked type I collagen matrix suggests that KD of *Mmp14* or *Sh3pxd2b* impairs ECM degradation [55, 56]. Finally, the reduced organoid size in type I collagen gel upon KD could additionally be caused by a reduction in cell proliferation. The latter two mechanisms are likely linked, as IMCD cells isolated from *Mmp14* KO mice were previously reported to have a proliferative defect that depended on MMP14's collagenolytic activity [57]. The fact that KD of either *Mmp14* or *Sh3pxd2b* caused a similar effect *in vitro* suggests that a shared pathway is affected. Given its role in ECM remodelling, podosome function is the most likely candidate. Retinoids could act through stimulating podosome formation, as we demonstrated that 13-cis retinoic acid stimulated trafficking of MMP14 S466P to podosomes *in vitro*. This could explain the therapeutic effect of 13-cis retinoic acid in acne treatment, although additional tissue-specific effects are likely involved [58, 59]. Future studies should repeat our experiments with sebocytes to assess whether these processes could affect the sebaceous gland. Such sebocyte 3D model could potentially be used in screening for anti-acne therapeutics.

Apart from acne, the WS phenotype is characterised by craniofacial dysmorphism and generalised osteopenia [1, 6]. The majority of the affected craniofacial skeletal elements are of neural crest (NC) origin, the primary embryonic structure contributing to the development of the face [60-62]. After their induction during embryogenesis, NC cells delaminate and migrate extensively, for which ECM remodelling is essential [60, 61, 63-65]. As MMP14 is involved in ECM remodelling and invasive cell motility, we hypothesised that impaired cranial NC migration underlies the WS craniofacial phenotype [4, 5, 21, 66]. To test this hypothesis, and to study the underlying cause of osteopenia in WS, we decided to use a zebrafish model. Zebrafish form the same skeletal tissues as humans, which furthermore develop in a similar manner. This especially holds true for the zebrafish skull bones, the majority of which is of NC origin [67-70].

Zebrafish have two well-conserved MMP14 paralogs, Mmp14a and Mmp14b, which are expressed in the head mesenchyme during development [71-76]. The rapid external development and optical transparency of zebrafish embryos furthermore enable live *in vivo* imaging of NC cells [77-79]. In **Chapter 4**, we used the CRISPR/Cas9 approach to successfully knock out (KO) both *mmp14a* and *mmp14b* in zebrafish. Our *mmp14a/b* KO fish recapitulated essential aspects of the WS phenotype, including stunted growth, gradually worsening craniofacial anomalies, hyperkyphosis, osteopenia (albeit limited to the skull), and a shortened lifespan [1, 6]. In contrast to our hypothesis, NC induction, delamination, pharyngeal arch invasion, and differentiation into larval craniofacial cartilage elements were unaffected by *mmp14a/b* KO. Previously described craniofacial defects in *mmp14a* of *mmp14b* morphants are likely off-target effects of the used Morpholino oligonucleotides [74, 75, 80]. In our *mmp14a/b* KO larvae, skeletal mineralisation during larval-to-juvenile metamorphosis was unaffected [81]. Adult mutant fish showed impaired endochondral/perichondral and intramembranous ossifying bones of both NC and mesodermal origin, further arguing against an NC-specific problem [68, 82]. Affected bones generally contained relatively little amounts of bone matrix with altered collagen content, clusters of multinucleated cells, and a relatively volumous, disorganised cartilage core. In mice, loss of MMP14 impairs timely cartilage removal during both ossification types and is accompanied by excessive absorption of mineralised bone matrix, resulting in similar bone abnormalities as those observed in our *mmp14a/b* KO fish [51, 83]. Although it was previously demonstrated *in vitro* that MMP14 stimulated osteoblast differentiation and inhibited osteoclast differentiation and activation, additional assays should shed light on the exact processes and cell types involved in the observed skeletal phenotype in the *mmp14a/b* KO fish [84-86]. In addition, it is still unknown to what extent impaired podosome function is involved in the resulting human, murine and zebrafish phenotype. The generation of various combinations of *mmp14a/b* KO, *sh3pxd2b* KO and *mmp2* KO zebrafish might shed light on how their protein products interact, at least in fish, in bone remodelling. Finally, the newly generated *mmp14a/b* KO fish could be a suitable model for development of novel therapeutics addressing not only the WS bone phenotype, but also low bone density in general.

