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Novel therapeutic interventions for pseudoachondroplasia

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Abstract

Pseudoachondroplasia (PSACH), a severe short-limbed dwarfing condition, is associated with life-long joint pain and early onset osteoarthritis. PSACH is caused by mutations in cartilage oligomeric matrix protein (COMP), a pentameric matricellular protein expressed primarily in cartilage and other musculoskeletal tissues. Mutations in COMP diminish calcium binding and as a result perturb protein folding and export to the extracellular matrix. Mutant COMP is retained in the endoplasmic reticulum (ER) of growth plate chondrocytes resulting in massive intracellular COMP retention. COMP trapped in the ER builds an intracellular matrix network that may prevent the normal cellular clearance mechanisms. We have shown that accumulation of intracellular matrix in mutant-COMP (MT-COMP) mice stimulates intense unrelenting ER stress, inflammation and oxidative stress. This cytotoxic stress triggers premature death of growth plate chondrocytes limiting long-bone growth. Here, we review the mutant COMP pathologic mechanisms and anti-inflammatory/antioxidant therapeutic approaches to reduce ER stress. In MT-COMP mice, aspirin and resveratrol both dampen the mutant COMP chondrocyte phenotype by decreasing intracellular accumulation, chondrocyte death and inflammatory marker expression. This reduction in chondrocyte stress translates into an improvement in long-bone growth in the MT-COMP mice. Our efforts now move to translational studies targeted at reducing the clinical consequences of MT-COMP and painful sequelae associated with PSACH.

Keywords

Anti-inflammatory; Antioxidant; Matricellular protein

1. Pseudoachondroplasia – the skeletal dysplasia

The first clinical and radiographic description of pseudoachondroplasia (PSACH) was reported in 1959 [1]. Since then, numerous studies of PSACH provide a comprehensive understanding of the natural history of the disorder [2–9]. PSACH babies are indistinguishable from other newborns during the first year of life because they have a normal birth length and weight. Diminished linear growth and/or a waddling gait are the first signs that alert the health care practitioner and/or parents that there is a growth problem.

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Radiographic examination leads to a diagnosis by the age of 18–24 months based on characteristic x-ray findings including shortening of all the long bones, small abnormal epiphyses, widened and irregular metaphyses, small, underossified capital femoral epiphyses and platyspondyly [3,7,9–11]. During childhood, limb shortening, brachydactyly, widened joints and joint laxity become obvious and lower limb abnormalities develop, ranging from genu varus to genu valgum or a combination of both [9,11]. Lower extremity abnormalities generally require surgical interventions (osteotomies); the timing of the procedures depends on the extent of joint laxity and the degree of deformity. The average adult height is 3′9″–3′11″ which is equivalent to the height of an average 6 year old (https://ghr.nlm.nih.gov/condition/pseudoachondroplasia). However, stature is variable with some being as tall as 4′10″. Early onset osteoarthritis occurs in young adults and produces significant discomfort. This affects all the major joints necessitating joint replacements usually starting with hip replacements in the second to third decades [4,7,11,12]. A recent natural history study found that pain starts in early childhood and is a significant problem for which there is no systematic or standard pain treatments [9,13]. Chronic pain, the most debilitating feature of PSACH, compromises mobility ultimately limiting physical activity and quality of life [7].

PSACH is an autosomal dominant disorder, that occurs as a (*de novo*) new event in 70–80% of families with the remaining cases being inherited from an affected parent [10,13,14]. Although autosomal recessive inheritance was reported based on recurrence in siblings of unaffected parents, these cases were subsequently shown to result from germline mosaicism. Affected individuals have a 50% risk of passing the mutation to their offspring in each pregnancy and prenatal diagnosis is available using molecular testing. Prenatal ultrasound will not detect PSACH since skeletal abnormalities develop postnatally overtime. Prenatal molecular diagnosis will establish affection status for familal cases.

2. Mutations in cartilage oligomeric matrix protein (COMP) cause PSACH

PSACH was first described as an rough endoplasmic reticulum (rER) storage disorder in 1972 based on electron micrography studies of iliac crest biopsies [3,15,16]. These studies revealed retention of a lamellar-appearing material in massively dilated ER cisternae of growth plate chondrocytes [17–24]. In 1995, mutations in COMP were shown to cause PSACH and the stored ER material was identified as COMP [10, 15]. Since then, more than 200 mutations have been identified with ≅99% found in the the highly conserved calcium-binding repeat domains indicating that this domain is extremely sensitive to genetic alterations (LOVD Mendelian genes https://grenada.lumc.nl/LOVD2/mendelian_genes/variants_statistics.php) [9,25–27]. Approximately 30% of cases result from deletion of one of five sequential aspartic acid residues at position 469–473 and is denoted as the D469del mutation [10].

