



MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

Novel mTORC1 Mechanism Suggests Therapeutic Targets for COMPopathies



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Accepted for publication
September 17, 2018.

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Cartilage oligomeric matrix protein (COMP) is a large, multifunctional extracellular protein that, when mutated, is retained in the rough endoplasmic reticulum (ER). This retention elicits ER stress, inflammation, and oxidative stress, resulting in dysfunction and death of growth plate chondrocytes. While identifying the cellular pathologic mechanisms underlying the murine mutant (MT)-COMP model of pseudoachondroplasia, increased midline-1 (MID1) expression and mammalian target of rapamycin complex 1 (mTORC1) signaling was found. This novel role for MID1/mTORC1 signaling was investigated since treatments shown to repress the pathology also reduced Mid1/mTORC1. Although ER stress—inducing drugs or tumor necrosis factor α (TNF α) in rat chondrosarcoma cells increased *Mid1*, oxidative stress did not, establishing that ER stress— or TNF α -driven inflammation alone is sufficient to elevate MID1 expression. Since MID1 ubiquitinates protein phosphatase 2A (PP2A), a negative regulator of mTORC1, PP2A was evaluated in MT-COMP growth plate chondrocytes. PP2A was decreased, indicating de-repression of mTORC1 signaling. Rapamycin treatment in MT-COMP mice reduced mTORC1 signaling and intracellular retention of COMP, and increased proliferation, but did not change inflammatory markers IL-16 and eosinophil peroxidase. Lastly, mRNA from tuberous sclerosis—1/2-null mice brain tissue exhibiting ER stress had increased Mid1 expression, confirming the relationship between ER stress and MID1/mTORC1 signaling. These findings suggest a mechanistic link between ER stress and MID1/mTORC1 signaling that has implications extending to other conditions involving ER stress. (*Am J Pathol* 2019, 189: 132–146; <https://doi.org/10.1016/j.ajpath.2018.09.008>)

Cartilage oligomeric matrix protein (COMP) is a large, pentameric extracellular matrix protein that is a member of the thrombospondin gene family. These proteins have adhesive properties that are known to mediate cell–cell and cell–matrix interactions.^{1–7} Reported functions of COMP include: i) production, maintenance, and homeostasis of cartilage, ii) regulation of type II collagen fibril assembly, and iii) enhancement of chondrocyte attachment and proliferation.^{8–14} Interestingly, more is known about the results of mutant (MT)-COMP in growth plate chondrocytes than the functions of wild-type COMP. Mutations in COMP cause pseudoachondroplasia (PSACH), a severe dwarfing condition characterized by disproportionate short stature, joint laxity, pain, and early-onset osteoarthritis.^{2,10,15–36} PSACH is not evident at birth, being identified by 2 years

of age, when slow linear growth leads to diagnosis. The hallmark of PSACH growth plate chondrocytes is massive retention of MT-COMP in rough endoplasmic reticulum (ER) cisternae.²⁰ MT-COMP prematurely assembles into an ordered matrix composed of types II and IX collagen and matrilin-3 and other extracellular matrix proteins, resulting in intracellular protein accumulation.^{30,37,38} Approximately 97% of COMP pentamers are predicted to contain at least

Supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH grant RO1-AR057117-05 (J.T.H. and K.L.P.); and the Leah Lewis Family Foundation (J.T.H.).

Disclosures: None declared.

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one mutant subunit causing a dominant negative effect that results in the protein being trapped in the ER.³⁹ Ultimately, the rough ER of affected chondrocytes enlarge, filling the cytoplasmic space and being cytotoxic to growth plate chondrocytes.^{2,20,32,34}

Previously, a doxycycline-inducible mouse model that expresses MT-COMP (D469del) in growth plate chondrocytes, and that recapitulates the clinical phenotype and chondrocyte pathology of PSACH, was generated, validated, and used to identify the pathologic mechanisms.^{38,40,41} Those studies showed that the retention of MT-COMP induced ER stress, initiating a self-perpetuating stress loop involving oxidative stress and inflammation, which led to DNA damage, necroptosis, and loss of growth plate chondrocytes.^{40–42} Although intracellular retention was detected as early as embryonic day 15, the loss of growth plate chondrocytes affected only postnatal long bone growth.^{38,41} By postnatal day (P) 14, COMP intracellular retention stimulated detectable chondrocyte loss that peaked between P21 and P28, when inflammation and oxidative stress are also at their highest levels.^{38,41}

A number of novel observations related to changes in mRNA expression were made during the investigation of the development of the pathology in the MT-COMP growth plate chondrocytes.^{38,40,41} Of particular interest was the up-regulation of a microtubule stabilizer, midline 1 (MID1),⁴³ which was elevated in the transcriptome of MT-COMP mice from P1 to P28 at all ages. The maximal levels of *Mid1* expression were correlated with high levels of intracellular retention of MT-COMP and chondrocyte death,⁴¹ suggesting a link between the MID1 and MT-COMP pathologic processes. Recent work has shown that persistent ER stress stimulates the stabilization of the microtubule network in an effort to maintain cellular viability.^{44,45} Moreover, this finding was intriguing since MID1 is a stimulator of mammalian target of rapamycin complex 1 (mTORC1) signaling. This association led to the assessment of the role of MID1 in the MT-COMP chondrocyte pathology.

Material and Methods

Generation of Bigenic Mice and CHOP-Null Bigenic Mice

The bigenic MT-COMP mice were generated using two plasmids, pTRE-COMP (coding sequence of human COMP+FLAG tag driven by the tetracycline responsive element promoter) and pTET-On-Col II (reverse tetracycline-controlled transactivator coding sequence driven by a type II collagen promoter) as previously described.^{38,41} Standard breeding was used to generate bigenic animals and bigenic animals in the CCAAT/enhancer-binding protein—homologous protein (CHOP)-null background. Genotypes of the transgenic offspring were verified by PCR.⁴⁶ All mice in these studies were

male. Mice were pre-/postnatally administered 500 ng/mL doxycycline through drinking water. All animal studies were approved by the Animal Welfare Committee at the University of Texas Health Science Center (Houston, TX).

Microarray Analysis

Total RNA was extracted from both hind-limb knee joints and purified using TRIzol and RNeasy columns (Qiagen, Hilden, Germany). RNA (300 ng) was amplified using the Total Prep RNA Amplification Kit (Illumina, San Diego, CA), and microarray analysis was performed as previously described.⁴¹ A *P* value of <0.05 was considered significant.

IHC Analysis

Hind limbs from MT-COMP and C57BL/6 control mice were collected and tibial growth plates analyzed as previously described.³⁸ Briefly, the limbs were fixed in 95% ethanol, and pepsin (1 mg/mL in 0.1 N HCl) was used for antigen retrieval. MID1 (catalog number SC 55247; goat polyclonal M-16; Santa Cruz Biotechnologies, Dallas, TX; 1:400), protein phosphatase (PP)-2A (catalog number GTX 113523; rabbit polyclonal; Genetex, Irvine, CA; 1:200), tumor necrosis factor (TNF)- α (catalog number ab6671; rabbit polyclonal; Abcam, Cambridge, UK; 1:200), TNF-related apoptosis-inducing ligand (TRAIL; catalog number ab42243; rabbit polyclonal; Abcam; 1:200), mTOR (catalog number 2976S; rabbit monoclonal; Cell Signaling Technology, Danvers, MA; 1:200), phosphorylated S6 ribosomal protein (pS6; catalog number 2215S; rabbit polyclonal; Cell Signaling Technology; 1:200), AKT (catalog number 3787S; rabbit monoclonal; Cell Signaling Technology; 1:200), glioma-associated oncogene homolog 2 (zinc finger protein) (Gli2; catalog number PA1-28838; rabbit polyclonal; Invitrogen, Carlsbad, CA; 1:300), and parathyroid hormone—related peptide (PTHrP; catalog number SC-20728; rabbit polyclonal; Santa Cruz Biotechnologies; 1:300) antibodies were incubated with sections overnight at 4°C for immunostaining. Species-specific secondary antibodies were incubated with sections for 50 minutes. Coverslips were mounted with ProLong Gold antifade reagent (Molecular Probes, Eugene, OR).