Samenvatting

In 2007 beschreven wij twee broers met een multisysteemaandoening, bestaande uit progressieve mitraalklepinsufficiëntie, osteopenie, thoracale hyperkyfose, craniofaciale dysmorfie, dermale fibrose, en ernstig nodulocysteus acne [1]. Recent toonden wij aan dat deze patiënten homozygoot zijn voor een niet eerder gerapporteerde c.332G>A missense mutatie in *MMP14*. Dit gen codeert voor matrix metalloproteinase 14, een membraangebonden endopeptidase dat vooral structurele componenten van de extracellulaire matrix (ECM) hydrolyseert en tot expressie komt in diverse weefsels, waaronder de huid en bot [2-5]. Het is om meerdere redenen aannemelijk dat deze mutatie pathogeen is. Zoals getoond in **Hoofdstuk 1**, voorspelde *in silico* analyse dat de resulterende p.R111H substitutie schadelijk is. Eerder werd een homozygote MMP14 p.T17R mutatie geïdentificeerd in twee patiënten met Winchester syndroom (WS), die qua fenotype sterk lijken op onze patiënten [6, 7]. Ten derde hebben de muismodellen *Sabe* en *Cartoon*, met respectievelijk homozygote MMP14 p.R92C en p.S466P mutatie, skeletafwijkingen vergelijkbaar met die van onze patiënten [8, 9]. Ten vierde is er een sterke gelijkenis tussen het fenotype van onze patiënten en dat van mensen met Frank-Ter Haar syndroom (FTHS) dan wel multicentrische osteolyse, nodulose, en arthropathie (MONA). FTHS en MONA worden veroorzaakt door homozygote mutaties in respectievelijk *SH3PXD2B* en *MMP2* [10-16]. Deze twee genen coderen voor eiwitten die direct samenwerken met MMP14 in de vorming van podosomen, gespecialiseerde membraanuitstulpingen die betrokken zijn bij ECM remodellering en invasieve celmotiliteit [17-20]. Om deze vier redenen hebben wij onze patiënten gediagnosticeerd met WS, hoewel de pathogeniciteit van de MMP14 p.R111H mutatie nog bevestogd moest worden. In dit proefschrift probeerden wij te ontrafelen hoe mutatie van *MMP14* leidt tot het WS phenotype.

In **Hoofdstuk 2** hebben wij onderzocht of onze nieuw ontdekte p.R111H mutatie daadwerkelijk pathogen is, en hoe deze en eerder gerapporteerde mutaties MMP14 beïnvloeden. MMP14 wordt gesynthetiseerd als een latent pre-proenzym, waarvan de activiteit gereguleerd wordt door verschillende processen waaronder proteolytische activatie van het zymogeen en blootstelling op het celoppervlak [2, 3, 21-24]. Om het plasmamembraan te bereiken bevat pro-MMP14 een signaalpeptide (SP) aan zijn aminoterminus voor insertie in het endoplasmatisch reticulum (ER) membraan tijdens translatie [2, 3]. Naast het SP ligt een prodomein, dat het nabij gelegen katalytisch domein inactief houdt [2, 3, 21, 25-27]. Na ER insertie wordt pro-MMP14 geactiveerd door sequentiële hydrolyse van zijn aminoterminus door MMPs en proproteine convertasen (PCs) en wordt actief MMP14 vervolgens naar het plasmamembraan getransporteerd [22, 28-32]. Al de bovengenoemde mutaties zijn aanwezig op plekken die cruciaal worden geacht voor activatie en/of transport van MMP14: het SP (p.T17R), de PC herkenningsmotieven (p.R92C en p.R111H), en het hemopexine (Hx) domein

