The skeletal phenotype of Achondrogenesis type 1A is caused exclusively by cartilage defects

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Summary Statement

Achondrogenesis type-1A (ACG1A) is caused by mutations in the cis-Golgin GMAP-210. Conditional inactivation of GMAP-210 reveals that the ACG1A skeletal phenotype is solely due to impaired protein trafficking by chondrocytes.

Abstract

Inactivating mutations in the ubiquitously expressed membrane trafficking component GMAP-210 (encoded by Trip11) cause Achondrogenesis type 1A (ACG1A). ACG1A is surprisingly tissue specific, mainly affecting cartilage development. Bone development is also abnormal, but since chondrogenesis and osteogenesis are closely coupled, this could be a secondary consequence of the cartilage defect. A possible explanation for the tissue specificity of ACG1A is that cartilage and bone are highly secretory tissues with a high usage of the membrane trafficking machinery. The perinatal lethality of ACG1A prevented investigating this hypothesis. We therefore generated mice with conditional Trip11 knockout alleles, and inactivated Trip11 in chondrocytes, osteoblasts, osteoclasts and pancreas acinar cells, all highly secretory cell types. We discovered that the ACG1A skeletal phenotype is solely due to absence of GMAP-210 in chondrocytes. Mice lacking GMAP-210 in osteoblasts, osteoclasts and acinar cells were normal. When we inactivated Trip11 in primary chondrocyte cultures, GMAP-210 deficiency affected trafficking of a subset of chondrocyte-expressed proteins rather than globally impairing membrane trafficking. Thus, GMAP-210 is essential for trafficking specific cargoes in chondrocytes but is dispensable in other highly secretory cells.
Introduction

The evolution of membrane bound intracellular compartments in eukaryotic cells enabled the efficient separation of cell functions, e.g., nucleus for DNA replication, mitochondria for respiration and endoplasmic reticulum (ER) for protein synthesis and folding. It also created the need for communication between different sub-cellular compartments. This interaction, and the interaction between the cell and its external environment, is mainly achieved through membrane trafficking. Membrane trafficking is a basic, life-essential function performed by all eukaryotic cells. It is essential for maintaining cellular homeostasis and plays a role in most, if not all, biological processes. A typical membrane trafficking event contains four steps (Bonifacino and Glick, 2004; Cai et al., 2007; Pfeffer, 2007): (1) cargo selection and the formation of transport vesicles at the donor compartment (Bonifacino and Lippincott-Schwartz, 2003; D’Arcangelo et al., 2013; Faini et al., 2013; Jackson, 2014; Kirchhausen, 2000; Kirchhausen et al., 2014; McMahon and Mills, 2004; Venditti et al., 2014); (2) vesicle delivery to the target compartment by diffusion or by motor-mediated transport along a cytoskeletal track (Fokin et al., 2014; Hancock, 2014; Hendricks et al., 2010; Roberts et al., 2013; Tumarello et al., 2013); (3) tethering/capturing of the vesicle at the target compartment (Balderhaar and Ungermann, 2013; Brown and Pfeffer, 2010; Chia and Gleeson, 2014; Munro, 2011) and (4) fusion of the vesicle membrane with the target membrane, releasing the cargo into the lumen of the target compartment (Hong and Lev, 2014; Jena, 2011).

Insights into membrane trafficking have come from work performed in cell lines and in single-cell organisms such as yeast. However, these systems do not encompass the diversity of cell types present in multi-cellular organisms and may not model what occurs in vivo (Apodaca and Brown, 2014). These limitations are relevant to the autosomal recessive skeletal dysplasia Achondrogenesis type 1A (ACG1A), which we previously reported is caused by loss-of-function mutations in TRIP11 (Smits et al., 2010). Although TRIP11 (Thyroid receptor interacting protein 11) was initially considered a potential co-activator of thyroid hormone nuclear receptor β (Lee et al., 1995), subsequent studies provided strong evidence for its role in membrane trafficking. The protein was re-named GMAP-210 (Golgi-microtubule-associated protein of 210 kDa), since it binds microtubuli and localizes to the Golgi apparatus (GA) (Infante et al., 1999; Ramos-Morales et al., 2001). The GA, made of stacks of flattened membranous cisternae, is the main protein sorting and post-translational modification center of a eukaryotic cell. It receives cargo (mainly from the ER) on its cis-side, proteins then acquire their post-translational modifications while moving through the medial Golgi, before being sorted into transport vesicles, destined for specific destinations in or outside the cell, at the trans-Golgi. Since GMAP-210 contains a central coiled-coil domain and a GRAB (GRIP related ARF1 binding) domain it has been classified as a member of the Golgin
protein family (Infante et al., 1999; Ramos-Morales et al., 2001). Golgins function as tethering factors, capturing transport vesicles and aiding their fusion with their target organelles (Gillingham and Munro, 2016; Witkos and Lowe, 2017). Knock-down experiments using small interfering RNAs implicate GMAP-210 in ER to Golgi transport (Roboti et al., 2015). This role in ER vesicle tethering was elegantly demonstrated by directionally localizing GMAP-210 from the Golgi to the mitochondria. This resulted in the redirection of ER-derived vesicles to this organelle (Wong and Munro, 2014). Golgins also function to maintain the organization of the Golgi and in vitro studies have shown that GMAP-210 plays an essential role in maintaining Golgi structure (Rios et al., 2004).

The lack of early embryonic lethality and the predominantly skeletal phenotype in humans and mice missing GMAP-210 was surprising since the protein is ubiquitously expressed and thought to be essential for cell function based on in vivo studies (Follit et al., 2008; Smits et al., 2010). We found that many cell types in GMAP-210 deficient mice had normal appearing Golgi. However, we did observe massive ER swelling and precocious cell death in growth plate chondrocytes along with impaired bone formation. Because bone formation (i.e., ossification) depends on cartilage formation, we could not determine if the bone defect was cell autonomous or secondary to the cartilage defect. Also, the perinatal lethality that occurs in GMAP-210 deficient humans and mice precludes the assessment of the protein’s postnatal roles in other tissues. Thus, it could not be determined, in global deficiency humans and mice, whether GMAP-210 is essential in cells that produce abundant extracellular matrix or that secrete large volumes of cargo, and whether chondrocytes use GMAP-210 to traffic all extracellular matrix proteins or only a subset of cargoes.