Treatments to Induce ER and Oxidative Stress in RCS Cells

Rat chondrosarcoma (RCS) cells (300,000 per 22.1-mm well) were treated with tunicamycin, thapsigargin, or peroxynitrite. ER stress was induced by either tunicamycin or thapsigargin. Tunicamycin was added to culture media (0.1, 0.5, 1, 1.5, and 2 mg/mL) and incubated for 8 hours. Thapsigargin treatment was 36 hours of exposure of 0.001, 0.05, 0.4, or 0.8 μ mol/L thapsigargin. Oxidative stress was induced by peroxynitrite treatment of 5, 50, 200, or 1000 mmol/L for 4 hours. RCS cells were harvested after

treatments and RNA prepared for RT-PCR analysis. All experiments were repeated in triplicate.

TNF α Treatment of RCS Cells to Evaluate *Mid1* and *Trail* Responses

RCS cells (300,000 per 22.1-mm well) were treated with 200 ng/mL murine TNF α (PeproTech, Rocky Hill, NJ) for 24 or 72 hours. RCS cells were harvested after TNF α treatment, and RNA was prepared for RT-PCR analysis.

mRNA Quantification

RNA was prepared as previously described.⁴⁰ Quantitative real-time RT-PCR was performed utilizing the ABI-7900 RT-PCR system (Applied Biosystems, Foster City, CA). Each assay was replicated three times, and each sample was measured in triplicate. The data were normalized to *Gapdh* (percentage of the normalizer transcript). The following primers were used in this study: *MT-COMP*, 5'-GCAAT-GACACCATCCCAGAG-3' (forward) and 5'-CTTGTCA-TCGTCGTCCTTGTAGTC-3' (reverse); *Chop*, 5'-CCAGC-AGAGGTCACAAGCAC-3' (forward) and 5'-CGCACT-GACCACTCTGTTTC-3' (reverse); *Gadd34*, 5'-AATCAG-GACCCTGAGATTCT-3' (forward) and 5'-CTGGTCC-TGCCAGACAG-3' (reverse); *Gadd45a*, 5'-CCGAAAG-GATGGACACGGTG-3' (forward) and 5'-TTATCGGGGT-CTACGTTGAGC-3' (reverse); *Ero1b*, 5'-CAAGGAAGC-CAACCTCCTT-3' (forward) and 5'-GTGTTTCGTCCACT-GAAGAAC-3' (reverse); *Mid1*, 5'-CACTATACTGTGC-ATGGCCTAC-3' (forward) and 5'-TCGATGAGCAGATT-TGGGATC-3' (reverse); *Trail*, 5'-ACTCCAAAATCG-GACTAGCTTG-3' (forward) and 5'-TCTCAAAGGTTCT-CAAAGTCACC-3' (reverse); *Gapdh*, 5'-AGTTCAACGG-CACAGTCAAG-3' (forward) and 5'-TACTCAGCAC-CAGCATCACC-3' (reverse).

Human Chondrocyte Nodule Culture

Cartilage nodules were established and maintained from control and D469del, G427E, and D511Y chondrocytes as previously described.^{2,33} The nodules were collected after 6 weeks in culture, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for immunohistochemistry analysis.

Generation of RCS Cells That Express Human D469del-MT-COMP

RCS cells expressing human D469del-MT-COMP were generated using the Lenti-X-Tet-On advanced inducible expression system according to the manufacturer's protocol (Takara Bio Company, Mountain View, CA). Briefly, RCS cells were infected with high-level lentiviral preparations of pLVX-Tet-On advanced vector or pLVX-Tight-human D469del-MT-COMP vector; at a multiplicity of infection

value of 1 to ensure that single-colony integrants could be isolated. Cells were cultured for 3 days in nonselective media. Puromycin and G418 were then added to the media for the selection of stable integrants. Single colonies were moved to separate dishes and grown in standard culture media with G418 and puromycin for these experiments. The expression of human D469del-MT-COMP in RCS cells was validated by Western blot with anti-Flag antibody for the recognition of the tagged D469del-MT-COMP (catalog number F7425 rabbit polyclonal; Sigma-Aldrich, Saint Louis, MO; 1:5000).

Mid1 siRNAs and Transfection

RCS cells expressing human D469del-MT-COMP were transfected with various concentrations of *Mid1* siRNAs using Mission siRNA transfection reagent following the manufacturer's recommendations (catalog number S1452; Sigma-Aldrich). All siRNAs target rat *Mid1* NM_022972 and were purchased from Sigma-Aldrich (*Mid1* siRNA 84: SASI_Rn01_00087200 targets beginning at nucleotide 1528; *Mid1* siRNA 86: SASI_Rn01_00087197 targets beginning at nucleotide 1051; *Mid1* siRNA 88: SASI_Rn01_00087196 targets beginning at nucleotide 899; *Mid1* siRNA 90: SASI_Rn01_00087198 targets beginning at nucleotide 903). RCS cells were harvested after treatments and RNA prepared for RT-PCR analysis.

MID1 Overexpression

RCS cells expressing human D469del-MT-COMP were transfected with 1.5 μ g of MID1-overexpression plasmid pCS2-xMID1⁴⁷ (kindly provided by Dr. Makoto Suzuki) using Eugene 6 reagent (Promega, Madison, WI) according to the manufacturer's protocol. After treatments, RCS cells were harvested and RNA prepared for RT-PCR analysis.

Results

MT-COMP Increases MID1 Expression

To identify the molecular mechanisms that underlie the MT-COMP chondrocyte pathology, transcriptome analysis was performed.⁴¹ RNA was collected from the hind-limb knee joints of MT-COMP and control mice at P1, P7, P14, P21, and P28 and subjected to microarray expression analysis. *Mid1* transcript level was significantly increased in MT-COMP mice at all ages compared to controls (Figure 1A). This increase was confirmed by quantitative real-time RT-PCR (data not shown), and immunostaining of MT-COMP growth plate chondrocytes showed increases in Mid1 protein in MT-COMP compared to the controls at all ages (Figure 1, B–I).

It was next determined whether this increase in MID1 expression was also present in human PSACH chondrocytes. Cartilage nodules grown in three-dimensional