(p.S466P) [2, 3, 7-9, 28-30, 33-35]. Wij hebben het effect van deze mutaties op hydrolyse, transport en activiteit van MMP14 onderzocht aan de hand van een nieuw ontwikkeld *in vitro* model. Dit model bestond uit fibroblasten die wild-type (WT) dan wel mutant MMP14 met twee verschillende labels aan respectievelijk hun amino- en carboxylterminus tot expressie brachten. Zoals verwacht, werd het WT fusie-eiwit sequentieel gehydrolyseerd in de biosynthetische-exocytaire route en bevond zich uiteindelijk op het celoppervlak als functioneel actief enzym. Dit leverde tevens het eerste directe bewijs voor verwijdering van het SP van MMP14 in het ER *in vitro*. De p.T17R mutatie verstoorde ER insertie en daaropvolgende hydrolyse en transport van MMP14. Daarentegen verliep de hydrolyse en het transport van MMP14 R111H schijnbaar normaal. De p.R111H mutatie verstoorde de katalytische activiteit en het stimulerende effect op celmigratie van MMP14 *in vitro* echter deels, wat suggereert dat R^{111} een cruciaal residu is voor MMP14 activatie. Het is mogelijk dat de verminderde activiteit veroorzaakt wordt door een subtiele afwijking in prodomeinhydrolyse, die wij niet konden detectered door middel van Western blot. Vergeleken met de door Winchester et al. beschreven patiënten, hadden onze patiënten een relatief mild fenotype; dit correleerde het beste met de mate van pro-MMP2 activatie door mutant MMP14 *in vitro* [6]. Onze resultaten bevestigden de pathogeniciteit van een nieuw, hypomorf *MMP14* allel, dat een milde vorm van WS veroorzaakt. Vergeleken met de WS patiënten, hebben de *Mmp14* mutante muizen een ernstiger fenotype. In overeenstemming hiermee verstoorde de p.R92C mutatie het intracellulair transport en de activiteit van MMP14 *in vitro* dramatisch. Dit impliceert dat het R^{89}-R-P-R-C^{93} PC herkenningsmotief een belangrijkere rol speelt in deze processen dan eerder werd aangenomen [23, 31, 36]. Hoewel het *Sabe* en *Cartoon* phenotype nagenoeg identiek zijn aan elkaar en de respectievelijke mutaties de pro-MMP2 activatie door MMP14 ernstig verstoorden, behield MMP14 S466P zijn vermogen tot hydrolyse van gelatine en stimulatie van celmotiliteit *in vitro* [8, 9]. Een mogelijke verklaring voor deze discrepantie is dat in tegenstelling tot MMP14 R92C, enig MMP14 S466P het celmembraan bereikt, en daarmee functies die onafhankelijk zijn van homodimerisatie gespaard blijven [37-40]. Homodimerisatie kan verstoord zijn door een dosis-effect, of door een direct effect van de p.S466P mutatie op het Hx domein [41]. Daarnaast zou de p.S466P mutatie de substraatspecificiteit van MMP14 kunnen veranderen, wat de proteolyse van gelatine, maar niet van pro-MMP2, vergemakkelijkt. Samengevat hebben wij aangetoond dat de bovengenoemde vier mutaties de functionele activiteit van MMP14 elk op een unieke manier verstoren, hetgeen restulteert in een vergelijkbaar klinisch fenotype.

Eén aspect van het WS en FTHS fenotype dat onze belangstelling heeft is acne [1, 6, 10, 11, 42]. Hoewel het alom bekend is dat acne gekenmerkt wordt door cysteuze talgklieren oftewel comedonen, is het onbekend waarom dergelijke cysteuze veranderingen optreden. De talgklier is in principe een holle, vertakte epitheliale structuur die

gescheiden is van de type I collageen-rijke dermis door een basaal membraan [43]. Vorming van dergelijke structuren hangt doorgaans af van *de novo* lumenformatie en vertakkende morfogenese [44]. Planaire celpolariteit (PCP) en ECM remodellering spelen een cruciale rol in deze twee processen en kunnen, indien verstoord, leiden tot cystevorming [45-48]. Opvallend genoeg zijn meerdere celtypen afhankelijk van de katalytische activiteit van MMP14 voor vertakkende morfogenese, en tevens van celmembraanlocalisatie van MMP14 voor PCP [49-52]. Daarom hypothetiseerden wij dat verstoorde luminogenese en vertakkende morfogenese van de talgklier door verminderde functie van MMP14 of SH3PXD2B (gezien hun hierboven beschreven directe interactive) ten grondslag kan liggen aan comedovorming. Aangezien acne in onze patiënten succesvol werd behandeld met 13-cis retinoïnezuur, hypothetiseerden wij dat dit medicament het veronderstelde defect in vertakkende morfogenese zou kunnen corrigeren [1]. In **Hoofdstuk 3** beschrijven wij een pilotstudie die het effect van *Mmp14* of *Sh3pxd2b* knockdown (KD) dan wel retinoïden op lumenvorming en vertakkende morfogenese van epitheelcellen *in vitro* onderzoekt [53, 54]. We toonden aan dat KD van deze genen lumenvorming in gereconstrueerd basaal membraan (Matrigel^{TM}) verstoorde en vertakkende morfogenese in type I collageengel verminderde, terwijl retinoïden beide processen direct stimuleerden. Verstoorde lumenvorming door KD van *Mmp14* of *Sh3pxd2b* impliceert dat PCP afwijkend is [52]. Verder suggereert de verkorte tubuluslengte in gecrosslinkte type I collageenmatrix dat KD van *Mmp14* of *Sh3pxd2b* de ECM degradatie verstoort [55, 56]. Tenslotte kan de KD de celdeling inhiberen, wat de kleinere organoïden in type I collageengel kan verklaren. De laatste twee mechanismen zijn waarschijnlijk gekoppeld, aangezien eerder is aangetoond dat IMCD cellen geïsoleerd uit *Mmp14* KO muizen *in vitro* een proliferatief defect hadden door verlies van de collagenolytische activiteit van MMP14 [57]. Het feit dat KD van *Mmp14* of *Sh3pxd2b* eenzelfde effect veroorzaakt *in vitro* suggereert dat een gemeenschappelijke functionele pathway aangedaan is. Vanwege haar rol in ECM remodellering, is podosoomfunctie de meest waarschijnlijke kandidaat. Retinoïden zouden hun effect kunnen uitoefenen door podosoomvorming te stimuleren, aangezien we in **Hoofdstuk 2** hebben aangetoond dat 13-cis retinoïnezuur het transport van MMP14 S466P naar podosomen *in vitro* stimuleerde. Dit kan het therapeutisch effect van 13-cis retinoïnezuur in de behandeling van acne verklaren, hoewel aanvullende weefselspecifieke effecten waarschijnlijk ook een rol spelen [58, 59]. Toekomstig onderzoek dat onze experimenten met sebocyten herhaalt, zal moeten uitwijzen of deze processen een rol kunnen spelen in de morfogenese van talgklieren. Een dergelijk 3D sebocytmodel kan mogelijk gebruikt worden in screening naar anti-acne farmaca.