COMP is a homopentameric protein that has a bouquet appearance on rotary shadowing with the *N*-terminal domain joining the five subunits [28]. Each COMP monomer has four distinct domains: *N*-terminal pentamerization domain, epidermal growth factor (EGF)-like domain (four repeats), a type 3 calcium-binding domain (7 repeats) and a C-terminal lectin-like globular region [29]. Mutations in the calcium-binding domain interfere with the number of bound calciums, which is predicted to disrupt protein folding of each arm thereby

having a dominant negative effect on the protein [30]. Chondrocytes from three PSACH patients with different mutations, [D469del, G427E and D511Y], all show similar intracellular retention of COMP in the endoplasmic reticulum [31,32]. Crystallographic studies show that the type 3 calcium binding domain wraps around the calcium metal scaffold in a unique 3D structure and mutations in this domain are predicted to cause a local collapse of the 3D structure [33]. Indeed, functional studies confirm that mutations disrupt calcium binding and protein folding with the mutant arms measuring longer that the wild type arms [28].

3. Function of COMP

COMP was first isolated from cartilage and thought to be cartilage-specific but was later shown to be relatively abundant in other musculoskeletal tissues such as tendon, ligament and smooth muscle [18,29, 34–43]. COMP is a secreted glycoprotein found in extracellular matrices (ECM) and is best characterized in the pericellular and territorial matrices surrounding growth plate and articular chondrocytes [21,40]. COMP, the fifth member of the thrombospondin gene family; also designated as, TSP-5, binds to a number of proteins in the ECM with the C-terminal domain a hub for interaction(s) with other ECM proteins [44–46]. For example, COMP binds to matrillins-1, –3 and –4 (MATN) [19,45], interacts with glycosaminoglycan (GAG) side chains of aggrecan within the type 3 repeats and the C-terminal domain [47]. The C-terminal globular domain binds to types I, II, IX, XII and XIV collagens [44,46,48,49] and may enhance mechanical strength of the ECM and serve as anchor plaques/points for protein complexes. COMP has also been shown, *in vitro*, to increase the speed at which type II collagen fibrils assemble by potentially positioning free collagen molecule close to each other [50]. Other work suggests that the interaction of COMP with collagens organizes the collagen ECM network (fibril morphology and density) and that COMP is critical for collagen secretion [51]. Interestingly, this observation is consistent with iliac crest growth plate and ligament PSACH biopsies showing varied collagen fibril structure, diameters [52] and fused fibrils [53]. Moreover, a recent study of COMP null mice showed showing alterations in collagen fibrils in skin and tendon [51] while an earlier study did not [54]. These studies support the idea that collagen assembly in the matrix is affected by the absence of COMP in the matrix either by knocking it out or because of retention within the chondrocytes.

Fibronectin binds to integrins, which are anchored in the cellular membrane, and fibronectin also interacts with COMP at the C-terminal domain [55]. COMP directly facilitates chondrocyte attachment through interaction specifically with α5β1 and α5β3 integrins [56]. The interaction of these integrins with the RGD sequence in the calcium binding repeats are critical for chondrocyte attachment [56]. Granulin epithelin precursor (GEP), a growth factor, interacts with the EGF domain of COMP and this interaction stimulates chondrocyte proliferation [57]. The precise function of the EGF domain in this context is unclear but the domain includes six cysteine residues involved in disulphide bonds which are common in secreted proteins (http://smart.embl.de/smart/do_annotation.pl?DOMAIN=SM00181). Collectively these findings suggests that COMP may be a structural component of cartilage also involved in regulating chondrocyte function.

The specific role of COMP in different tissues is not well defined. Weight bearing equine tendons produce more COMP than non-weight bearing ones suggesting that COMP may play a role in withstanding mechanical stress consistent with the presence of a mechanosensitive region in the promoter [58]. In contrast, COMP null mice manifest only minor alterations in articular cartilage with exercise [59]. However, COMP null mice have a diminished skin fibrotic response during healing [51] and enlarged atherosclerotic plaques [60]. Moreover, COMP expression is up-regulated during human skin healing of photodamage and many fibrotic conditions of the skin, liver and lung suggesting that COMP may be an important component in fibrosis regardless of whether the fibrotic process is a component of healing or pathology [51,61–67].