To address these aforementioned issues we generated mice carrying conditional Trip11 null alleles. Herein we detail studies in which we inactivated Trip11 in vivo specifically in chondrocytes, osteoblasts, the osteoclast encompassing hematopoietic lineage, and exocrine pancreatic acinar cells. We also inactivated Trip11 in primary cultured chondrocytes in vitro and used proteomics to determine whether chondrocytes require GMAP-210 for all extracellular matrix proteins or only a subset of cargoes. We found that Trip11 inactivation in chondrocytes replicates the ACG1A phenotype, whereas there is no apparent phenotype when GMAP-210 is absent in osteoblasts, osteoclasts or pancreatic acinar cells. Our data demonstrate that the skeletal phenotype of ACG1A is caused exclusively by chondrocyte defects and that Trip11 is dispensable in several other cell types that extensively utilize the membrane trafficking machinery. Furthermore, we found that absence of GMAP-210 does not lead to intracellular accumulation of all secreted proteins, but only affects the secretion of a select group of cartilage extracellular matrix proteins.
Results

The Trip11cko allele is a true loss-of-function allele after Cre-recombination.

Animals homozygous for the conditional allele (i.e., Trip11cko/cko) were indistinguishable from wild-type and heterozygous (Trip11cko/+ ) mice, indicating the inserted LoxP sites did not impair Trip11 expression. In contrast, when the conditional allele was Cre-recombined (i.e., Trip11−/−) and transmitted via the germline, homozygous Trip11−/− offspring had the same skeletal dysplasia previously observed in mice with other loss-of-function alleles (Fig. 1A), featuring a short trunk, short limbs and a domed skull with a protruding tongue. Skeletal preparations of E17.5 Trip11−/− embryos confirmed the smaller ribcage, shorter vertebral column and reduced length of the appendicular bones. Similar to ACG1A, a delay in mineralization of the vertebral column, sternum and the intramembranous skull bones was present in Trip11−/− embryos (Fig. 1B-G).

Alcian blue stained sections through the humeri of E15.5 mutant embryos showed a delay in the formation of the primary ossification center (Fig. 2A). Furthermore, chondrocytes in mutant embryos had a swollen appearance, with massive expansion of the ER and disruption of the Golgi apparatus (GA) evident by electron microscopy (EM) (Fig. 2B). Cell lysates obtained from mouse embryonic fibroblasts generated from E13.5 Trip11−/− embryos lacked immuno-detectable GMAP-210, compared to Trip11+/+ and Trip11+/− littermates (Fig. 2C).

These data indicate that the Trip11cko allele becomes a loss-of-function allele after Cre-recombination.

Specific inactivation of Trip11 in cartilage using Col2a1-Cre yields a phenotype identical to Achondrogenesis type 1A

Mice lacking GMAP-210 in chondrocytes were generated using the Col2a1-Cre transgene. Tg:Col2a1-Cre;Trip11cko/−;ROSA26mTmG/+ newborn mice were present at the expected Mendelian frequency and died shortly after birth with a severe chondrodysplasia similar to that observed in Trip11−/− mice (Fig. 3A). Newborn Tg:Col2a1-Cre;Trip11cko/−;ROSA26mTmG/+ pups had short limbs, a short snout and a domed skull. Skeletal preparations revealed bones with a reduced length in the extremities, a small ribcage, and a delay in mineralization of the vertebral column and the skull bones (Fig. 3B-G).

Histology analysis of the humeri of E15.5 mutant embryos demonstrated delayed formation of the primary ossification center and areas where columnar chondrocytes were swollen (Fig. 4A). At P0, widespread chondrocyte swelling was observed in the humeri of mutant pups; furthermore, hypertrophic zones were absent and the humeri featured an abnormally thick bone collar (Fig. 4B).
Electron microscopy revealed that P0 mutant humeral chondrocytes had ER swelling and loss of GA stacking (Fig. 4C). Interestingly, decreased lung alveolar formation, which had been observed in mice with global loss-of-function mutations in Trip11 (Follit et al., 2008; Smits et al., 2010) was also observed in Tg:Col2a1-Cre;Trip11\textsuperscript{cko/-};ROSA26\textsuperscript{mTmG/+} mice (Fig. 4D). Thus, the alveolar phenotype appears secondary to the small ribcage, rather than a primary lung cell phenotype. The American Thoracic Society has reported that alveolar deficiency has been observed in other conditions characterized by insufficient thoracic volume (ad hoc Statement Committee, 2004). The alveolar insufficiency is the likely cause of death of Tg:Col2a1-Cre;Trip11\textsuperscript{cko/-};ROSA26\textsuperscript{mTmG/+} mice.

Combined these data show that the skeletal phenotype of cartilage specific Trip11 null mice is identical to that of global Trip11 null mice.

Absence of GMAP-210 does not impair osteoblast and osteoclast function

Humans and mice with global deficiency of GMAP-210 have significantly reduced ossification of vertebral bodies and skull bones. That both endochondral (vertebral bodies) and intramembranous bones (skull) show defects, implies a direct role for GMAP-210 in bone formation. However a delay in mineralization of these skeletal elements is also observed in cartilage specific Trip11 knockout mice, suggesting that the delay is a secondary consequence of the cartilage phenotype. To investigate whether GMAP-210 has cell-autonomous functions in bone we specifically inactivated Trip11 in osteoblasts using the Bglap-Cre transgene and, as osteoclasts derive from the hematopoietic lineage, in hematopoietic stem cells using the Vav1-Cre transgene.

Tg:Bglap-Cre;Trip11\textsuperscript{cko/-};ROSA26\textsuperscript{mTmG/+} offspring were born at the expected Mendelian frequency and appeared normal (Fig. 5A). Skeletal preparations of E17.5 mutant and control embryos showed that Trip11 inactivation in osteoblasts had no affect on the size or the mineralization of either endochondral or intramembranous bones (Fig. 5B-G). Histologic sections of tibiae from P0 osteoblast specific knockout pups revealed similar amounts of trabecular and cortical bone when compared to control littermates (Fig. 6A). EM studies revealed no swelling of ER cisternae in mutant osteoblasts, however they did feature an abnormal GA stack structure (Fig. 6C). Despite the abnormal GA, when analyzed at 6 weeks of age osteoblast specific knockout mice had the same µCT bone measurements as their littermate controls (Fig. 6B and Fig. S1A).

To confirm that the Bglap-Cre transgene recombined the Trip11\textsuperscript{cko} allele, we recovered cells from calvariae of P5 pups. We used the ROSA26\textsuperscript{mTmG} allele to flow sort primary osteoblasts, which now fluoresce green, from non-osteoblasts, which fluoresce red. When western blots were
immuno-detected using an anti-GMAP-210 antibody, no protein was detected in osteoblasts from Tg:Bglap-Cre;Trip11\textsuperscript{cko/-};ROSA26\textsuperscript{mTmG/+} mice (Fig. 6D).