Naast acne wordt het WS fenotype gekenmerkt door craniofaciale dysmorfie en gegeneraliseerde osteopenie [1, 6]. De meeste aangedane craniofaciale skeletelementen zijn afgeleid van de van neurale lijst (NL), de voornaamste embryonale structuur die bij-

draagt aan de ontwikkeling van het gelaat [60-62]. Na hun inductie, delamineren NL cellen en migreren ze uitvoerig, waarvoor ECM remodellering essentieel is [60, 61, 63-65]. Aangezien MMP14 betrokken is bij ECM remodellering en invasieve celmotiliteit, hypothetiseerden wij dat verstoorde NL migratie ten grondslag ligt aan de craniofaciale dysmorfie van WS [4, 5, 21, 66]. Om deze hypothese te testen en de onderliggende oorzaak van osteopenie bij WS te bestuderen, besloten wij een zebravismodel te ontwikkelen. Zebravissen vormen dezelfde skeletweefsels als mensen, die zich bovendien op een vergelijkbare wijze ontwikkelen. Dit geldt met name voor de schedelbeenderen van de zebravis, waarvan de meerderheid is afgeleid van de NL [67-70]. Zebravissen hebben twee goed geconserveerde MMP14 paralogen, Mmp14a en Mmp14b, die tijdens de ontwikkeling in het mesenchym van de kop tot expressie komen [71-76]. De snelle uitwendige ontwikkeling en optische transparantie van het zebravisembryo maken bovendien live *in vivo* beeldvorming mogelijk [77-79]. In **Hoofdstuk 4** hebben wij de CRISPR/Cas9 techniek gebruikt voor knockout (KO) van zowel *mmp14a* als *mmp14b* in zebravissen. Onze *mmp14a/b* KO vissen toonden essentiële aspecten van het WS fenotype, inclusief verminderde groei, geleidelijk verergerende craniofaciale afwijkingen, hyperkyfose, osteopenie (hoewel beperkt tot de schedel), en een verkorte levensduur [1, 6]. In tegenstelling tot onze hypothese, waren NL inductie, delaminatie, farynxbooginvasie, en differentiatie tot craniofaciaal kraakbeen onaangetast door *mmp14a/b* KO. Zoals eerder gesuggereerd, zijn voorheen beschreven craniofaciale defecten in *mmp14a* en *mmp14b* morfanten waarschijnlijk aspecifieke neveneffecten van de gebruikte Morfolino oligonucleotiden [74, 75, 80]. In onze *mmp14a/b* KO larven was de skeletmineralisatie tijdens metamorfose onaangetast [81]. Volwassen mutanten hadden een verstoorde enchondrale/perichondrale en intramembraneuze ossificatie van zowel NL- als mesoderm-afgeleide beenderen, wat verder tegen een NL-specifiek problem pleit [68, 82]. Aangedane botten bevatten in het algemeen relatief weinig botmatrix met een afwijkende collageeninhoud en clusters multinucleaire cellen, en een relatief volumineuze, ongeorganiseerde kraakbeenkern. In muizen verstoort verlies van MMP14 de tijdige verwijdering van kraakbeen tijdens beide vormen van ossificatie, wat gepaard gaat met overmatige resorptie van gemineraliseerde botmatrix. Dit resulteert in vergelijkbare botafwijkingen als aanwezig in onze *mmp14a/b* KO vissen [51, 83]. Hoewel *in vitro* is aangetoond dat MMP14 de osteoblastdifferentiatie stimuleert en osteoclastdifferentiatie en -activatie inhibeert, zal aanvullend onderzoek uit moeten wijzen welke processen en celtypes betrokken zijn in het skeletfenotype van onze *mmp14a/b* KO vissen [84-86]. Verder is het nog onbekend in welke mate afwijkende podosoomfunctie betrokken is in het WS fenotype. Analyse van zebravissen met verschillende combinaties van *mmp14a/b*, *sh3pxd2b* en/of *mmp2* KO kan helpen om de samenwerking van de respectievelijke eiwitproducten in botremodellering te begrijpen. Tenslotte kan de *mmp14a/b* KO vis gebruikt worden voor de ontwikkeling van medicatie voor botafwijkingen in WS alsook osteopenie in het algemeen.