4. Towards understanding the PSACH chondrocyte pathology

4.1. Mutant (MT-COMP) mouse

The bigenic MT-COMP or (wild-type) WT-COMP mice express COMP in response to doxycycline (DOX) administration and were generated from breeding two mouse lines (Fig. 1). The TRE-COMP transgenic mouse line contains the coding sequence of human COMP gene with FLAG-tag driven by the tetracycline responsive element (TRE) promoter. The TET-On-Col II mouse expresses the transactivator protein (rtTA) driven by a type II collagen promoter [68]. Western blot analysis confirmed protein expression in cartilage (COMP and FLAG antibodies) and tissue specificity of expression was validated using RTPCR [69].

Limb length and intracellular retention of MT-COMP in growth plate chondrocytes was assessed in MT-COMP mice administered DOX (500 ng/ml) pre- and postnatally [69]. Consistent with the human PSACH phenotype, newborn MT-COMP and control mice have normal birth parameters. By 1 week postnatal life, MT-COMP mice are smaller than controls and the difference in size becomes more evident with age with obvious short limbs by 2 weeks of age (Fig. 2) [70]. In contrast, WT-COMP mice show no phenotypic difference from control mice and limb lengths are equivalent (Fig. 3). MT-COMP mice remain smaller than control mice throughout adulthood [71]. Intracellular retention of MT-COMP in growth plate chondrocytes is a progressive process beginning in the prenatal period (E15), when only a small number of growth plate chondrocytes showing MT-COMP in the ER [72]. Intracellular retention increases over time, peaking at P21 with almost every chondrocyte containing MT-COMP [72]. The progressive nature of intracellular retention and the accompanying chondrocyte death in the growth plate mirror the progressive loss of long-bone growth.

4.2. COMP mutations cause inflammatory processes that triggers chondrocyte death

WT-COMP monomeric subunits are immediately assembled into a pentameric protein followed by rapid folding of the five arms and transport to Golgi for posttranslational modification and finally export to ECM [73]. In contrast, pentameric proteins with mutant subunits cannot fold correctly and are retained in the ER creating dominant negative effects [17,20]. Mutant COMP stalled in the ER appears to participate in premature intracellular assembly of extracellular matrix composed of MT-COMP, types IX and II collagens and matrilin-3 (MATN3) as well as other ECM proteins [32,69,74]. Adding a BM40 signal

peptide hastens the rate at which mutant COMP moves through the ER, decreasing intracellular accumulation and resulting in a very mild phenotype in mice [75–77]. It is likely that the unique repertoire of proteins synthesized by chondrocytes promotes intracellular matrix formation. Interestingly, some studies have suggested that MT-COMP is secreted in tendon, ligament and COS-7 (green monkey kidney cells) which would likely alter the pathological consequences of MT-COMP and manifest in poor matrix quality [17,18,23,78]. In chondrocytes, the stalled COMP and intracellular COMP matrix activates the unfolded protein response (UPR), which functions to either refold or degrade the misfolded protein [17, 20]. Misfolded proteins in the ER that cannot be refolded should be transported to the cytosol by retrotranslocation where ubiquitin is added and the protein is degraded by the proteasome [79] (Fig. 4A). We have previously shown that this mechanism fails [32,69]. This failure likely results because the MT-COMP intracellular matrix complex may not be able to fit through the pore in the ER preventing clearance of MT-COMP from the ER (Fig. 4B). Using deconvolution microscopy, we have shown intracellular matrix in the ER of MT-COMP mice and human PSACH chondrocytes indicating that this complex may be blocking translocation from the ER (Fig. 4C). Moreover, the reduction of ubiquitination in the MT-COMP growth plate supports this theory (Fig. 4D–E). The presence of intracellular matrix may explain why the UPR clearance mechanisms are unable to prevent the massive intracellular protein accumulation in PSACH growth plate chondrocytes [3,9,11, 12,18,20–22].

We have demonstrated that CHOP/DDIT3 (CCAAT-enhancer-binding protein homologous protein/DNA-Damage-Inducible Transcript 3), a component of the UPR, plays an essential role in MT-COMP chondrocyte pathology [72,80]. CHOP is a multifunctional transcription factor involved in sensing ER stress [81]. CHOP is stimulated through the activation of PRKR-like ER kinase (PERK) one of the three branches of the unfolded protein response (UPR) [82]. Chronic activation of CHOP results in pathophysiological conditions that promote cell death by restarting protein translation and generating reactive oxygen species (ROS) [81]. CHOP restarting protein translation can generate additional stress in the ER [81]. MT-COMP expressed in rat chondrosarcoma cells (RCS) stimulated CHOP and restarted protein translation as monitored by changes in the phosphorylation state of translation initiation factor 2 (eIF2α) [80]. CHOP also activates an ER oxidase (ERO1), which generates hyper-oxidizing environment and stimulates oxidative stress [81, 83]. In the MT-COMP mice, a number of mRNAs involved in oxidative stress including ERO1 are upregulated and we have demonstrated that RCS cells expressing MT-COMP have excessive oxidative stress by measuring the conversion of a fluorescent probe by ROS [72,80]. In the MT-COMP mice, the restart of general protein translation stimulates additional ER stress and the oxidizing environment of the ER leads to more ER stress, oxidative stress and an inflammatory process.