Mice lacking GMAP-210 in hematopoietic stem cell derivatives, which include osteoclasts, (i.e. Tg:Vav1-Cre;Trip11\textsuperscript{cko/-};ROSA26\textsuperscript{mTmG/+}), were born at the expected Mendelian frequency and appeared normal (Fig. 7A). Skeletal preparations of P0 mutant (Tg:Vav1-Cre;Trip11\textsuperscript{cko/-};ROSA26\textsuperscript{mTmG/+}) and control pups (Tg:Vav1-Cre;Trip11\textsuperscript{cko/+};ROSA26\textsuperscript{mTmG/+}) showed that Trip11 inactivation throughout the hematopoietic lineage had no affect on the size or the mineralization of either endochondral or intramembranous bones (Fig. 7B-G). Furthermore, histological analysis of sections through the humeri of mutant and control P0 pups showed normal amounts of trabeculae, cortical bone and bone marrow (Fig. 8A). By EM, osteoclasts lacking GMAP-210 exhibited no swelling of the ER cisternae and had normal GA architecture (Fig. 8B). The absence of a bone phenotype was further confirmed by µCT on 8-week-old control and mutant mice (Fig. 8B and Fig. S1B).

Since the Vav1-Cre transgene is also active in immunoglobulin-producing lymphocytes, we analyzed IgG levels in serum from mutant and control 8-week-old mice. Coomassie blue staining of SDS-PAGE separated serum did not reveal any differences in the IgG band intensity between mutant and control mice implying that inactivation of Trip11 does not impair IgG secretion (Fig. S2).

To confirm that the Vav1-Cre transgene recombined the Trip11\textsuperscript{cko} allele, we isolated hematopoietic precursors from the bone marrow of mutant and control 8-week-old mice and extracted protein. A Western blot using a GMAP-210 specific antibody showed the total absence of GMAP-210 in Tg:Vav1-Cre;Trip11\textsuperscript{cko/-};ROSA26\textsuperscript{mTmG/+} hematopoietic precursors (Fig. 8D).

Together these data show that absence of GMAP-210 does not interfere with either osteoblast or osteoclast function.

A subset of secreted proteins is retained in GMAP-210 deficient chondrocytes.

Massive expansion of ER cisternae is a prominent feature in GMAP-210 deficient chondrocytes. Chondrocytes produce large amounts of extracellular matrix proteins, so ER swelling could be a consequence of globally impaired membrane trafficking. However, osteoblasts, which also produce abundant extracellular matrices, have normal appearing ER when GMAP-210 is absent. Similarly, the ER of the highly secretory osteoclasts is also unaffected by the absence of GMAP-210. Furthermore, when we inactivated Trip11 specifically in the acinar cells of the adult pancreas, which secrete massive amounts of digestive enzymes, using the Mist1-CreERT2 transgene, we did not observe any swelling of the ER cisternae in these cells (Fig. S3).
Therefore, to determine whether the ER retention in chondrocytes is due to impaired trafficking of specific proteins, we cultured primary chondrocytes from mice with conditional alleles, inactivated the alleles *ex vivo* and then compared these chondrocytes intracellular proteomes to those of controls. Pellet cultures of primary chondrocytes from Tg:Cag-Cre/Esr1;*Trip11*^cko/−;*ROSA26*TmG/^+^ mice and control littermates were treated with 4-OH tamoxifen for 2 weeks while they were actively producing cartilage matrices. We confirmed GMAP-210 was depleted in the cells by western blot (Fig. 9A). Histologic sections through the pellet cultures demonstrated that they contained differentiated chondrocytes that had produced a glycosaminoglycan rich matrix that stains with Alcian blue (Fig. 9B). Interestingly, by EM, experimental and control chondrocytes exhibited ER swelling. However, Golgi swelling and disappearance of the GA stack structure was only evident in the chondrocytes lacking GMAP-210 (Fig. 9C). For mice with global GMAP-210 deficiency, we previously observed that Perlecan (HSPG2) was retained within their chondrocyte ER (Smits et al., 2010). Therefore, we performed western blotting to immunodetect Perlecan in chondrocytes from the pellet culture experiments; we observed that Perlecan levels also increased in chondrocytes whose *Trip11* expression had been inactivated *ex vivo* (Fig. 9A).

We next compared the proteomes of chondrocyte pellet cultures in which GMAP-210 had, or had not, been depleted *ex vivo*. A total of 7636 different proteins were detected (Table S1). Comparison between mutant and control cultures confirmed the reduction in GMAP-210 levels in mutant cultures and revealed a statistically significant (i.e. passing both student’s t-test and Benjamini-Hochberg FDR) reduction in COL10A1 protein levels, indicating that, similar to what we observed *in vivo*, chondrocytes lacking GMAP-210 fail to undergo hypertrophic differentiation *in vitro* (Fig. 9D). In addition to GMAP-210 and COL10A1, the intracellular levels of 62 proteins were significantly lower in GMAP-210 depleted chondrocytes, while the intracellular levels of 100 proteins were significantly higher in GMAP-210 depleted chondrocytes. Of the latter group, 39 proteins have an established or predicted role in membrane trafficking and/or Golgi/ER function (Table S2).

With regard to cartilage extracellular matrix proteins, only CHADL (chondroadherin-like protein) had a statistically significant increased intracellular protein abundance in GMAP-210 depleted chondrocytes after controlling for multiple hypothesis testing. However, using student’s t-test, Perlecan, COL9A2, Aggrecan, Matrillin-4 and Nidogen-2 were significantly increased in GMAP-210 depleted chondrocytes compared to controls while Nidogen-1 and Decorin were significantly decreased (Fig. 9E). We confirmed the increased retention of Perlecan and COL9A2 in GMAP-210 depleted cells by western blot (Fig. 9A and Fig. S4). Interestingly, the intracellular levels of many abundant cartilage extracellular matrix proteins, including COL2A1, COL9A1, Fibromodulin, Cartilage oligomeric matrix protein, Biglycan, Link protein, Matrillin-3, and Asporin, were not
significantly affected by GMAP-210 depletion, (Fig. S5A). Furthermore, the intracellular levels of more broadly expressed extracellular matrix proteins, including COL1A1 and Laminins beta 1 and 2, were also unaffected.

These findings show that inactivation of Trip11 despite impairing GA structure does not result in a general secretion defect in chondrocytes, but instead affects the secretion of a specific set of proteins.

Discussion

Despite its importance for cellular function, most studies on membrane trafficking have been performed in yeast or cultured cells. Only recently have studies began to address this critical process in complex environments such as tissues or multi-cellular organisms like fish and mammals. Thus, there remains limited knowledge regarding cell-type and tissue-specific aspects of membrane trafficking. Tissue specificity in membrane trafficking is exemplified in the lethal skeletal dysplasia ACG1A, which is caused by mutations in TRIP11, the gene encoding the ubiquitously expressed cis-Golgin GMAP-210. Despite the ubiquitous expression of this vesicle-tethering factor, humans lacking GMAP-210 survive to birth with a phenotype that appears limited to the skeletal system. GMAP-210 has no closely related family members. Two other cis-Golgins have been identified in the human and mouse genomes (GM-130 and Golgin-160), but both of these golgins are ubiquitously expressed, making compensation by other cis-Golgins an unlikely explanation for the absence of a phenotype in other tissues (Munro, 2011). In this study we have made use of mice carrying conditional Trip11 null alleles to gain insight into how tissue specificity is accomplished in ACG1A.