References

1. Van Steensel MA, Ceulen RP, Delhaas T, de Die-Smulders C. Two Dutch brothers with Borrone dermato-cardio-skeletal syndrome. American Journal of Medical Genetics Part A. 2007;143a(11):1223-6.
2. Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, et al. A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature. 1994;370(6484):61-5.
3. Takino T, Sato H, Yamamoto E, Seiki M. Cloning of a human gene potentially encoding a novel matrix metalloproteinase having a C-terminal transmembrane domain. Gene. 1995;155(2):293-8.
4. Ohuchi E, Imai K, Fujii Y, Sato H, Seiki M, Okada Y. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. The Journal of Biological Chemistry. 1997;272(4):2446-51.
5. d'Ortho MP, Will H, Atkinson S, Butler G, Messent A, Gavrilovic J, et al. Membrane-type matrix metalloproteinases 1 and 2 exhibit broad-spectrum proteolytic capacities comparable to many matrix metalloproteinases. European Journal of Biochemistry. 1997;250(3):751-7.
6. Winchester P, Grossman H, Lim WN, Danes BS. A new acid mucopolysaccharidosis with skeletal deformities simulating rheumatoid arthritis. The American Journal of Roentgenology, Radium Therapy, and Nuclear Medicine. 1969;106(1):121-8.
7. Evans BR, Mosig RA, Lobl M, Martignetti CR, Camacho C, Grum-Tokars V, et al. Mutation of membrane type-1 metalloproteinase, MT1-MMP, causes the multicentric osteolysis and arthritis disease Winchester syndrome. American Journal of Human Genetics. 2012;91(3):572-6.
8. Curtain MM; Donahue LR. 2007. A possible new mutation to Mmp 14 MGI Direct Data Submission. MGI: J:127164. Updated Nov 2012. The Jackson Laboratory. URL: informatics.jax.org/downloads/Reference_texts/J127164.pdf.
9. Du X, Moresco EMY, Murray A, Beutler B. record for cartoon, updated Dec 12, 2013. MUTAGENETIX ™, B. Beutler and colleagues, center for the Genetics of Host Defense, UT Southwestern Medical Center, Dallas, TX. URL: mutagenetix. utsouthwestern.edu.
10. Frank Y, Ziprkowski M, Romano A, Stein R, Katznelson MB, Cohen B, et al. Megalocornea associated with multiple skeletal anomalies: a new genetic syndrome? Journal de Genetique Humaine. 1973;21(2):67-72.
11. Ter Haar B, Hamel B, Hendriks J, de Jager J. Melnick-Needles syndrome: indication for an autosomal recessive form. American Journal of Medical Genetics. 1982;13(4):469-77.
12. Maas SM, Kayserili H, Lam J, Apak MY, Hennekam RC. Further delineation of Frank-ter Haar syndrome. American Journal of Medical Genetics Part A. 2004;131(2):127-33.
13. Iqbal Z, Cejudo-Martin P, de Brouwer A, van der Zwaag B, Ruiz-Lozano P, Scimia MC, et al. Disruption of the podosome adaptor protein TKS4 (SH3PXD2B) causes the skeletal dysplasia, eye, and cardiac abnormalities of Frank-Ter Haar Syndrome. American Journal of Human Genetics. 2010;86(2):254-61.
14. Martignetti JA, Aqeel AA, Sewairi WA, Boumah CE, Kambouris M, Mayouf SA, et al. Mutation of the matrix metalloproteinase 2 gene (MMP2) causes a multicentric osteolysis and arthritis syndrome. Nature Genetics. 2001;28(3):261-5.
15. Zankl A, Bonafe L, Calcaterra V, Di Rocco M, Superti-Furga A. Winchester syndrome caused by a homozygous mutation affecting the active site of matrix metalloproteinase 2. Clinical Genetics. 2005;67(3):261-6.
16. Zankl A, Pachman L, Poznanski A, Bonafe L, Wang F, Shusterman Y, et al. Torg syndrome is caused by inactivating mutations in MMP2 and is allelic to NAO and Winchester syndrome. Journal of Bone and Mineral Research. 2007;22(2):329-33.
17. Nakahara H, Howard L, Thompson EW, Sato H, Seiki M, Yeh Y, et al. Transmembrane/cytoplasmic domain-mediated membrane type 1-matrix metalloprotease docking to invadopodia is required for