The inflammatory processes, in the MT-COMP mice, were identified and characterized by transcriptome profiling, RTPCR analysis and immunostaining [70–72]. In the MT-COMP growth plate, IL1 and TNFα are induced by IL16 and IL18 [70]. The pro-inflammatory cytokine, IL-16, attracts eosinophils in cooperation with CCR5 [84,85] and this is the origin of the increase in eosinophil-related mRNAs in the MT-COMP growth plate [70]. Our findings confirm similar reports showing that inflammation can induce both oxidative and

ER stress [83,86,87]. As shown in Fig. 5, chronic ER stress, inflammation, oxidative stress and DNA damage drive MT-COMP growth plate chondrocytes to necroptosis, a programmed form of necrosis stimulated by inflammation [72]. This becomes a self-perpetuating pathological process with each cellular stress exacerbating the others [83,88–91]. The relentless stress depletes the pool of growth plate chondrocytes necessary for long-bone growth and results in a dwarfed phenotype in the MT-COMP mice similar to that observed in humans.

4.3. Therapeutic interventions

Our first therapeutic approach to treat PSACH in our MT-COMP mouse was administration of ER stress reducing, drugs valproate, lithium and phenylbutyric acid from birth until 4 weeks after birth. While all three drugs decreased intracellular MT-COMP retention, chondrocyte death and inflammatory markers, these drugs were not well tolerated by either control or MT-COMP mice and caused reduced limb length and in some cases reduced viability [70]. Given the negative side effects of ER stress reduction drugs, we next focused on interrupting the pathological loop between inflammation, ER and oxidative stresses in MT-COMP chondrocytes [92]. This treatment approach is supported by other work that achieved successful reduction of ER stress with antioxidant therapy (butylated hydroxyanisole) [87] and NSAIDS treatments (diclofenac, indomethacin, ibuprofen, aspirin or ketoprofen) [93]. MT-COMP mice were treated with aspirin, ibuprofen, resveratrol, GSE, turmeric or CoQ10 from birth to 4 weeks [92]. Each of these therapies produced dampening of the MT-COMP chondrocyte phenotype [92]. Aspirin and resveratrol treatment dramatically reduced intracellular retention of MT-COMP in growth plate chondrocytes (Fig. 6A–D) resulting in less chondrocyte death as demonstrated by decreased TUNEL staining (Fig. 6E–H) and decreased TNFα, IL-1β, OSM (oncostatin M) and IL-16, all markers of inflammatory process associated with MT-COMP chondrocyte pathology (Fig. 6I–X). IL-16 has been implicated in the risk for knee OA [94] and OSM expression is associated with OA [95] suggesting that this inflammatory process elicited by MT-COMP may play a role in early joint erosion associated with PSACH. These antioxidant/anti-inflammatory treatments restored chondrocyte proliferation in MT-COMP growth plates, and most importantly, femoral length increased with aspirin or resveratrol treatment compared to the untreated MT-COMP mouse [92]. Grape see extract (GSE), turmeric and CoQ10 (antioxidants) reduced intracellular retention of MT-COMP to a lesser extent than aspirin or resveratrol [92]. Interestingly, some extracellular MT-COMP was observed with resveratrol and turmeric treatments [92] suggesting that decreases in retention with drug treatments are in part due to some export of MT-COMP. Treatments with GSE, CoQ10, and turmeric reduced TUNEL staining to a lesser extent than aspirin or resveratrol treatments in the MT-COMP growth plates but caused disorganization in control growth plates compared to untreated controls [92]. The alteration in chondrocyte organization makes these therapies less desirable than aspirin or resveratrol.