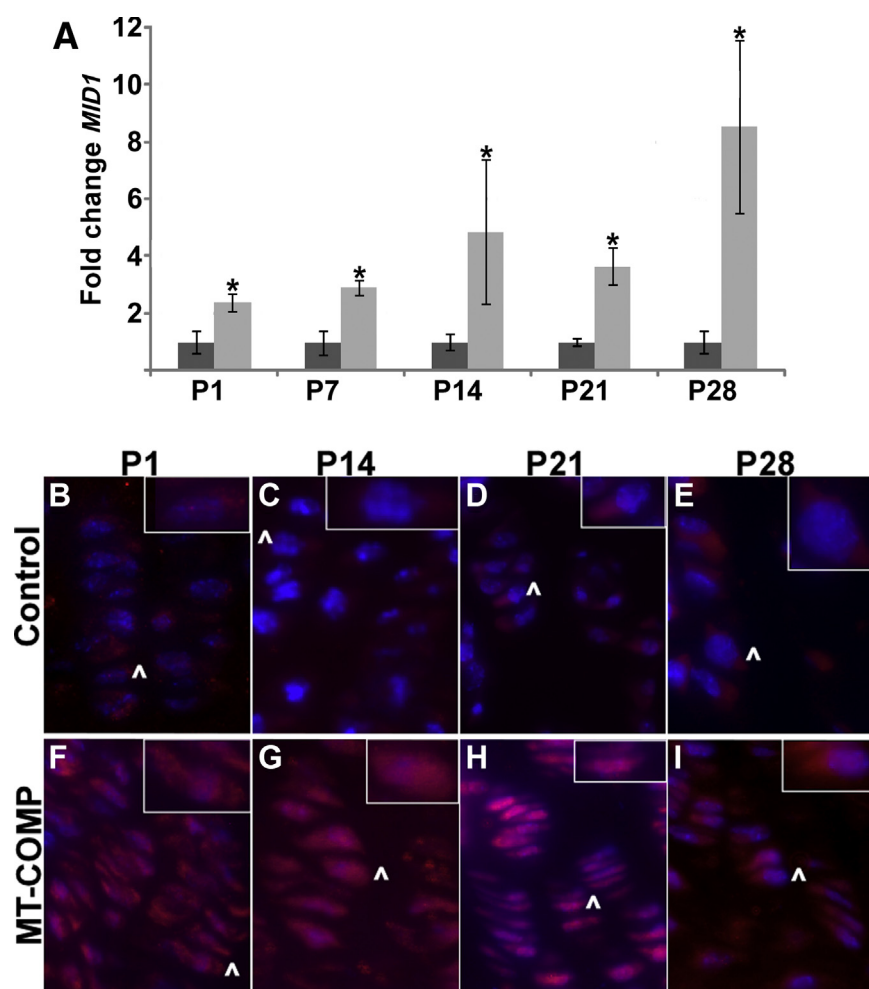


Figure 1 Midline 1 (MID1) is up-regulated in mutant cartilage oligomeric matrix protein (MT-COMP) mouse growth plate chondrocytes. *Mid1* mRNA level was assessed by microarray and Mid1 protein by immunohistochemistry from P1 to P28. **A:** *Mid1* expression in C57BL/6 mice (control; dark gray bars) was set to 1 and compared with the level of *Mid1* expression in the MT-COMP mice (light gray bars). Growth plate chondrocytes from P1 to P14 show more *Mid1* mRNA in the MT-COMP chondrocytes compared to control. **B–I:** Immunostaining of control C57BL/6 and MT-COMP growth plates with Mid1 (red) and DAPI (blue) stained nuclei from P1 to P28. **Arrowheads** mark the cells that are enlarged in the **insets**. Data are expressed as means $\pm$ SD. $n = 3$ mice per group (**A**). * $P < 0.05$. Original magnification: $\times 400$ (**B–I**); $\times 800$ (**insets**).

culture as previously described were used in these experiments.^{24,29,33,48} The chondrocytes were obtained from iliac crest biopsies from PSACH patients with D469del, G427E, and D511Y *COMP* mutations. These chondrocytes retain MT-COMP in the ER, causing ER stress and recapitulating the chondrocyte pathology observed in chondrocytes from PSACH growth plate biopsies.^{24,29,33,48} MID1 immunostaining was increased in the PSACH chondrocytes when compared to control chondrocytes (Figure 2, A–L). These results indicate that increased MID1 expression is associated with the human MT-COMP/PSACH chondrocyte pathology.

Mid1 Is Increased by ER Stress and TNF α /TRAIL Inflammation

MID1 plays a role in microtubule stability,⁴³ and recent findings indicate that chronic ER stress results in the stabilization of the microtubule network to sustain cellular viability.^{44,45} Excessively low or high microtubule stability is detrimental to cell viability, and chemotherapies exploit this microtubule stability to reduce cancer cell viability.⁴⁹ In

these experiments, it was tested whether three stressors (ie, ER stress, oxidative stress, or inflammation) involved in the MT-COMP pathology increases *Mid1* *in vitro*. RCS cells, well-characterized cells that maintain a chondrogenic phenotype,⁵⁰ were used in these experiments because the delivery of stressor molecules to the growth plate is not feasible. Tunicamycin and thapsigargin were used to stimulate ER stress. Tunicamycin has been reported to inhibit N-linked glycosylation in the ER and to cause protein accumulation in the ER, thereby activating the unfolded protein response.⁵¹ In contrast, thapsigargin has been reported to inhibit the sarco-/endoplasmic reticulum Ca²⁺-ATPase, depleting Ca²⁺ in the ER and stimulating ER stress.⁵¹ In the present study, RCS cells treated with tunicamycin (0.1 to 2 μ g/mL for 8 hours) had a 2- to 3.5-fold increase in *Mid1* mRNA (Figure 2M and Supplemental Figure S1), whereas thapsigargin (0.001 to 0.8 μ mol/L for 36 hours) treatment increased *Mid1* 2- to 20-fold, accompanied by an increase in *Chop* (Figure 2M and Supplemental Figure S1). These results demonstrate that ER stress increases *Mid1* levels in chondrocytes and suggest that ER stress generated by MT-COMP intracellular

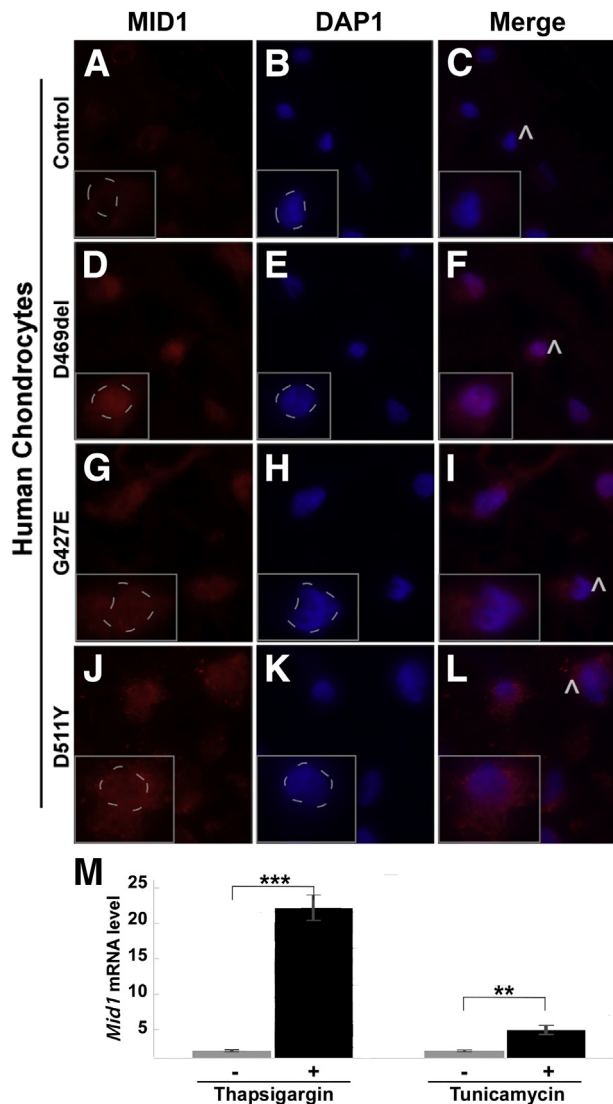


Figure 2 Midline (MID)-1 is increased in human pseudoachondroplasia (PSACH) chondrocytes and by endoplasmic reticulum stress in rat chondrosarcoma (RCS) cells. **A–L:** Chondrocytes from control and three different PSACH patients [with D511Y, G427E, and D469del cartilage oligomeric matrix protein (COMP) gene mutations] were grown in a three-dimensional culture system as previously described^{2,33} to generate nodules that were immunostained with MID1 antibody. DAPI (blue signal) marks the nuclei of chondrocytes. **Arrowheads** indicate the cells that are enlarged in the **insets**; **dashed circles** indicate the nuclei in the **insets**. **M:** *Mid1* mRNA levels in RCS cells treated with thapsigargin or tunicamycin (gray bars) are compared to that in untreated cells (control; black bars). Data are expressed as means $\pm$ SD. $n > 100$ cells (**A–L**); $n = 9$ (**M**). $**P < 0.005$, $***P < 0.0005$. Original magnification: $\times 600$ (**A–L**); $\times 1500$ (**insets**).

retention increases MID1 levels in MT-COMP growth plate chondrocytes. Oxidative stress is also involved in the MT-COMP chondrocyte pathology. Peroxynitrite, an endogenous peroxide, was used to generate free-radical intermediates in RCS cells, and *Mid1* mRNA levels were measured.⁵² Peroxynitrite treatment (5 to 1000 $\mu\text{mol/L}$ for 4 hours) showed no significant increase in *Mid1* mRNA (Supplemental Figure S1), suggesting that increases in *Mid1* result from ER stress but not oxidative stress.