cell invasion. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(15):7959-64.
18. Buschman MD, Bromann PA, Cejudo-Martin P, Wen F, Pass I, Courtneidge SA. The novel adaptor protein Tks4 (SH3PXD2B) is required for functional podosome formation. Molecular Biology of the Cell. 2009;20(5):1302-11.
19. Murphy DA, Courtneidge SA. The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. Nature Reviews Molecular Cell Biology. 2011;12(7):413-26.
20. Gawden-Bone C, Zhou Z, King E, Prescott A, Watts C, Lucocq J. Dendritic cell podosomes are protrusive and invade the extracellular matrix using metalloproteinase MMP-14. Journal of Cell Science. 2010;123(Pt 9):1427-37.
21. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annual Review of Cell and Developmental Biology. 2001;17:463-516.
22. Remacle AG, Rozanov DV, Baciu PC, Chekanov AV, Golubkov VS, Strongin AY. The transmembrane domain is essential for the microtubular trafficking of membrane type-1 matrix metalloproteinase (MT1-MMP). Journal of Cell Science. 2005;118(Pt 21):4975-84.
23. Remacle AG, Rozanov DV, Fugere M, Day R, Strongin AY. Furin regulates the intracellular activation and the uptake rate of cell surface-associated MT1-MMP. Oncogene. 2006;25(41):5648-55.
24. Koziol A, Martin-Alonso M, Clemente C, Gonzalo P, Arroyo AG. Site-specific cellular functions of MT1-MMP. European Journal of Cell Biology. 2012;91(11-12):889-95.
25. Springman EB, Angleton EL, Birkedal-Hansen H, Van Wart HE. Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proceedings of the National Academy of Sciences of the United States of America. 1990;87(1):364-8.
26. Van Wart HE, Birkedal-Hansen H. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proceedings of the National Academy of Sciences of the United States of America. 1990;87(14):5578-82.
27. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. Nature Reviews Molecular Cell Biology. 2007;8(3):221-33.
28. Massova I, Kotra LP, Fridman R, Mobashery S. Matrix metalloproteinases: structures, evolution, and diversification. FASEB Journal. 1998;12(12):1075-95.
29. Yana I, Weiss SJ. Regulation of membrane type-1 matrix metalloproteinase activation by proprotein convertases. Molecular Biology of the Cell. 2000;11(7):2387-401.
30. Rozanov DV, Deryugina EI, Ratnikov BI, Monosov EZ, Marchenko GN, Quigley JP, et al. Mutation analysis of membrane type-1 matrix metalloproteinase (MT1-MMP). The role of the cytoplasmic tail Cys(574), the active site Glu(240), and furin cleavage motifs in oligomerization, processing, and self-proteolysis of MT1-MMP expressed in breast carcinoma cells. The Journal of Biological Chemistry. 2001;276(28):25705-14.
31. Golubkov VS, Chekanov AV, Shiryaev SA, Aleshin AE, Ratnikov BI, Gawlik K, et al. Proteolysis of the membrane type-1 matrix metalloproteinase prodomain: implications for a two-step proteolytic processing and activation. The Journal of Biological Chemistry. 2007;282(50):36283-91.
32. Golubkov VS, Cieplak P, Chekanov AV, Ratnikov BI, Aleshin AE, Golubkova NV, et al. Internal cleavages of the autoinhibitory prodomain are required for membrane type 1 matrix metalloproteinase activation, although furin cleavage alone generates inactive proteinase. The Journal of Biological Chemistry. 2010;285(36):27726-36.
33. Mori H, Tomari T, Koshikawa N, Kajita M, Itoh Y, Sato H, et al. CD44 directs membrane-type 1 matrix metalloproteinase to lamellipodia by associating with its hemopexin-like domain. The EMBO Journal. 2002;21(15):3949-59.
34. Atkinson SJ, Roghi C, Murphy G. MT1-MMP hemopexin domain exchange with MT4-MMP blocks enzyme maturation and trafficking to the plasma membrane in MCF7 cells. The Biochemical Journal. 2006;398(1):15-22.