In a second approach, we tested whether systemic administration would deliver COMP targeted antisense oligonucleotides (ASO) to growth plate chondrocytes and decrease MT-COMP growth plate chondrocyte pathology [96]. We found that ASO1 was successfully delivered reducing steady-state levels of cartilage oligomeric matrix protein mRNA

dampening intracellular retention of MT-COMP. Interestingly, although ASO1 was designed against human COMP, mutant human COMP mRNA was reduced 38% and wild-type mouse COMP mRNA was reduced 60% [96]. Reduction in COMP retention in chondrocytes, led to a reduction of inflammatory markers, interleukin-16 (IL-16) and chitinase-like 3 (Chil3/YM1 a macrophage protein), the number of TUNEL positive chondrocytes, and a partial restoration of DNA proliferation [96]. These exciting novel results demonstrate that ASOs can be delivered and knock down mRNAs in growth plate and articular cartilages and open the door for a new antisense treatment approach for PSACH and potentially other human cartilage disorders.

PSACH is caused by different heterozygous COMP mutations (the vast majority in the type 3 calcium binding repeats) that all initiate and perpetuate dominant negative effect. Based on our findings that MT-COMP intracellular accumulation elicits chronic ER stress stimulating inflammation and oxidative stress and thereby driving the chondrocytes to necroptosis [70,72], we have identified avenues for a mechanistic-based therapeutic approach in the MT-COMP mice [92]. We show two different anti-inflammatory/antioxidant therapeutics that interrupt the MT-COMP stimulated feed-forward stress loop in growth plate chondrocytes. Suppressing cellular stress in chondrocytes improves chondrocyte longevity most likely by reducing the pressure on the ER thereby permitting normal UPR clearance mechanisms to function. This exciting therapeutic approach using over-the-counter medications, which are easy to access, administer and are well tolerated have the potential to greatly improve the quality of life for PSACH individuals. Importantly, the time between preclinical studies and clinical use is abbreviated for over-the-counter medications especially those that are well tolerated. This is an advantage for future trials in PSACH. Additionally using a knock-down approach, we demonstrate a reduction in PSACH chondrocyte pathology by eliminating the source of the dominant negative effect. The latter approach is novel and demonstrates that ASOs can reach the relatively avascular growth plate. Using multiple approaches, we show that reduction of PSACH chondrocyte pathology can be accomplished in our murine model. These therapeutic approaches are a significant advance towards developing human PSACH treatment targeted at alleviating the chronic joint pain that diminishes quality of life and mobility. Our findings set the foundation for testing a wide variety of drugs and therapeutic approaches including antisense technology for PSACH and other cartilage-related conditions.

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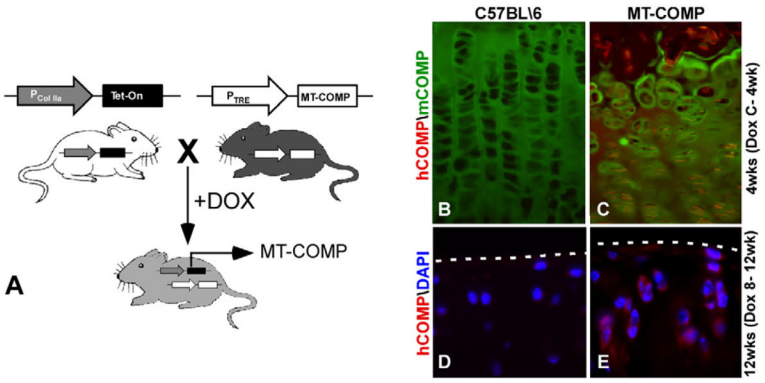

Fig. 1.

MT-COMP transgenic mouse. (A) MT-COMP bigenic mouse line was generated using the Tet-On system with the tetracycline responsive element (TRE) driving human D469del-MT-COMP expression. The type II collagen promoter (Col IIa) drives rtTA (Tet-On) expression in chondrocytes. (B) In the presence of doxycycline (DOX), the bigenic MT-COMP mouse expresses mutant human D469del-COMP in growth plate and articular chondrocytes as visualized using human-specific COMP antibody. MT-COMP (hCOMP in red) expression is detected in the murine growth plate chondrocytes at 4 weeks with administration of DOX from conception to 4 weeks (C-4wk) (C), whereas mouse COMP (mCOMP in green) is primarily extracellular (B and C). Similarly, MT-COMP is found in articular cartilage chondrocytes of 12 week mice administered DOX from 8 to 12 weeks (E) but not in controls (D). The articular cartilage border is marked by a dashed line. DAPI staining of nuclei are shown in blue (D and E).