MT-COMP expression has been reported to be associated with the inflammatory process driven by $\text{TNF}\alpha$ and $\text{IL-1}\beta$.^{42,53} $\text{TNF}\alpha$ /TRAIL drive MID1 expression in asthma and esophageal eosinophilia.^{54,55} It was determined whether $\text{TNF}\alpha$ increases *Trail* and *Mid1* independent of MT-COMP expression in RCS cells, as this $\text{TNF}\alpha$ increase in MID1 was previously shown in the epithelial cells lining the airway.⁵⁵ $\text{TNF}\alpha$ treatment (200 ng/mL) in RCS cells for 72 hours increased both *Trail* and *Mid1* mRNAs by 33- and 16-fold, respectively (Figure 3K). Consistent with these findings, both $\text{TNF}\alpha$ and TRAIL immunostaining were also increased in MT-COMP mice compared to controls (Figure 3, A–J). These results demonstrate that ER stress and $\text{TNF}\alpha$ /TRAIL increased *Mid1* in chondrocytes *in vitro*, and this finding is consistent with the up-regulation of *Mid1* observed in MT-COMP mice growth plate chondrocytes.

Increased MID1 Coincides with a Decrease in PP2A Level and Increased mTORC1 Signaling in MT-COMP Chondrocytes

Since MID1 acts as a negative regulator of PP2A in many cell types,^{56,57} it was evaluated whether MID1 has a similar function in MT-COMP growth plate chondrocytes in mice. MID1 has been established as a ubiquitin ligase that targets PP2A for degradation,^{56,57} and therefore high levels of MID1 would be expected to decrease PP2A. PP2A was diminished in the MT-COMP growth plate, consistent with the role of MID1 as a negative regulator of PP2A (Figure 4, A–J).^{58,59}

MID1 is known to regulate mTORC1 but not mTORC2.⁶⁰ Since MID1 is up-regulated in chondrocytes expressing MT-COMP and MID1 stimulates mTORC1, mTORC1 signaling and phosphorylated (p)-AKT were assessed in MT-COMP mice growth plates. pS6⁶¹ and pmTOR were used as readouts for mTORC1 signaling and pAKT for activated AKT. MT-COMP growth plates at P28 showed strong pS6, pAKT, and pmTOR expression levels, which were absent in the controls (Figure 4, K–Y). MID1 modulates mTORC1 through at least two mechanisms: by regulating 3-phosphoinositide-dependent protein kinase (PDK)-1 upstream of AKT and mTORC1,⁶² and regulating PP2A levels, which influences mTOR/regulatory associated protein of mTOR complex formation.⁶⁰ These findings indicate that mTORC1 signaling is stimulated in MT-COMP mouse growth plate chondrocytes by increased levels of pAKT and repression of PP2A, which inhibits mTORC1 formation.

The tuberous sclerosis (TSC) mouse models, which lack *Tsc1*, *Tsc2*, or both, exhibit ER stress and increased mTORC1 signaling in their brain tissue.^{58,59} These TSC mice were used to test whether *Mid1* was increased in the context of elevated ER stress and mTORC1 signaling in TSC mice (Figure 4Z). *Mid1* mRNA was increased three- to fivefold in all TSC mice, similar to the increase observed in the MT-COMP mice (Figure 4Z and

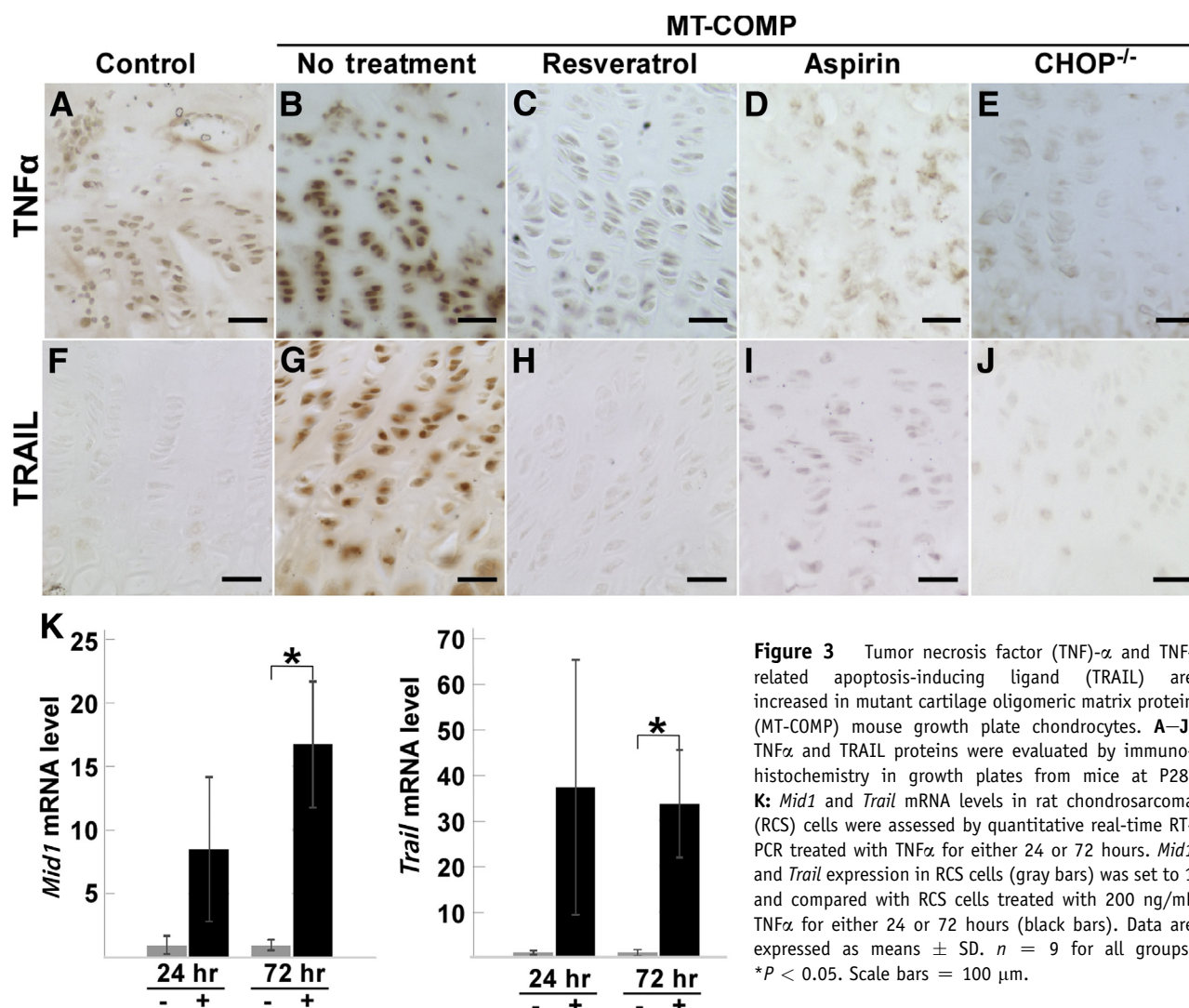


Figure 3 Tumor necrosis factor (TNF)- α and TNF-related apoptosis-inducing ligand (TRAIL) are increased in mutant cartilage oligomeric matrix protein (MT-COMP) mouse growth plate chondrocytes. **A–J:** TNF α and TRAIL proteins were evaluated by immunohistochemistry in growth plates from mice at P28. **K:** *Mid1* and *Trail* mRNA levels in rat chondrosarcoma (RCS) cells were assessed by quantitative real-time RT-PCR treated with TNF α for either 24 or 72 hours. *Mid1* and *Trail* expression in RCS cells (gray bars) was set to 1 and compared with RCS cells treated with 200 ng/mL TNF α for either 24 or 72 hours (black bars). Data are expressed as means $\pm$ SD. $n = 9$ for all groups. * $P < 0.05$. Scale bars = 100 μ m.