We found that inactivation of Trip11 specifically in chondrocytes completely recapitulates the ACG1A phenotype. Tg:Col2a1-Cre;Trip11\textsuperscript{cko/−};ROSA26\textsuperscript{mTmG/+} mice display a severe and lethal skeletal dysplasia whose features are identical to ACG1A. This includes the delay in mineralization of the vertebral column and the bones of the skull, thus showing that these features are a secondary consequence of the cartilage phenotype and not a cell autonomous bone phenotype. This was further confirmed by the finding that Trip11 inactivation specifically in bone forming osteoblasts or bone resorbing osteoclasts (indirectly through inactivation in hematopoietic precursors) did not result in any detectable bone phenotype. Our data therefore show that the skeletal phenotype of ACG1A is caused exclusively by the absence of GMAP-210 in chondrocytes and that this ubiquitously expressed cis-Golgin is not essential in osteoblasts and osteoclasts.

Chondrocytes secrete a large volume of protein to form their abundant extracellular matrix. However, our data would seem to exclude GMAP-210 causing a general dysfunction in protein
trafficking since other highly secretory cell types such as osteoblasts, osteocytes, pancreatic acinar cells, and B-lymphocytes appear to function normally in the absence of GMAP-210. Thus the effect of GMAP-210 deficiency more likely depends upon the type of cargo produced by a cell, rather than that cell’s cargo volume. We addressed cargo specificity by comparing the proteomes of primary chondrocyte pellet cultures in which GMAP-210 was either absent or present. We found that lack of GMAP-210 results in the intracellular accumulation of proteins with an established or predicted role in membrane trafficking and/or Golgi/ER function, confirming that GMAP-210 depletion has a disruptive effect on the membrane trafficking process. However, despite this, absence of GMAP-210 does not lead to a general secretion defect, but instead affects secretion of a subset of extracellular matrix proteins. The affected cargoes do not share a common feature, however three of them (CHADL, Aggrecan and COL9A2) are highly and specifically expressed by chondrocytes, with Aggrecan being the most highly expressed protein in chondrocytes (using immunofluorescence microscopy, we previously did not observed intracellular retention of Aggrecan in vivo (Smits et al., 2010), however retention may have been masked by the extremely high abundance of Aggrecan in cartilage). Because of the chondrocyte specific expression of these three proteins it is tempting to conclude that their intracellular accumulation is mainly responsible for the ACG1A phenotype. Creating double knockout (i.e., Trip11/Chadl, Trip11/Acan, or Trip11/Col9a2) embryos and then looking for an improvement in the skeletal phenotype could be a way to conclusively assess whether intracellular accumulation of a specific cargo is responsible for the ACG1A phenotype.

Recently, multiple studies have investigated the mechanism by which GMAP-210 recognizes ER derived vesicles. This would occur through its N-terminal Amphipathic Lipid Packing Sensor (ALPS) domain. The ALPS is a membrane-binding domain sensitive to membrane curvature, i.e. it is able to bind curved membranes, but has a much lower affinity for flat membranes. It allows GMAP-210 to distinguish between round small transport vesicles and larger flatter membrane structures such as Golgi cisternae (Drin et al., 2007; Drin et al., 2008; Wong et al., 2017). It has been suggested that the ALPS motif binds to the surface of lipid membranes by the random insertion of its hydrophobic residues into so-called lipid packing defects (i.e. voids) present in lipid membranes (Magdeleine et al., 2016; Vamparys et al., 2013; Vanni et al., 2013). The properties of the ER membrane (low sterol, low electrostatics and high level of unsaturation) in combination with a high curvature would lead to a high level of packing defects on ER to Golgi transport vesicles. This would enable efficient binding of these vesicles to GMAP-210 (Magdeleine et al., 2016; Vanni et al., 2013). As the membranes of vesicles derived from the trans side of the Golgi, or other organelles, have a different composition with less packing defects, they would not be recognized by GMAP-210 (Klemm et al., 2009; Magdeleine et al., 2016). Based on these studies it was proposed
that GMAP-210 sorts transport vesicles at the cis-Golgi based on size and lipid composition but would not be able to distinguish vesicles containing different cargos (Magdeleine et al., 2016). One would expect that if GMAP-210 functions as a broad filter for transport vesicles, its absence would lead to a general secretion defect. This is contradicted by our proteome data, which shows that only a select few cartilage extracellular matrix proteins accumulate intracellularly in the absence of GMAP-210. This suggests that mechanisms must exist through which GMAP-210 is capable of recognizing transport vesicles containing a specific cargo.

In summary, we have shown that the skeletal phenotype of ACG1A is caused exclusively by a chondrocyte defect. Furthermore, our data indicate that the defective cartilage is not the result *per se* of the high volume of cargo secreted by chondrocytes, but is instead a consequence of the role of GMAP-210 in the intracellular transport of a subset of cargo, which includes proteins specifically expressed by chondrocytes.

**Materials and Methods**

*Generation of mice carrying Trip11 conditional knockout alleles*

All animal experiments were done under protocols reviewed and approved by Childrens Hospital Boston Institutional Animal Care and Use Committee.

A targeting vector, using pBluescript II KS(+) (Promega) as backbone, was constructed in which the first exon of *Trip11* (which contains the ATG) was flanked with LoxP sites (Fig. S5B). To avoid disrupting important upstream regulatory sequences, the locations of the LoxP sites were chosen based on low evolutionary conservation of the DNA sequence between species. The 5’ LoxP site was inserted 671 basepairs (bp) upstream of exon 1. An Frt site flanked neomycin selection cassette immediately followed by the 3’ LoxP site was inserted 1,506 bp downstream of exon 1. Five kb and 3 kb 5’ and 3’ homology arms were used, respectively. A thymidine kinase negative selection cassette was inserted downstream of the 3’ homology arm.

The targeting vector was electroporated in 129SV ES cells and correctly targeted neomycin resistant colonies were confirmed by long-range PCR (Roche Long template PCR system) using two primer pairs. One pair had a forward primer (5’-GCATCGCATTGTCTGAGTAGGTGTC-3’) located in the neomycin selection cassette and a reverse primer located outside of the 3’ arm of homology (5’-GAAAGAAACTGAAGCAGGGGAGCTGAG-3’). The other pair had a forward primer located outside the 5’ arm of homology (5’-CTCCAGGTCTGTCCTGTGAGGGATG-3’) and a reverse primer that anchored within the 5’ LoxP site (5’-CATTGGGGGTGGGGTTGACGAATAAC-3’ (ATAAC = LoxP sequence)). One of several correctly targeted clones with normal karyotypes was used to generate the mouse strain containing the conditional allele. Once germline transmission
was achieved, the neomycin selection cassette was excised using FLPeR mice (Jackson laboratories) (Farley et al., 2000). Thus, mice used in the experiments described below have exon 1 of Trip11 floxed, and no longer have a neomycin cassette. This allele is designated as Trip11cko, and is genotyped using a primer pair that flanks the 3’ LoxP site (FP: 5’-GGAAAGACTAGCTAGAGATTGAAC-3’; RP: 5’-TGGCTCTTTACTGGACACATGAAG-3’).