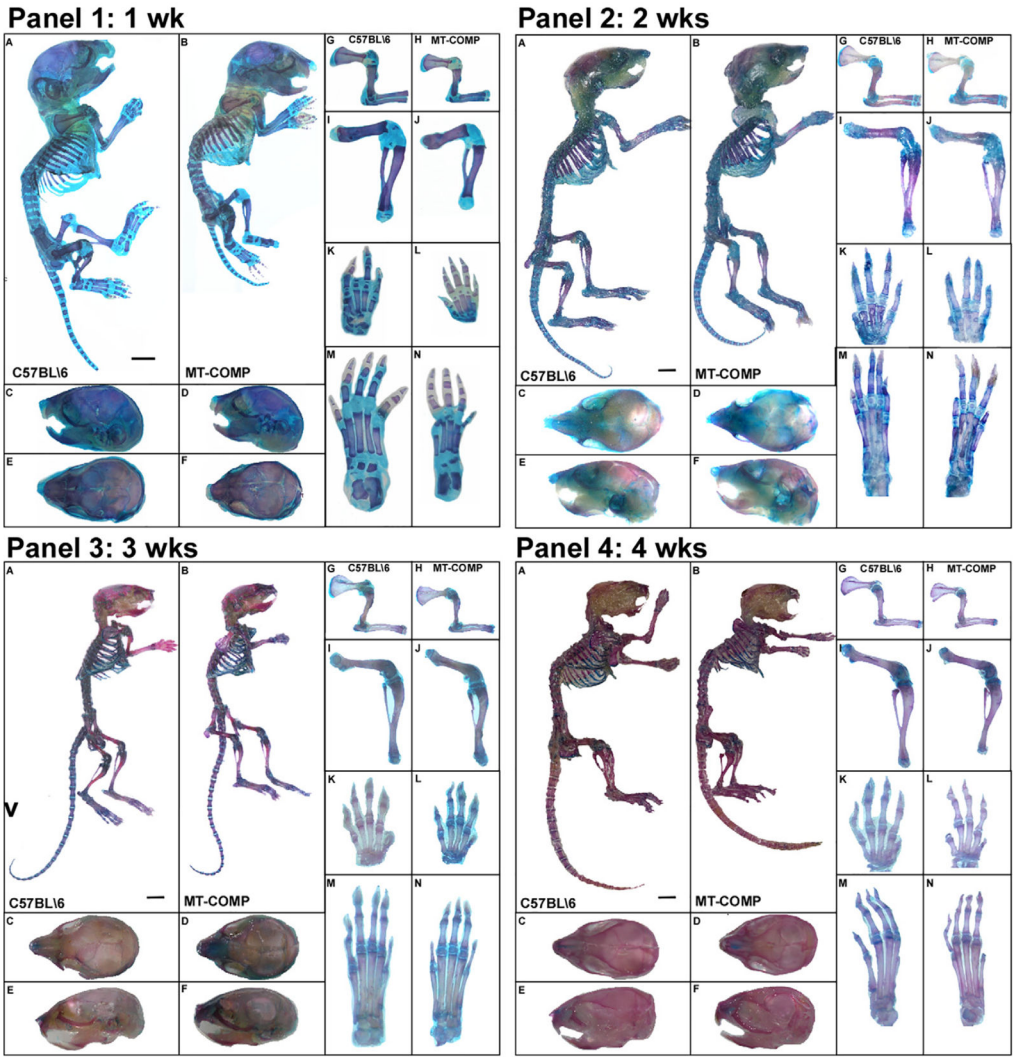

Fig. 2.

MT-COMP causes skeletal growth retardation. MT-COMP and control (C57BL\6) mice are compared in all panels. Skeletons were stained with alizarin (red) and alcian blue to visualize bone and cartilage, respectively. MT-COMP mice are shorter than controls by P7 (Panel 1) and remain shorter into adulthood [71] (Panels 2–4). All long bones and paws are markedly reduced (G–N Panels 1–4). Skull lengths are reduced primarily due to a decrease in the snout length. Adapted from Posey 2014 [70]. (Bar = 0.1 cm).