Figure 1A), linking *Mid1* up-regulation with excessive mTORC1 signaling.⁶³

mTORC1 Signaling and MT-COMP Growth Plate Pathology Is Reduced by Rapamycin Treatment

Rapamycin treatment in MT-COMP mice decreased COMP intracellular retention and mTORC1 signaling, but not inflammatory markers (Figure 5, G–I). Rapamycin decreased mTORC1 signaling in the MT-COMP growth plates, as shown by the reduction in pS6 (Figure 5, A–C). Additionally, intracellular retention of COMP was decreased (Figure 5, D–F) and chondrocyte proliferation was increased in the MT-COMP mice (Figure 5, J–L). The inflammatory markers IL-16 and eosinophil peroxidase (data not shown) were not reduced in MT-COMP growth plates by rapamycin treatment (Figure 5). Similarly, rapamycin ameliorated the growth plate abnormalities in *Tsc1*-null mice by repressing mTORC1 signaling.⁶³ In *Tsc1*-null mice, excessive mTORC1 results in short stature.⁶³ MID1 was increased with MT-COMP

expression, and mTORC1 signaling was stimulated directly by MID1 and indirectly through MID1 depletion of PP2A, an mTORC1 brake. These findings indicate that in the context of ER-stress growth plate pathology reducing mTORC1 signaling is therapeutic in mice.

Excessive mTORC1 Signaling Alters Levels of GLI2 and PTHrP, Which Modulate Murine Growth Plate Chondrocytes

Recent work has shown that mTORC1 regulates growth plate chondrocyte maturation. Ablation of the *Tsc1* gene resulted in excessive mTORC1 signaling in mice, causing chondrodysplasia/dwarfing.⁶³ mTORC1 regulated PTHrP, coordinating chondrocyte growth, proliferation, and differentiation/hypertrophy.⁶³ This regulation occurred through the mTORC1 downstream S6 kinase- β_1 phosphorylation of GLI2, stimulating the transcription of PthrP.⁶³ PTHrP controls bone development by regulating chondrocyte differentiation. To determine whether Gli2 and PTHrP are altered

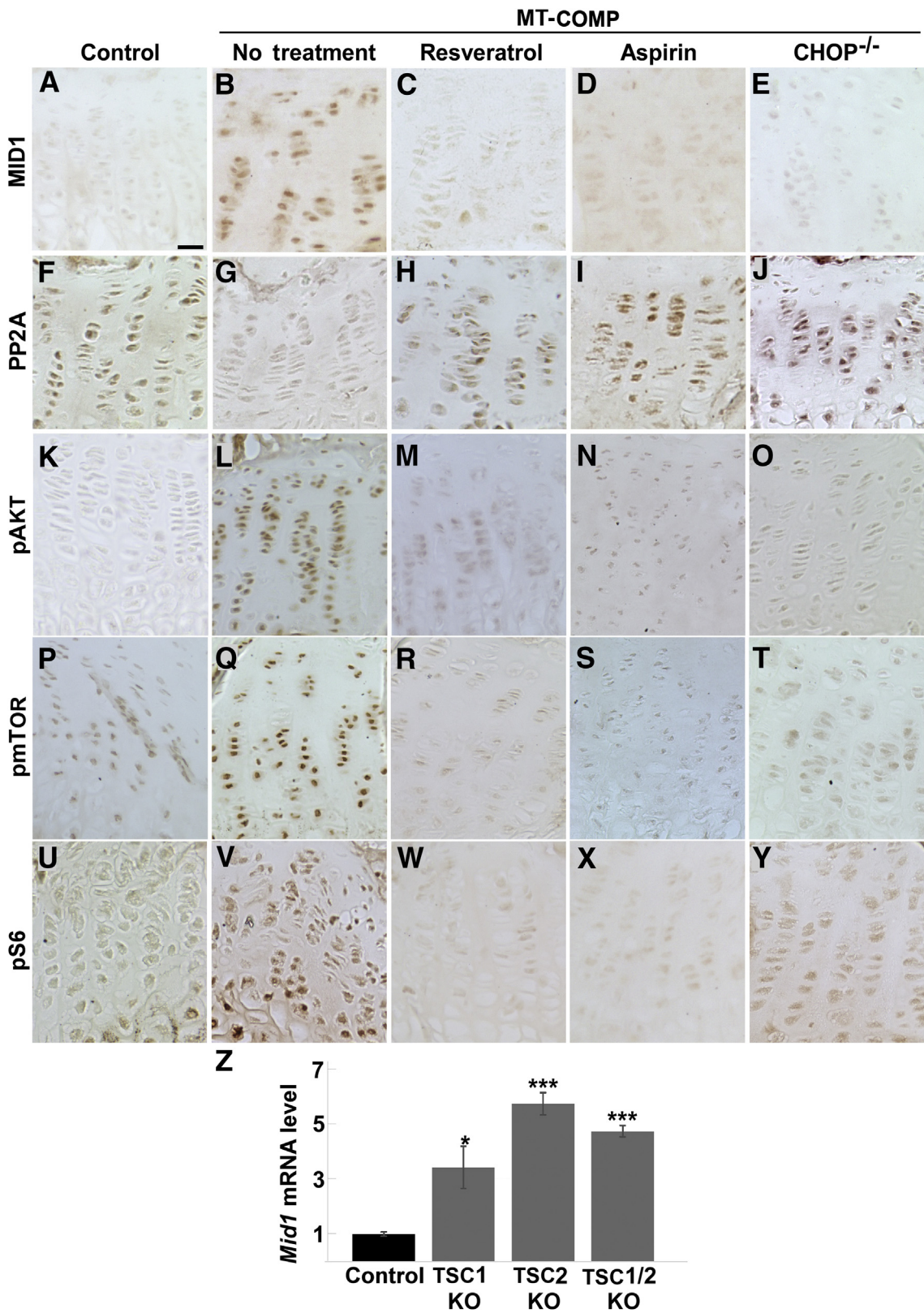


Figure 4 Increased midline 1 (MID1) decreases protein phosphatase (PP)-2a in mutant cartilage oligomeric matrix protein (MT-COMP) mouse growth plate chondrocytes. **A–Y:** MID1 (**A–E**), PP2A (**F–J**), phosphorylated AKT (**K–O**), phosphorylated mammalian target of rapamycin (pmTOR; **P–T**), and phosphorylated S6 ribosomal protein (pS6; **U–Y**) assessed by immunohistochemistry in P28 control (C57BL/6), MT-COMP with no treatment, MT-COMP treated with aspirin or resveratrol, and MT-COMP/CHOP^{-/-} growth plate chondrocytes. AKT, mTOR, and pS6 are all components of the mTORC1 signaling pathway. **Z:** RT-PCR performed on RNA extracted from tuberous sclerosis complex (TSC)-1-null, TSC2-null, TSC1/2-double null, and control mouse brains. Data are expressed as means ± SD. *n* > 3 (**A–Y**); *n* = 3 (**Z**). **P* < 0.05, ****P* < 0.0005 versus control. Scale bar = 100 μm (all images).