We generated a global Trip11 knockout allele, here designated as Trip11−, using the Ella-Cre transgene (Jackson laboratories) (Lakso et al., 1996) to excise the floxed exon 1. Mice with this Trip11− allele are identified using a primer pair with a forward primer located upstream of the 5’ LoxP site (5’-AGTCTCTGGATTTGATCTTCAGCAC-3’) and a reverse primer located downstream of the 3’ LoxP site (5’-TGGCTCTTTACTGGACACATGAAG-3’).

Although we targeted Trip11 in 129SV ES cells, we maintained the Trip11cko and Trip11− alleles by crossing them into C57BL/6 mice.

**Tissue-specific and ex vivo inactivation of Trip11**

We conditionally inactivated Trip11 in chondrocytes, osteoblasts, hematopoietic stem cells and exocrine pancreatic cells, using Col2a1-Cre (kindly provided by Dr. Olson, Harvard School of Dental Medicine), Bglap-Cre (Jackson Laboratories, stock # 019509), Vav1-Cre (kindly provided by Dr. Orkin, Childrens Hospital Boston) and Mist1-CreERT2 (kindly provided by Dr. Konieczny, Purdue University) respectively (Ovchinnikov et al., 2000; Stadtfeld and Graf, 2005; Tuveson et al., 2006; Zhang et al., 2002). Since heterozygous Trip11 knockout mice are viable, the different Cre alleles were bred to produce Tg:Col2a1-Cre;Trip11cko/−;ROSA26mTmG/+; Tg:Bglap-Cre;Trip11cko/−;ROSA26mTmG/+; Tg:Vav1-Cre;Trip11cko/−;ROSA26mTmG/+ mice and Tg:Mist1-CreERT2;Trip11cko/−;ROSA26mTmG/+ mice, so that Cre-mediated recombination of only one Trip11 allele is needed to knockout GMAP-210 function in a cell. Tg:Col2a1-Cre;Trip11cko/+;ROSA26mTmG/+, Tg:Bglap-Cre;Trip11cko/+;ROSA26mTmG/+, Tg:Vav1-Cre;Trip11cko/+;ROSA26mTmG/+ and Tg:Mist1-CreERT2;Trip11cko/+;ROSA26mTmG/+ littermates served as controls. For inactivation of Trip11 using the Mist1-CreERT2 transgene, mice were injected intraperitoneally with tamoxifen (Sigma) (0.1mg per g of mouse weight; tamoxifen stock: 10mg/ml in corn oil) 3 times with the injections separated by one day. The ROSA26mTmG allele (Jackson laboratories, stock # 007576) was used to visualize and/or sort Cre-recombined cells based on their having enhanced green fluorescence protein expression (Muzumdar et al., 2007). In order to inactivate Trip11 in primary chondrocyte pellet cultures ex vivo, we used Tg:Cag-Cre/Esr1 mice (Jackson laboratories, stock # 004682) (Hayashi and McMahon, 2002) to produce offspring with the genotype Tg:Cag-Cre/Esr1;Trip11cko/−;ROSA26mTmG/+ from which we isolated chondrocytes.
Skeletal preparation

Alcian blue and Alizarin red staining of intact skeletons were performed as previously described (Smits et al., 2004; Smits et al., 2001). In short, embryos or newborn pups were humanely sacrificed and placed in hot tap water for 10 minutes. The skin was carefully removed using forceps and the animals were eviscerated. Fixation was done in 95% ethanol for 3 days after which the bodies were stained in Alcian blue staining solution (0.015% Alcian blue 8GX (Sigma), 20% Acetic Acid, 76% Ethanol) for 24 hours. The bodies were subsequently washed three times with 95% ethanol (24 hours for each wash). Bodies were cleared in 1% KOH for 24 hours and stained for 12 hours in Alizarin Red staining solution (0.005% Alizarin Red (Sigma), 2% KOH). Bodies were then cleared in 2% KOH for 24 hours. The clearing process was completed using the following ratios of 2% KOH to glycerol (80:20, 60:40, 40:60 and 20:80). Each clearing step was performed for 24 hrs. Pictures of the stained skeletons were taken with Nikon’s NIS-element AR 4.20.01 software, using a Nikon SMZ18 stereomicroscope (10x objective, 0.75x lens) equipped with a Nikon digital sight DS-Ri1 camera.

Histology

Forelimbs of embryos and pups were fixed with 4% paraformaldehyde in PBS for 24 hours and subsequently demineralized for 7 days in 10% EDTA. Samples were dehydrated using a graded series of ethanol (30%, 50%, 70%, 95% and 2 times 100%; 12 hours for each step). Samples were then cleared with xylene (2x for 45 minutes) and saturated in paraffin (2x for 1 hour). After embedding, 7 µm sections were cut.

For Alcian blue staining, sections were deparaffinized with xylene (2x 3 minutes), rehydrated using a graded series of ethanol (2x 100% for 1 minute, 1x 95%, 70%, 50%, 30% ethanol for 30 seconds). Sections were placed in 3% Acetic acid for 3 minutes and stained in Alcian blue staining solution (1% Alcian blue 8GX (Sigma) in 3% Acetic acid) for 30 minutes. Next, sections were washed for 10 minutes under running tap water and counterstained with Nuclear fast red (0.1% Nuclear fast red (Sigma) in 5% Aluminum sulfate) for 5 minutes. Subsequently the sections were washed under running tap water for 1 minute and dehydrated with a graded series of ethanol (1x 30%, 50%, 70% and 95% ethanol for 30 seconds followed by 2x 100% ethanol for 1 minute). Sections were then cleared with xylene (2x for 1 minute), dried and mounted using Permount (Sigma).

Pictures of the stained sections were taken with Nikon’s NIS-element AR 4.20.01 software, using a Nikon Eclipse 80i microscope (10x objective combined with a 10x or 20x lens) equipped
with a Nikon digital sight DS-Ri1 camera. Images of control and mutant samples were adjusted identically for brightness, contrast and sharpness (Adobe photoshop CS6).