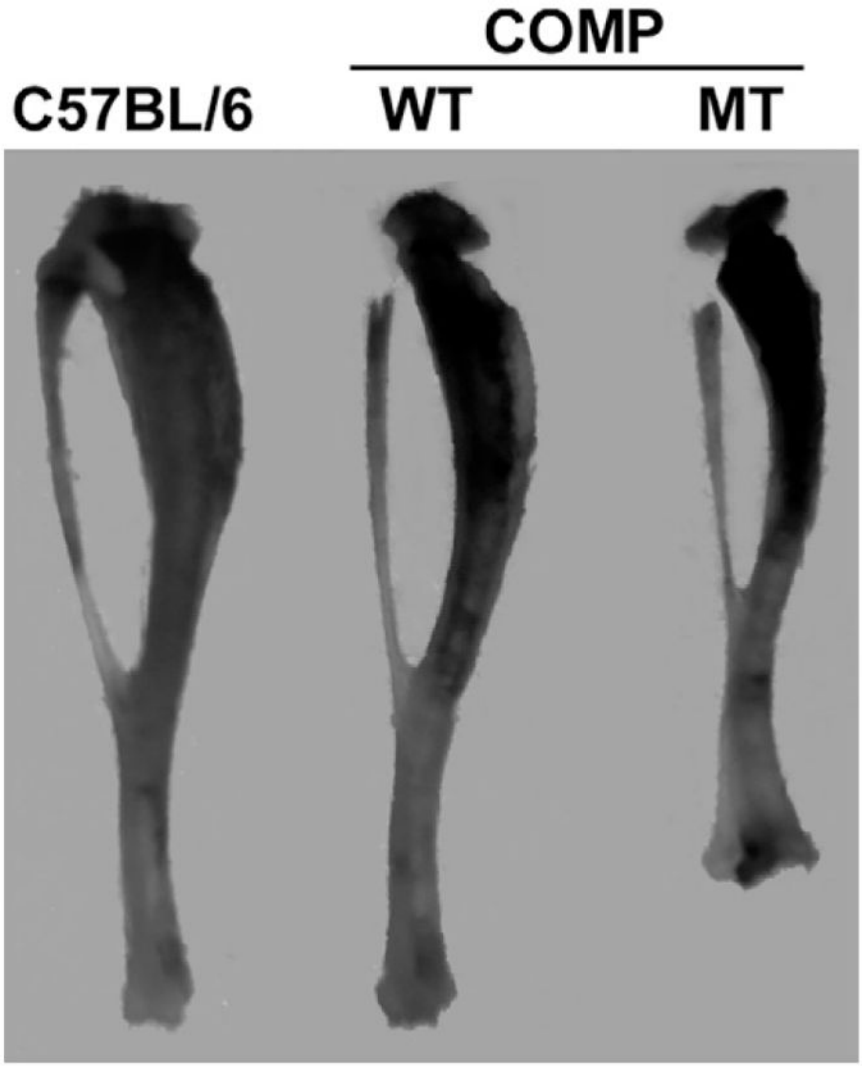

Fig. 3.

MT-COMP tibias are abnormally modeled. MT-COMP tibias are short and have metaphyseal flaring similar to that seen in PSACH. Overexpression of WT-COMP does not affect long bone morphology or length.

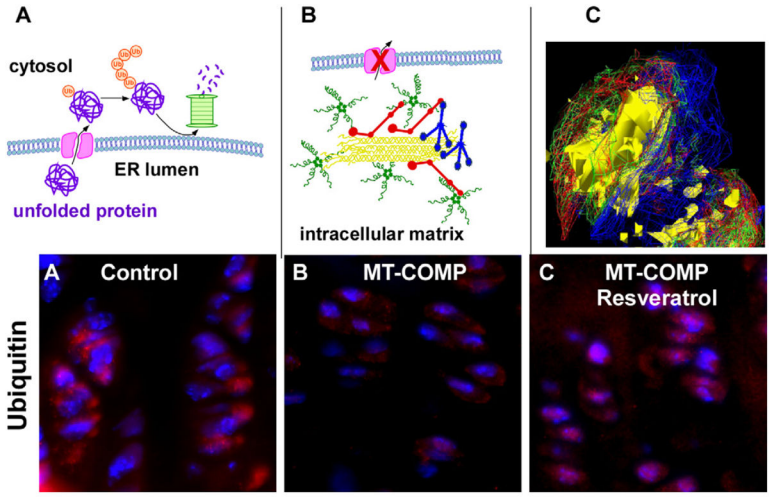

Fig. 4.

MT-COMP promotes the assembly of intracellular ER network in chondrocytes. (A) Schematic model showing how misfolded proteins in the ER are exported to the cytosol by retrotranslocation where ubiquitin (Ub) is added signaling proteasomal degradation [79]. (B) Model depicting how retained MT-COMP interacts with other matrix proteins in the ER generating a matrix network that is likely not able to fit through the retrotranslocation ER pore that leads to the cytosol. (C) Deconvolution microscopy shows intracellular matrix in chondrocyte ER composed of MT-COMP (green), matrilin-3 (blue), types 2 (yellow) and 9 (red) collagens. Adapted from Posey 2009 [69]. Ubiquitin immunohistochemistry of control (D), MT-COMP (E) and resveratrol treated MT-COMP growth plate chondrocytes (F).