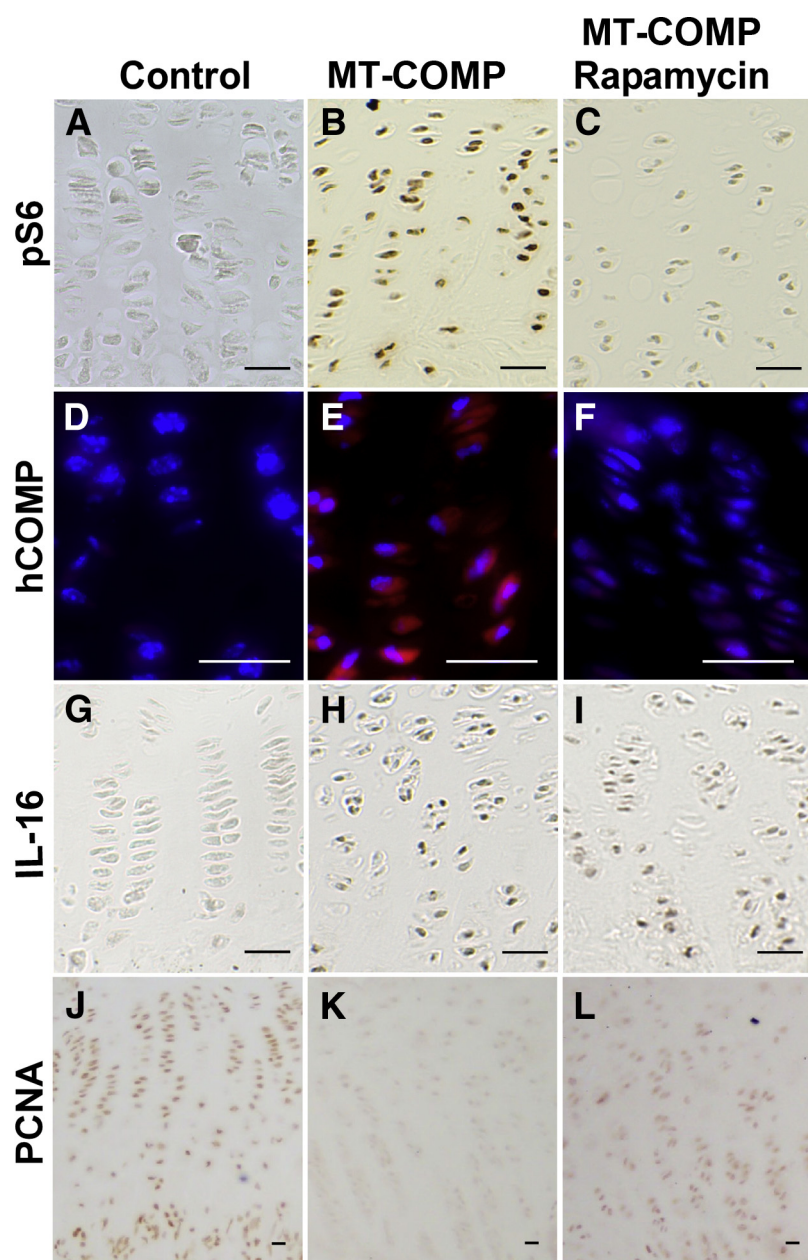


Figure 5 Rapamycin treatment of mutant cartilage oligomeric matrix protein (MT-COMP) mice decreases intracellular retention of COMP and mammalian target of rapamycin complex 1 (mTORC1) signaling but not inflammation. Control, untreated MT-COMP, and rapamycin-treated MT-COMP growth plates at P28 immunostained with antibodies for phosphorylated S6 ribosomal protein (pS6) (A–C), human (h) COMP (D–F), IL-16 (G–I), and DNA proliferation cell nuclear antigen (PCNA) (J–L). Rapamycin treatment reduces pS6 and intracellular COMP and improves chondrocyte proliferation. The inflammatory marker IL-16 is not reduced by rapamycin treatment. Scale bars = 100 μ m.

in MT-COMP mice, growth plates were assessed at P28 in MT-COMP mice. pS6, a readout for mTORC1 signaling, was increased (Figure 5B). GLI2 and PTHrP expression levels were also increased, consistent with elevated mTORC1 signaling in MT-COMP growth plates compared to controls (Figure 6). This finding suggests that excessive mTORC1/GLI2/PTHrP stimulated by MT-COMP expression represses growth plate chondrocyte hypertrophy.^{64–66}

Anti-Inflammatory or Antioxidant Treatments or Elimination of CHOP-ER Stress Response Decreases *Mid1* Expression

Aspirin or resveratrol treatments have been reported to reduce intracellular accumulation of COMP, decrease

inflammation markers and chondrocyte death, and increase proliferation and limb length.⁵³ Additionally, the absence of CHOP, a key component of MT-COMP ER stress, in MT-COMP/CHOP^{−/−} mice was reported to have diminished the negative effects of MT-COMP expression.^{40,41} The expression levels of MID1 and PP2A in MT-COMP growth plates in mice treated with aspirin or resveratrol or loss of CHOP were next assessed. All of these therapeutic interventions restored normal levels of MID1 and PP2A in the growth plate (Figure 4, A–J), confirming that MID1 increases are related to MT-COMP chondrocyte pathology. Aspirin or resveratrol treatments or *Chop* gene ablation, all of which have been reported to dampen the MT-COMP chondrocyte pathology,⁵³ also decreased the expression levels of TNF α and TRAIL, which drive increases in MID1

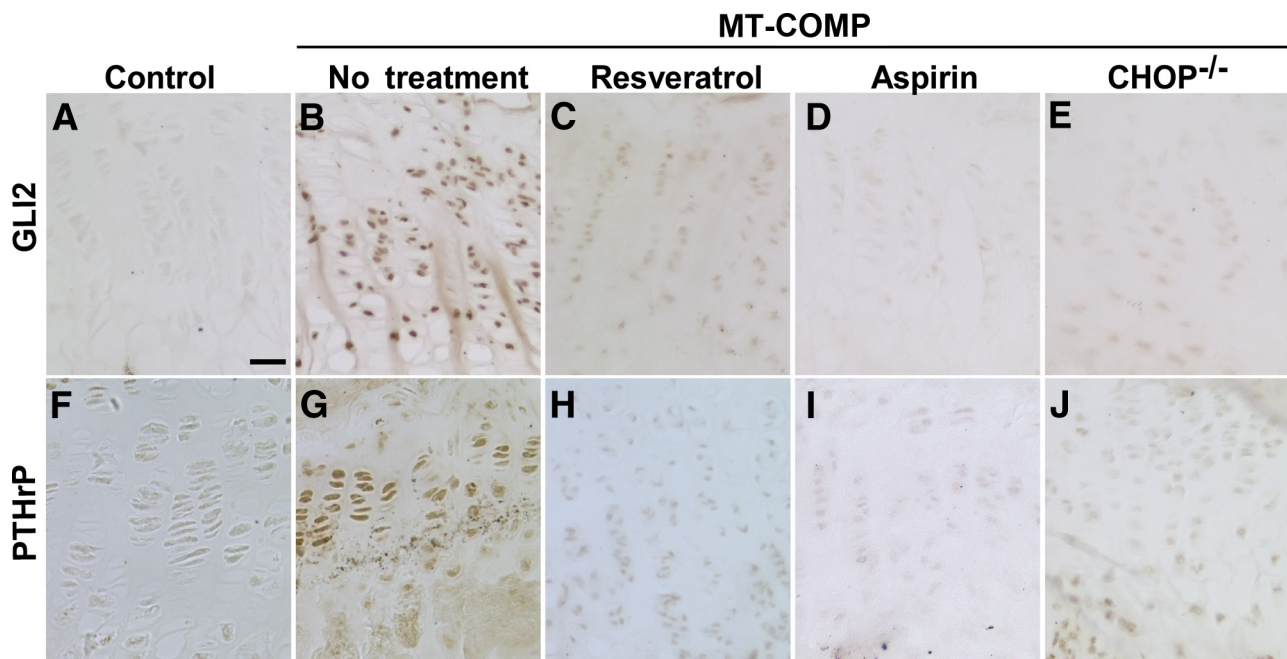


Figure 6 Glioma-associated oncogene homolog 2 (Gli2) and parathyroid hormone-related protein (PTHrP) are increased with mammalian target of rapamycin complex 1 (mTORC1) signaling in mutant cartilage oligomeric matrix protein (MT-COMP) growth plates. Control (C57BL/6), untreated MT-COMP, MT-COMP treated with resveratrol or aspirin, and MT-COMP/CHOP^{-/-} growth plates from P28 mice were immunostained for phosphorylated Gli2 (A–E) and PTHrP (F–J). Scale bar = 100 μ m (all images). CHOP, CCAAT/enhancer-binding protein homologous protein.

(Figure 3, A–J). These treatments also normalized mTORC1 signaling (Figure 4), likely due to decreases in the PP2A level. Additionally, resveratrol or aspirin treatment or elimination of CHOP (MT-COMP/CHOP^{-/-}) normalized Gli2 and PTHrP levels (Figure 6), also likely due to down-regulation of mTORC1 signaling.

Overexpression of MID1 Decreases *Chop* mRNA Levels in the Presence of MT-COMP

To define the relationship between MID1 and CHOP, MID1 was overexpressed and knocked down in RSC cells expressing MT-COMP. Overexpression of MID1 in MT-COMP RSC cells (Figure 7A) was accompanied by decreased *Chop* mRNA levels (Figure 7B). MT-COMP expression has been associated with increases in *Chop*, growth arrest, and DNA damage-inducible proteins 34 and 45a and ER oxidoreductin protein1 β mRNAs.⁴⁰ siRNA knockdown of *Mid1* in MT-COMP RSC cells resulted in increases in MT-COMP, *Chop*, *Gadd34*, *Gadd45a*, and *Erlb* (Figure 7, C–H).