**μCT measurements**

μCT measurements of tibia mid-shaft cortical bone and proximal tibial trabecular bone were performed as previously described (Cui et al., 2011; Sawakami et al., 2006). Briefly, the right tibiae from 6-8 week-old male mice were dissected from each carcass and preserved in 70% ethanol for μCT scanning on a Scanco Medical μCT 35 system. Specimens from mice with the *Bglap*-Cre transgene were scanned with an isotropic voxel size of 10 µm at 50 kV tube voltage, 120 mA and a 151-ms integration time and the regions of interest depicted in Fig. 7B were analyzed. Specimens from mice with the *Vav1*-Cre transgene were scanned with an isotropic voxel size of 7 µm using an X-ray tube potential of 55 kVp, an X-ray intensity of 0.145 mA and an integration time of 600 ms. A region beginning 1.4 mm distal to the proximal growth plate and extending 1.4 mm distally was selected for trabecular bone analysis. A second region 3.0 mm proximal to the tibia-fibula junction and 0.7 mm in length was selected for cortical analysis. A semi-automated contouring approach was used to distinguish cortical and trabecular bone. 3D renderings were generated and microstructural properties of bone were calculated using software supplied by the manufacturer.

**Electron microscopy**

Samples were fixed at 4ºC for 48 hr in 2% formaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate buffer, pH 7.4, decalcified for 1 week using 10% EDTA, and re-fixed for 24 hrs. After fixation and decalcification, specimens were treated with 1% osmium tetroxide, 1% uranyl acetate in maleate buffer, dehydrated in a graded ethanol series, treated with propylene oxide and embedded in Taab epon mixture at 60ºC. Blocks were sectioned at 95 nm on a Leica Ultracut microtome and viewed and imaged with a Philips Tecnai Spirit electron microscope (2,900x and 93,000x). Images displayed were adjusted for brightness and contrast to offset bleaching (Adobe photoshop CS6).

**Cultivation of primary mouse embryonic fibroblasts**

Mouse embryonic fibroblasts were prepared using E13.5 *Trip11*<sup>+/−</sup>, *Trip11*<sup>cko+/−</sup> and *Trip11*<sup>−/−</sup> embryos. Yolk sacs were used to confirm the genotypes. The head and internal organs were removed, then each body was placed in a well of a 24 well plate immersed in 1 ml 0.25% trypsin-EDTA (Life Technologies). Disaggregation was achieved by pulling each embryo through an 18G needle 5 times and then digesting for 30 minutes at 37ºC in a 5% CO<sub>2</sub> incubator. Cells were
vigorously pipetted to obtain a single cell solution, which was transferred to 20 ml DMEM/10% FBS with DNAse I (Roche) added to a final concentration of 100 µg/ml and incubated at 37°C for 15 minutes. Cells were then pelleted, washed in PBS, and plated in DMEM/10% FBS on gelatinized 10 cm dishes (one embryo/dish).

Cultivation and cell sorting of primary osteoblasts

Five-day-old Tg:Bglap-Cre;Trip11 cko/+;ROSA26 mTmG/+ and Tg:Bglap-Cre;Trip11 cko-;ROSA26 mTmG/+ littermates were decapitated and their heads placed in sterile PBS. Parietal calvarial bone was recovered and cleaned. After rinsing 3 times in PBS the bone was serially placed in digestion medium (0.625% Collagenase II (Life Technologies)/0.01% Trypsin (Fisher) in PBS) 3 times, for 15 minutes each at 37°C. Calvaria were moved to 1 ml growth medium (DMEM containing 10% fetal bovine serum (GemCell), 100 U/ml Penicillin (Life technologies), 100 µg/ml Streptomycin (Life Technologies)). Pre-osteoblasts were allowed to grow out of the calvaria and were cultured and expanded until they became confluent in 75 cm² culture flasks. Single cell suspensions were then made using digestion medium and GFP expressing cells were recovered by flow sorting (BL2-Aria IIU cell sorter).

Extraction of bone marrow hematopoietic precursors

Eight-week-old Tg:Vav1-Cre;Trip11 cko/+;ROSA26 mTmG/+ and Tg:Vav1-Cre;Trip11 cko-;ROSA26 mTmG/+ littermates were euthanized and their tibias and femurs recovered, washed 3 times in PBS containing 1000 U/ml Penicillin (Life Technologies) and 1 mg/ml Streptomycin (Life Technologies), and then placed in regular PBS. The epiphyses of the bones were cut off and the metaphyses were flushed with 10 ml of PBS using a 25G needle. The flushed cells were filtered through a 48 µm cell strainer and then collected by centrifugation (400 rcf for 5 minutes). Pelleted cells were re-suspended in 10 ml growth medium (alpha-MEM containing 10% fetal bovine serum (GemCell), 100 U/ml Penicillin and 100 µg/ml Streptomycin) and plated in 10 cm² culture dishes. Fibroblasts were allowed to attach for 12 hours to the culture dish at 37°C in a 5% CO₂ incubator after which non-adherent cells were collected, washed with PBS and used for western blotting.

Primary chondrocyte pellet cultures

Seven-day-old Trip11 cko/cko;ROSA26 mTmG/+ and Tg:Cag-Cre/Esr1;Trip11 cko-;ROSA26 mTmG/+ mice were euthanized, and their ribcages recovered and washed 3 times with 2 ml PBS containing 1000 U/ml Penicillin (Life Technologies) and 1 mg/ml Streptomycin (Life Technologies). Ribcages were cleaned of adherent tissue by incubating them in 2 ml digestion medium (DMEM/F12 (Life
Technologies) containing 3 mg/ml collagenase A (Roche), 100 U/ml Penicillin and 100 µg/ml Streptomycin) at 37°C for an hour in a 5% CO₂ incubator. Ribcages were then washed once with PBS, transferred to new digestion medium and incubated at 37°C in a 5% CO₂ incubator. At 4 hours and 6 hours, pipetting was performed to release cells from the digesting tissue. The single cell suspension was filtered through a 48 µm cell strainer into a 50 ml conical tube containing 10 ml complete medium (DMEM/F12 containing 10% fetal bovine serum (GemCell), 100 U/ml Penicillin, 100 µg/ml Streptomycin and 50 µg/ml Ascorbic acid (Sigma)). Cells were pelleted (400 rcf for 5 minutes), washed in PBS, repelleted and resuspended in complete medium containing 4-OH-tamoxifen (Sigma) at a final concentration of 1 µM. Cells were again pelleted, the cap of the 14 ml tube was loosened and the cell pellets were cultured at 37°C in a 5% CO₂ incubator for 14 days under continuous 4-OH-tamoxifen exposure. Medium was replaced every other day. After 14 days, pellets were dissociated by incubating them for 1 hour in digestion medium. Cells were washed once with PBS and used for mass spectrometry and western blot.