Fig. 5.

PSACH chondrocyte death results from intense cellular stress. Schematic showing that MT-COMP intracellular ER retention stimulates ER stress through CHOP-mediated UPR generating an inflammatory response. Prolonged ER stress also produces excessive ROS (reactive oxygen species) causing oxidative stress that further contributes to the inflammatory process and creates a self-perpetuating stress loop in chondrocytes. This results in the death of growth plate chondrocytes, which translates into diminished long-bone growth and skeletal dysplasia.

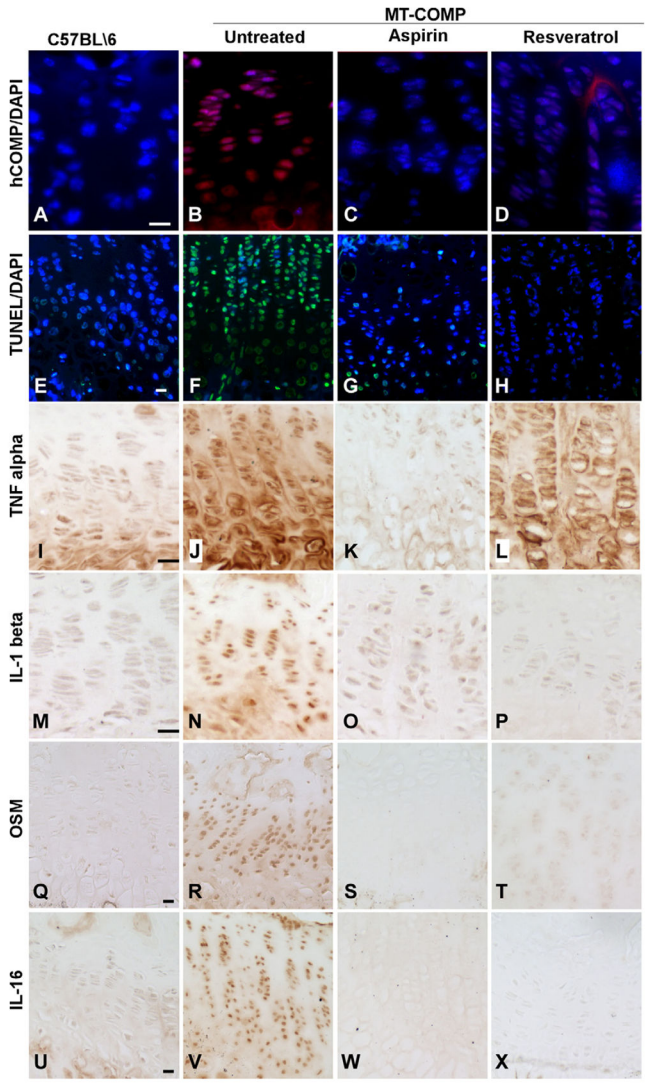

Fig. 6.

Anti-inflammatory and antioxidant treatments normalize MT-COMP growth plates. MT-COMP and control mice were treated with either aspirin or resveratrol from birth to 4 weeks and hind limbs were collected for analysis. Human-specific COMP antibody assessed ER retention in mouse growth plates chondrocytes. C57BL\6 growth plate chondrocytes were negative control because transgenic human MT-COMP is not present (A). Untreated MT-COMP growth plate chondrocytes show intracellular human COMP (B) and retention is decreased by aspirin or resveratrol (C–D). TUNEL assessment was used to assess cell death in growth plates. Basal level of apoptosis in the C57BL\6 growth plate is limited to a few hypertrophic chondrocytes (E). Most MT-COMP chondrocytes are TUNEL positive in the absence of treatment (F) but aspirin or resveratrol treatments showed reduced the number of TUNEL positive (green) chondrocytes (G and H). Inflammation markers, TNFα, IL-1β, OSM and IL-16, were assessed by immunostaining. TNFα is low in controls (I), elevated in MT-COMP growth plates (J) and decreased by aspirin and to a lesser degree resveratrol treatments (K and L). IL-1β signal shows a similar pattern of expression, low levels in

control (M) and treated MT-COMP (O and P) and elevated in untreated MT-COMP (N). OSM, a cytokine associated with OA, is elevated in untreated (R) and decreased in control (Q) and MT-COMP treated mice (S and T). IL-16, showed similar findings as other inflammatory markers (U–X). Adapted from Posey 2014 [70,71].