35. Lehti K, Lohi J, Juntunen MM, Pei D, Keski-Oja J. Oligomerization through hemopexin and cytoplasmic domains regulates the activity and turnover of membrane-type 1 matrix metalloproteinase. The Journal of Biological Chemistry. 2002;277(10):8440-8.
36. Seidah NG, Mayer G, Zaid A, Rousselet E, Nassoury N, Poirier S, et al. The activation and physiological functions of the proprotein convertases. The International Journal of Biochemistry & Cell Biology. 2008;40(6-7):1111-25.
37. Itoh Y, Takamura A, Ito N, Maru Y, Sato H, Suenaga N, et al. Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion. The EMBO Journal. 2001;20(17):4782-93.
38. Itoh Y, Ito N, Nagase H, Seiki M. The second dimer interface of MT1-MMP, the transmembrane domain, is essential for ProMMP-2 activation on the cell surface. The Journal of Biological Chemistry. 2008;283(19):13053-62.
39. Cao J, Kozarekar P, Pavlaki M, Chiarelli C, Bahou WF, Zucker S. Distinct roles for the catalytic and hemopexin domains of membrane type 1-matrix metalloproteinase in substrate degradation and cell migration. The Journal of Biological Chemistry. 2004;279(14):14129-39.
40. Dufour A, Sampson NS, Zucker S, Cao J. Role of the hemopexin domain of matrix metalloproteinases in cell migration. Journal of Cellular Physiology. 2008;217(3):643-51.
41. Zarrabi K, Dufour A, Li J, Kuscu C, Pulkoski-Gross A, Zhi J, et al. Inhibition of matrix metalloproteinase 14 (MMP-14)-mediated cancer cell migration. The Journal of Biological Chemistry. 2011;286(38):33167-77.
42. Borrone C, Di Rocco M, Crovato F, Camera G, Gambini C. New multisystemic disorder involving heart valves, skin, bones, and joints in two brothers. American Journal of Medical Genetics. 1993;46(2):228-34.
43. Williams BB, Cantrell VA, Mundell NA, Bennett AC, Quick RE, Jessen JR. VANGL2 regulates membrane trafficking of MMP14 to control cell polarity and migration. Journal of Cell Science. 2012;125(Pt 9):2141-7.
44. Kim HY, Nelson CM. Extracellular matrix and cytoskeletal dynamics during branching morphogenesis. Organogenesis. 2012;8(2):56-64.
45. Datta A, Bryant DM, Mostov KE. Molecular regulation of lumen morphogenesis. Current Biology. 2011;21(3):R126-36.
46. Lubarsky B, Krasnow MA. Tube morphogenesis: making and shaping biological tubes. Cell. 2003;112(1):19-28.
47. Andrew DJ, Ewald AJ. Morphogenesis of epithelial tubes: Insights into tube formation, elongation, and elaboration. Developmental Biology. 2010;341(1):34-55.
48. Marciano DK. A holey pursuit: lumen formation in the developing kidney. Pediatric Nephrology. 2017;32(1):7-20.
49. Hotary K, Allen E, Punturieri A, Yana I, Weiss SJ. Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3. The Journal of Cell Biology. 2000;149(6):1309-23.
50. Chun TH, Sabeh F, Ota I, Murphy H, McDonagh KT, Holmbeck K, et al. MT1-MMP-dependent neovessel formation within the confines of the three-dimensional extracellular matrix. The Journal of Cell Biology. 2004;167(4):757-67.
51. Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, et al. Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(8):4052-7.
52. Happe H, de Heer E, Peters DJ. Polycystic kidney disease: the complexity of planar cell polarity and signaling during tissue regeneration and cyst formation. Biochimica et Biophysica Acta. 2011;1812(10):1249-55.
53. Debnath J, Brugge JS. Modelling glandular epithelial cancers in three-dimensional cultures. Nature Reviews Cancer. 2005;5(9):675-88.