Discussion

This is the first study linking ER stress to MID1/mTORC1 signaling in chondrocytes and other cell types, a finding that has significant implications for cellular functions including autophagy, protein synthesis, and potentially cellular

viability. Moreover, these results identify new therapeutic targets for this pathologic process in a wide spectrum of ER-stress disorders. The pathologic mechanistic model shows that MT-COMP expression and ER retention stimulate unrelenting ER stress that initiates TNF α /TRAIL inflammation and MID1 up-regulation, resulting in increased mTORC1 signaling (Figure 8).

MT-COMP-induced chondrocyte death occurs through necroptosis (a form of cell death).^{40,41} This process begins with intracellular accumulation of MT-COMP, which induces ER stress and the unfolded protein response. The refolding and degradation branches of the unfolded protein response are not fully engaged by MT-COMP retention, and only the protein kinase RNA-activated-like endoplasmic reticulum kinase/CHOP branch proceeds beyond initial activation.⁴⁰ Reactive oxygen species are generated by CHOP/ERO1 β along with NADPH oxidase 4, creating DNA damage and the release of phosphorylated histone 2AX.⁴⁰ MT-COMP-induced mitochondrial dysfunction stimulates the release of cell death-inducing factor and its translocation to the nucleus, where it interacts with phosphorylated histone 2AX, inducing necroptosis.^{40,41} MID1, a microtubule stabilizer, which was increased, may be activated to sustain chondrocyte viability in this pathologic process.^{47,67} The ER is inextricably linked to microtubules, as microtubules are the transport highways used by the ER to export its cargo.⁶⁸ Persistent ER stress is known to stabilize the microtubule network, thereby providing resistance to apoptosis.^{69–77} Additionally, in our study, the

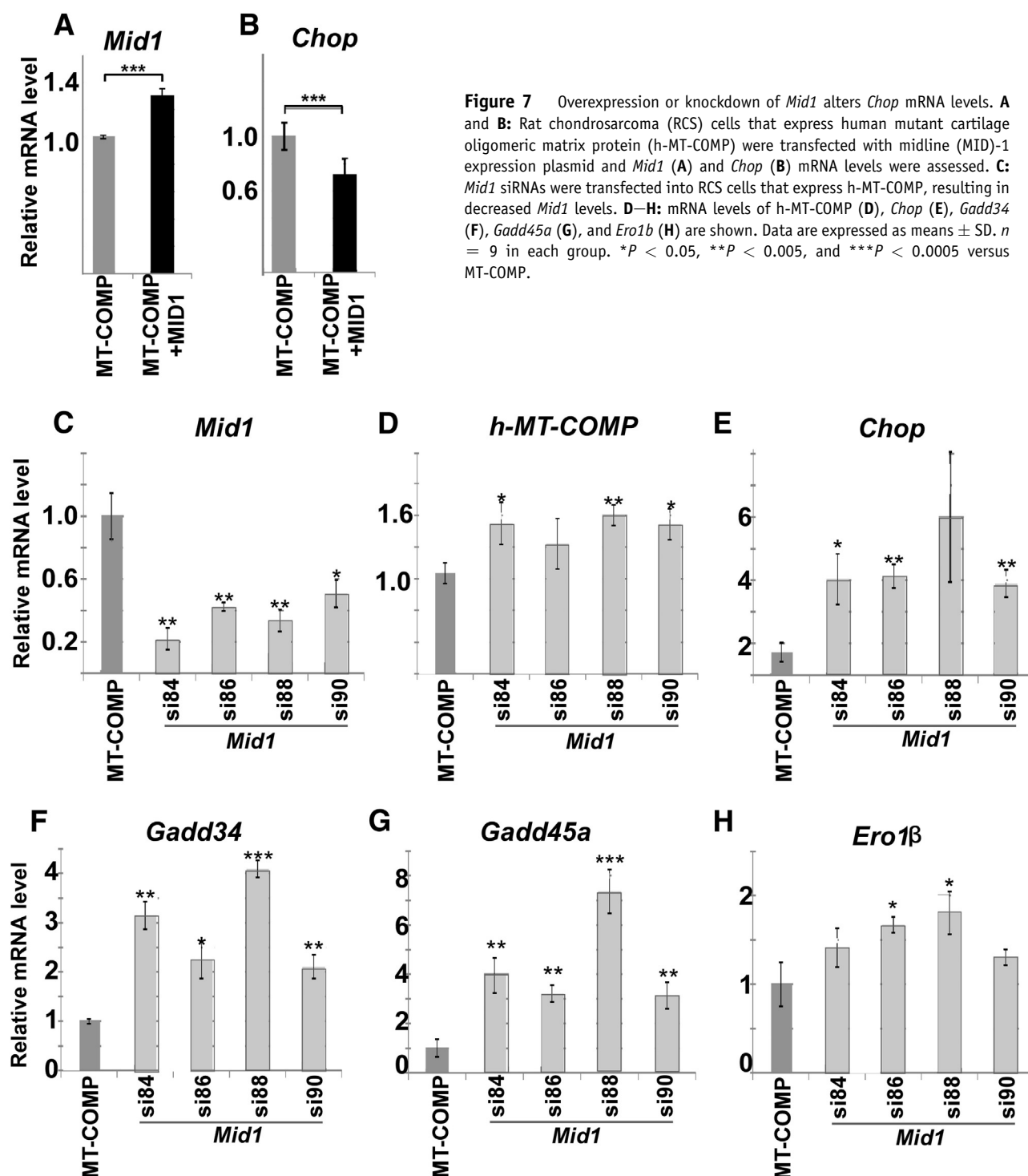


Figure 7 Overexpression or knockdown of *Mid1* alters *Chop* mRNA levels. **A** and **B**: Rat chondrosarcoma (RCS) cells that express human mutant cartilage oligomeric matrix protein (h-MT-COMP) were transfected with midline (MID)-1 expression plasmid and *Mid1* (**A**) and *Chop* (**B**) mRNA levels were assessed. **C**: *Mid1* siRNAs were transfected into RCS cells that express h-MT-COMP, resulting in decreased *Mid1* levels. **D–H**: mRNA levels of h-MT-COMP (**D**), *Chop* (**E**), *Gadd34* (**F**), *Gadd45a* (**G**), and *Ero1b* (**H**) are shown. Data are expressed as means $\pm$ SD. $n = 9$ in each group. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$ versus MT-COMP.

overexpression of MID1 was associated with a decrease in *Chop*, whereas knockdown of *Mid1* was associated with an increase in the stress-related transcripts *Chop*, *Gadd34*, *Gadd45a*, and *Ero1b*. It is not clear whether the increase in *Chop* when MID1 was knocked down was due to decreased *Mid1* or increased MT-COMP. MID1 increases stability and

translational efficiency of select mRNAs through MID1-associated sequence.⁶² However, *COMP* mRNA does not contain a MID1-binding domain, and the inverse relationship between MID1 and MT-COMP suggests that MID1 does not directly regulate *COMP* mRNA levels. Based on this information, increased MID1 in ER stress-related