**Western blotting**

Cells were lysed on ice in 1 ml mammalian lysis buffer (Promega) containing 1x Protease Inhibitor cocktail (Promega) for 10 minutes. Protein concentration was measured using the Bio-Rad protein DC assay. Ten µg of each sample was separated on a 3-8% Tris-Acetate SDS PAGE gel (Life Technologies) and transferred to Invitrolon 0.45 µm pore PVDF membranes (Life Technologies). Membranes were blocked for 30 minutes in Western breeze blocking buffer (Life Technologies). Primary antibodies were diluted in Western breeze antibody dilution buffer and incubated with the membranes for 1 hour at room temperature. Membranes were then washed 3 times for 5 minutes each with Western breeze wash buffer. Peroxidase-coupled secondary antibodies were diluted in Western breeze antibody dilution buffer and incubated with the membranes for 1 hour at room temperature followed by 3 washes. Membranes were rinsed x 2 with water and incubated for 5 minutes with Tropix CDP star substrate (Applied Biosystems). Immunoreactive bands were visualized using Hyblot CL autoradiography film (Denville).

Primary antibodies used: anti-TRIP11/GMAP-210 10G5 (LifeSpan Biosciences, LS-C20059) at a 1/500 dilution; anti-HSPG2 A76 (Abcam, ab26265) at a 1/500 dilution, anti-COL9A2 (Santa Cruz, sc-398130) at a 1/500 dilution, anti-ACTIN AC-15 (Sigma, A1978) at a 1/15,000 dilution and anti-TUBULIN (Sigma, T8203) at a 1/1000 dilution. Secondary antibodies used: peroxidase-coupled goat anti-mouse IgG (Thermo Scientific, 31320) at a 1/10,000 dilution.
**SDS-PAGE of plasma samples**

Blood was extracted using a 1ml syringe with an 18G needle from the heart of euthanized mice immediately after death. Blood samples were collected in 1ml MiniCollect Lithium/Heparin tubes (Greiner-bio), spun down at 400 rcf for 15 minutes to separate serum from plasma. Plasma aliquots were added to 1x SDS-PAGE loading buffer/denaturation solution (Thermo Scientific), denatured at 70°C for 10 minutes and separated on a 3-8% Tris-Acetate SDS-PAGE gel (Thermo Scientific). After electrophoresis, gels were washed 3 times 5 minutes with water, stained with Coomassie blue (SimplyBlue Safe stain, Thermo Scientific) and destained with water for 24 hrs.

**Sample preparation, quantitative mass spectrometry, and data analysis**

Chondrocyte cell pellets were resuspended in lysis buffer (8 M urea, 150 mM NaCl, 200 mM EPPS pH 8.5) with complete protease inhibitors (Roche), and lysed by syringe pumping (n\textsubscript{pumps} = 10) cells through a 25\textsubscript{g}8 gauge needle. Protein lysates were reduced (5 mM TCEP) and alkylated (15 mM iodoacetamide) before chloroform/methanol precipitation of the protein lysate. Proteins were resuspended in 200 mM EPPS pH 8.5 and digested with LysC overnight (Wako, room temperature), followed by a 6-hour trypsin digestion (Promega, 37°C). For each biological replicate, 100 µg of peptide was labeled with tandem mass tag (TMT, Thermo Scientific) reagents at a 2:1 (w/w) TMT:peptide ratio. After a 1.5 hours at room temperature the reaction was quenched (0.5% hydroxylamine) and the peptide samples were mixed. Mixed samples were fractionated by high-pH reverse phase chromatography for a final total of 12 pooled fractions spanning the gradient (Yang et al., 2012). Fractionated peptides were desalted by in-house made C\textsubscript{18} stage-tips and loaded on an in-house pulled C\textsubscript{18} analytical column (35cm total length with 100um, 2.6Å beads) before being injected into an Orbitrap Fusion Lumos mass spectrometer running an LC-MS/MS/MS top-10 data dependent method using synchronous-precursor-selection-MS\textsuperscript{3} for TMT quantification (McAlister et al., 2014). Peptide-spectral matches were determined using SEQUEST which identified 102693 total peptides across 12 pooled fractions (Eng et al., 1994). Peptide spectral matches were filtered to a peptide and protein false discovery rate (FDR) both less than 1%. Significance of protein quantitative results was determined based on a Student’s t-test, and multiple test correction of p-values was done according to Benjamini-Hochberg FDR less than 0.05 (Benjamini and Hochberg, 1995). Data analysis and quantitative proteomics plotting was done with the R programming language (https://www.R-project.org/).
Competing interests
No competing interests declared.