54. Giles RH, Ajzenberg H, Jackson PK. 3D spheroid model of mIMCD3 cells for studying ciliopathies and renal epithelial disorders. Nature Protocols. 2014;9(12):2725-31.
55. Artym VV, Matsumoto KK. Imaging cells in three-dimensional collagen matrix. Current Protocols in Cell Biology. 2010 Sep;Chapter 10:Unit 10.18.1-20.
56. Pohl M, Sakurai H, Bush KT, Nigam SK. Matrix metalloproteinases and their inhibitors regulate in vitro ureteric bud branching morphogenesis. American Journal of Physiology Renal Physiology. 2000;279(5):F891-900.
57. Riggins KS, Mernaugh G, Su Y, Quaranta V, Koshikawa N, Seiki M, et al. MT1-MMP-mediated basement membrane remodeling modulates renal development. Experimental Cell Research. 2010;316(17):2993-3005.
58. Ganceviciene R, Zouboulis CC. Isotretinoin: state of the art treatment for acne vulgaris. Journal der Deutschen Dermatologischen Gesellschaft. 2010;8 Suppl 1:S47-59.
59. Zouboulis CC. Isotretinoin revisited: pluripotent effects on human sebaceous gland cells. The Journal of Investigative Dermatology. 2006;126(10):2154-6.
60. Santagati F, Rijli FM. Cranial neural crest and the building of the vertebrate head. Nature Reviews Neuroscience. 2003;4(10):806-18.
61. Mayor R, Theveneau E. The neural crest. Development (Cambridge, England). 2013;140(11):2247-51.
62. Cordero DR, Brugmann S, Chu Y, Bajpai R, Jame M, Helms JA. Cranial neural crest cells on the move: their roles in craniofacial development. American journal of medical genetics Part A. 2011;155a(2):270-9.
63. Halloran MC, Berndt JD. Current progress in neural crest cell motility and migration and future prospects for the zebrafish model system. Developmental Dynamics. 2003;228(3):497-513.
64. Christian L, Bahudhanapati H, Wei S. Extracellular metalloproteinases in neural crest development and craniofacial morphogenesis. Critical Reviews in Biochemistry and Molecular Biology. 2013;48(6):544-60.
65. Erickson CA, Perris R. The role of cell-cell and cell-matrix interactions in the morphogenesis of the neural crest. Developmental Biology. 1993;159(1):60-74.
66. Sabeh F, Ota I, Holmbeck K, Birkedal-Hansen H, Soloway P, Balbin M, et al. Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. The Journal of Cell Biology. 2004;167(4):769-81.
67. Yelick PC, Schilling TF. Molecular dissection of craniofacial development using zebrafish. Critical Reviews in Oral Biology and Medicine. 2002;13(4):308-22.
68. Kague E, Gallagher M, Burke S, Parsons M, Franz-Odendaal T, Fisher S. Skeletogenic fate of zebrafish cranial and trunk neural crest. PloS One. 2012;7(11):e47394.
69. Schilling TF, Kimmel CB. Segment and cell type lineage restrictions during pharyngeal arch development in the zebrafish embryo. Development. 1994;120(3):483-94.
70. Witten PE, Huysseune A. A comparative view on mechanisms and functions of skeletal remodelling in teleost fish, with special emphasis on osteoclasts and their function. Biological Reviews of the Cambridge Philosophical Society. 2009;84(2):315-46.
71. Zhang J, Bai S, Zhang X, Nagase H, Sarras MP, Jr. The expression of novel membrane-type matrix metalloproteinase isoforms is required for normal development of zebrafish embryos. Matrix Biology. 2003;22(3):279-93.
72. Wyatt RA, Keow JY, Harris ND, Hache CA, Li DH, Crawford BD. The zebrafish embryo: a powerful model system for investigating matrix remodeling. Zebrafish. 2009;6(4):347-54.
73. Taylor JS, Braasch I, Frickey T, Meyer A, Van de Peer Y. Genome duplication, a trait shared by 22000 species of ray-finned fish. Genome Research. 2003;13(3):382-90.
74. Coyle RC, Latimer A, Jessen JR. Membrane-type 1 matrix metalloproteinase regulates cell migration during zebrafish gastrulation: evidence for an interaction with non-canonical Wnt signaling. Experimental Cell Research. 2008;314(10):2150-62.

75. Janssens E, Gaublomme D, De Groef L, Darras VM, Arckens L, Delorme N, et al. Matrix metalloproteinase 14 in the zebrafish: an eye on retinal and retinotectal development. PloS One. 2013;8(1):e52915.
76. Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degrave, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X.Q. and Thisse, C. (2001) Expression of the zebrafish genome during embryogenesis (NIH R01 RR15402). ZFIN Direct Data Submission. URL: http://zfin.org.
77. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Developmental Dynamics. 1995;203(3):253-310.
78. Schilling TF. The morphology of larval and adult zebrafish. Zebrafish. 2002;261:59-94.
79. Mackay EW, Apschner A, Schulte-Merker S. A bone to pick with zebrafish. BoneKEy Reports. 2013;2:445.
80. Boer EF, Jette CA, Stewart RA. Neural Crest Migration and Survival Are Susceptible to Morpholino-Induced Artifacts. PloS One. 2016;11(12):e0167278.
81. Parichy DM, Elizondo MR, Mills MG, Gordon TN, Engeszer RE. Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. Developmental Dynamics. 2009;238(12):2975-3015.
82. Nieto MA. The early steps of neural crest development. Mechanisms of Development. 2001;105(1-2):27-35.
83. Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, et al. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell. 1999;99(1):81-92.
84. Tang Y, Rowe RG, Botvinick EL, Kurup A, Putnam AJ, Seiki M, et al. MT1-MMP-dependent control of skeletal stem cell commitment via a beta1-integrin/YAP/TAZ signaling axis. Developmental Cell. 2013;25(4):402-16.
85. Chan KM, Wong HL, Jin G, Liu B, Cao R, Cao Y, et al. MT1-MMP inactivates ADAM9 to regulate FGFR2 signaling and calvarial osteogenesis. Developmental Cell. 2012;22(6):1176-90.
86. Hikita A, Yana I, Wakeyama H, Nakamura M, Kadono Y, Oshima Y, et al. Negative regulation of osteoclastogenesis by ectodomain shedding of receptor activator of NF-kappaB ligand. The Journal of Biological Chemistry. 2006;281(48):36846-55.