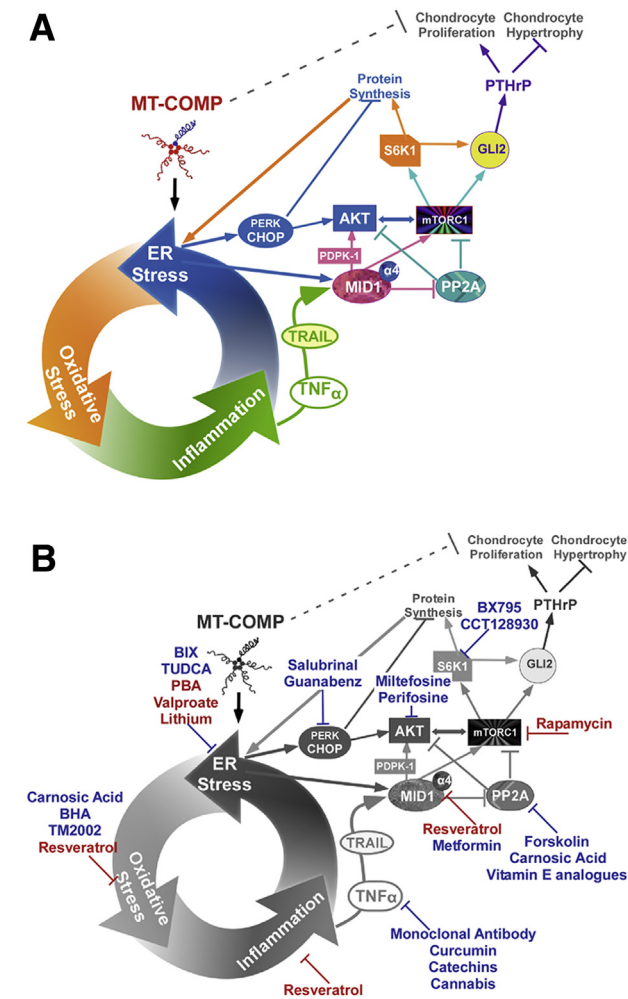


Figure 8 Model depicting the roles of midline 1 (MID1), mammalian target of rapamycin, complex 1 (mTORC1), and parathyroid hormone-related protein (PTHrP) in mutant cartilage oligomeric matrix protein (MT-COMP) chondrocyte pathology. **A:** MT-COMP expression elicits endoplasmic reticulum (ER) stress through PERK/CHOP, which leads to oxidative stress and inflammation. The inflammatory process driven in part by tumor necrosis factor (TNF)- α increases TNF-related apoptosis-inducing ligand (TRAIL) and MID1. CHOP down-regulates general protein synthesis as part of the unfolded protein response and up-regulates AKT. AKT and MID1 stimulate mTORC1 signaling, along with a decrease in the mTORC1 brake, protein phosphatase (PP)-2A. mTORC1 signaling drives protein synthesis through protein S6 kinase/phospho-S6 and likely generates additional ER stress. mTORC1 up-regulates glioma-associated oncogene homolog 2 (zinc finger protein) (GLI)-2 and PTHrP, which alters chondrocyte proliferation and hypertrophy. **B:** Resveratrol counteracts several processes involved in the MT-COMP chondrocyte pathology including oxidative stress, inflammation, and MID1/ $\alpha 4$ complex. Aspirin, a cyclooxygenase-2 inhibitor, dampens inflammation and diminishes the negative effects of MT-COMP expression (red). Rapamycin decreases intracellular retention of MT-COMP and mTORC1 signaling but does not improve inflammation and proliferation. Other drugs to potentially reduce the MT-COMP chondrocyte pathology are shown in blue. BHA, butylated hydroxyanisole; BIX, immunoglobulin heavy-chain-binding protein inducer X; CHOP, CCAAT/enhancer-binding protein-homologous protein; PBA, 4-phenylbutyrate; PDPK-1, protein 3-phosphoinositide-dependent protein kinase-1; PERK, protein kinase RNA-activated-like endoplasmic reticulum kinase TUDCA, tauroursodeoxycholic acid.

conditions likely slows but does not ultimately eliminate chondrocyte death.^{2,33,36,38,40,66,78}

It has been tested whether MID1/mTORC1 signaling is involved in other ER stress-related conditions, including Alzheimer, Huntington, and Parkinson diseases; amyotrophic lateral sclerosis; cancer; type II diabetes⁷⁹; and TSC.⁵⁸ Both PSACH and Alzheimer disease involve protein misfolding, oxidative stress, inflammation, and premature cell death.^{36,41,80–87} MID1 is elevated in Alzheimer-diseased brains compared to healthy controls.⁸⁸ Consistent with these findings, mTORC1 is increased and PP2A, which is negatively regulated by MID1, is decreased in Alzheimer disease.⁸⁹ Furthermore, in human TSC or mice lacking either Tsc1 or Tsc2 or both Tsc genes, unrestrained mTOR signaling generates ER stress with a concurrent increase in mTOR activation.⁹⁰ *Mid1* mRNA was increased in the brain tissue of Tsc1-, Tsc2-, and Tsc1/2-double null mice, providing further evidence that MID1/mTORC1 is linked to ER stress (Figure 4Z). Moreover, dampening mTORC1 signaling was reported to improve Parkinson disease outcomes in mice, preventing the development of dyskinesia.⁹¹ All together, these combined observations establish that up-regulation of MID1 accompanies elevated mTORC1 signaling in a variety of ER stress-related conditions.⁹²

mTORC1 is a master regulator of growth in response to nutritional status, energy levels, cellular stress, growth factors, and amino acids, and regulates general protein translation and autophagy.⁹³ Dysfunction of secretory cells or misfolded proteins (chondrocytes, β -islet cells, and neurons) are a significant cause of ER stress-related conditions. Secretory cells synthesize large amounts of exported proteins that are necessary for organ/tissue functioning. Since mTORC1 regulates general protein synthesis, increases in mTORC1 signaling may sustain protein synthesis during periods of ER stress, allowing cells to continue functioning. During ER stress, the unfolded protein response down-regulates general translation to allow clearance of misfolded protein(s). In contrast, mTORC1 stimulates general protein synthesis, which may ultimately exacerbate ER stress and inhibit ER clearance. Additionally, increased mTORC1 signaling represses autophagy, eliminating another mechanism to clear misfolded proteins and resolve ER stress. Collectively, these results indicate that increased MID1 during ER stress functions to prolong cellular survival as well as stimulates excess mTORC1 signaling, which ultimately may be detrimental.

Besides playing a role in ER stress-related disorders, mTORC1 signaling regulates PTHrP and GLI2 modulation of chondrocyte hypertrophy, and increased mTORC1 signaling is associated with osteoarthritis joint degeneration.⁹⁴ In the present study, PTHrP and GLI2 were both increased in growth plate chondrocytes in MT-COMP mice (Figure 6). This finding suggests that the lack of hypertrophic chondrocytes in the PSACH growth plate and the early-onset osteoarthritis in PSACH are additional consequences of increased mTORC1 signaling.

This work identifies the mTORC1 pathway as a potential therapeutic target in ER-stress conditions. It has been reported that aspirin and resveratrol treatments dampen MT-COMP chondrocyte pathology by reducing inflammation and oxidative stress.⁵³ In the present study, these treatments also reduced Mid1, TNF α , Trail, and mTORC1 signaling in mice. Although rapamycin targeting of mTORC1 in the MT-COMP mouse decreased pS6 and MT-COMP intracellular accumulation, and increased DNA proliferation, it did not reduce inflammation (eosinophil peroxidase or IL-16). This is an expected result since MID1 up-regulation is downstream of TNF α /TRAIL inflammation and therefore rapamycin may be more effective when used in combination with anti-inflammatory medications such as aspirin. However, there are significant side effects (suppression of the immune system, increased risks for infections and cancer, and impaired wound healing) with chronic rapamycin treatments that may limit their current therapeutic utility (Food and Drug Administration, https://www.accessdata.fda.gov/drugsatfda_docs/label/2010/021110s058lbl.pdf, last accessed September, 17, 2018).

Given the numerous conditions that involve ER dysfunction, a complete understanding all of the consequences of ER stress is crucial for the development of mechanism-driven therapeutic approaches. The results of this work show that TNF α inflammation, MID1, mTORC1 signaling, the microtubule network, protein synthesis, and autophagy form a complex, multifaceted response to protein accumulation in the ER when the clearance efforts fail, and MID1 may act as a prosurvival factor to extend chondrocyte life. Importantly, therapeutics interrupting the upstream self-perpetuating pathologic loop between ER stress, inflammation, and oxidative stress may be most effective.

Acknowledgments

We thank Frankie Chiu for technical assistance, and Dr. Makoto Suzuki for providing the MID1-overexpression plasmid.

K.L.P. designed the study, analyzed the data, performed literature search, generated the figures, and wrote the manuscript; F.C., A.C.V., and M.G.H. collected and analyzed data; M.J.G. collected data and reviewed the manuscript; J.T.H. designed the study, interpreted data, and wrote the manuscript.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.09.008>.

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