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References


Figure 1: Mice homozygous for the recombined Trip11 conditional allele (Trip11⁻/⁻) have a severe skeletal dysplasia. A) E17.5 wild-type (Trip11⁺/+⁻) and recombined (Trip11⁻/⁻) embryos. Note the short limbs, short snout, domed skull, protruding tongue and omphalocele in the recombined (i.e., knockout) embryo. B-G) Skeletal preparations of the embryos shown in A. Compared to the Trip11⁺/+⁻ embryo, the Trip11⁻/⁻ embryo has B) a small ribcage; C) decreased mineralization of the calvarium (arrow); D) absent mineralization of the sternum (arrow); E) absent mineralization of the vertebral bodies (arrow); F) Short forelimbs; and G) short hindlimbs. N=3, one representative result is shown.
Figure 2: Swelling of ER cisternae and disruption of the Golgi stack structure in chondrocytes of mice homozygous for a recombined Trip11 conditional allele (Trip11⁻/⁻). A) Alcian blue stained sections through the humeri of E15.5 wild-type (Trip11⁺/⁺) and knockout (Trip11⁻/⁻) embryos. Note the delay in the formation of the primary ossification center in the knockout. Bottom panels: higher magnification of columnar chondrocytes. Note the swollen appearance of chondrocytes in the knockout. B) Transmission electron microscopy pictures of epiphyseal chondrocytes from the humeri of E15.5 wild-type and knockout embryos (top: 2900x, bottom: 9300x). Note the increased size of ER cisternae in the knockout chondrocytes (top panels, arrow) and the disruption of the Golgi stack structure (bottom panel, arrow). C) Western blot with a GMAP-210 specific antibody on the cell lysates of mouse embryonic fibroblasts extracted from E13.5 wild-type, Trip11⁻/⁺ and knockout embryos. Note the complete absence of GMAP-210 protein in the knockout (-/-) cell lysate. N=3, one representative result is shown.
Figure 3: Inactivation of *Trip11* in chondrocytes recapitulates the skeletal dysplasia seen in germline knockout mice. A) Control (Tg:Col2a1-Cre;Trip11cko/+) and chondrocyte knockout (Tg:Col2a1-Cre;Trip11cko/-) newborn pups. Note the short snout, domed skull and short limbs in the chondrocyte knockout. B-G) Skeletal preparations of the pups shown in A. Compared to the control embryo, the embryo with the chondrocyte-specific deletion of *Trip11* has short C) forelimbs and D) hindlimbs; E) a small ribcage, F) delayed mineralization of the vertebral body (arrow) and G) decreased mineralization of the skull (arrows). N=3, one representative result is shown.
Figure 4: Specific inactivation of *Trip11* in chondrocytes causes swelling of ER cisternae and disruption of the Golgi stack structure. A) Alcian blue stained sections through the humeri of E15.5 control (*Trip11*^cko/+^) and chondrocyte knockout (Tg:*Col2a1*-Cre; *Trip11*^cko/-^) embryos. Inserts: higher magnification of columnar chondrocytes showing the delay in the formation of the primary ossification center and the swollen appearance of some chondrocytes in the humerus of the chondrocyte knockout. B) Alcian blue staining of sections through the humerus of P0 control (Tg:*Col2a1*-Cre; *Trip11*^cko/+^) and chondrocyte knockout (Tg:*Col2a1*-Cre; *Trip11*^cko/-^) pups. Inserts: higher magnification of columnar chondrocytes. Note the swollen appearance of chondrocytes in the humerus of the chondrocyte knockout. C) Transmission electron microscopy pictures of epiphyseal chondrocytes from the humerus of P0 control (Tg:*Col2a1*-Cre; *Trip11*^cko/+^) and chondrocyte knockout (Tg:*Col2a1*-Cre; *Trip11*^cko/-^) pups (top: 2900x, bottom: 9300x). Note the increased size of ER cisternae in the chondrocyte knockout (ER) and the disruption of the Golgi stack structure (arrow). D) Haematoxylin staining of sections through the lungs of P0 control (Tg:*Col2a1*-Cre; *Trip11*^cko/+^) and chondrocyte knockout (Tg:*Col2a1*-Cre; *Trip11*^cko/-^) pups. Bottom:
higher magnification of the alveoli. Note the impaired alveolar development in the mutant lung. N=3, one representative result is shown.
Figure 5: Specific inactivation of *Trip11* in osteoblasts does not result in a skeletal dysplasia. 
A) five-month-old control (Tg: *Bglap-Cre; Trip11*^cko/+; *ROSA26*^mTmG/+*) and osteoblast knockout (Tg: *Bglap-Cre; Trip11*^cko/-; *ROSA26*^mTmG/+*) mice are indistinguishable. B-F) Skeletal preparations of E17.5 control (Tg: *Bglap-Cre; Trip11*^cko/+; *ROSA26*^mTmG/+*) and osteoblast knockout (Tg: *Bglap-Cre; Trip11*^cko/-; *ROSA26*^mTmG/+*) embryos. B) Whole skeletal preparations show no size difference between control and osteoblast knockouts. C) Higher magnifications of the forelimbs D) hindlimbs, E) sternums F) lumbar vertebrae and G) skulls showing no differences between control and osteoblast knockout mice. N=3, one representative result is shown.
Figure 6: Specific inactivation of Trip11 in osteoblasts does not result in swelling of ER cisternae in their osteocyte descendants, but does disrupt Golgi stack structure. A) Alcian blue stained sections through the humeri of P0 control (Trip11<sup>cko/+</sup>;ROSA26<sup>mTmG/+</sup>) and osteoblast knockout (Tg:Bglap-Cre;Trip11<sup>cko/-</sup>;ROSA26<sup>mTmG/+</sup>) littermates. Left: low magnification of the primary ossification centers; Right: higher magnification of the primary spongiosa of the proximal growth plates. No differences between control and osteoblast knockout are observed. B) µCT of the tibias of 6 week old control (1: Trip11<sup>cko/+</sup>;ROSA26<sup>mTmG/+</sup>) and osteoblast knockout (Tg:Bglap-Cre;Trip11<sup>cko/-</sup>;ROSA26<sup>mTmG/+</sup>) mice shows the mice have similar trabecular and cortical bone architecture. C) Transmission electron microscopy pictures of calvarial osteocytes from P5 control (Trip11<sup>cko/+</sup>;ROSA26<sup>mTmG/+</sup>) and osteoblast knockout (Tg:Bglap-Cre;Trip11<sup>cko/-</sup>;ROSA26<sup>mTmG/+</sup>) pups. Top panels: magnification 2900x; bottom panels: magnification 9300x. Note the absence of swelling of ER cisternae in control and mutant osteocytes (white arrows), but note that Golgi cisternae (black arrows) are increased in size and not organized into stacks in the mutant. D) Western blot analysis of lysates from GFP sorted primary calvarial outgrowth cultures established
from 5 day old control (Tg:Bglap-Cre;Trip11^cko/+;ROSA26^mTmG/+)) and osteoblast knockout (Tg:Bglap-Cre;Trip11^cko/-;ROSA26^mTmG/+)) mice. (lane 1) GFP negative control cells; (lane 2) GFP positive control cells; (lane 3) GFP negative osteoblast knockout cells and (lane 4) GFP positive osteoblast knockout cells. Note the absence of GMAP-210 protein in GFP positive osteoblast knockout cells. Re-probing of the western blot with an anti-actin antibody serves as a loading control. N=3, one representative result is shown.
Figure 7: Specific inactivation of *Trip11* in the hematopoietic lineage does not affect viability or cause skeletal dysplasia. A) Eight week old male control (Tg:Vav1-Cre;*Trip11*cko/*;ROSA26mTmG/+)) and blood cell and osteoclast knockout (Tg:Vav1-Cre;*Trip11*cko/-;ROSA26mTmG/+) mice. No dwarfism is present in the blood cell and osteoclast knockout animal. B-F) Skeletal preparations of P0 control (Tg:Vav1-Cre;*Trip11*cko/+;ROSA26mTmG/+) and blood cell and osteoclast knockout (Tg:Vav1-Cre;*Trip11*cko/-;ROSA26mTmG/) newborns. B) Whole skeletal preparations showing the mice are indistinguishable. Higher magnifications of the C) forelimbs, D) hindlimbs, E) sternum, F) lumbar vertebrae and G) skulls showing no differences between control and blood cell and osteoclast knockout mice. N=3, one representative result is shown.
Figure 8: Inactivation of *Trip11* in osteoclasts does not result in swelling of ER cisternae and does not disrupt Golgi stack structure. A) Alcian blue stained sections through the humeri of P0 control (Tg:Vav1-Cre;Trip11<sup>cko/+</sup>;ROSA26<sup>mTmG</sup>/) and blood cell and osteoclast knockout (Tg:Vav1-Cre;Trip11<sup>cko/-</sup>;ROSA26<sup>mTmG</sup>/) pups. Bottom: higher magnification of the primary spongosia of the proximal growth plates. No differences between mutant and control can be observed. B) µCT of the tibias of 8-week-old control (Tg:Vav1-Cre;Trip11<sup>cko/+</sup>;ROSA26<sup>mTmG</sup>/) and mutant (Tg:Vav1-Cre;Trip11<sup>cko/-</sup>;ROSA26<sup>mTmG</sup>/) mice. No differences in the density of trabeculae or in the thickness of the bone collar can be observed between mutant and control mice. C) Transmission electron microscopy pictures of tibial osteoclasts from 8-week-old control and blood cell and osteoclast knockout mice. Left panels: magnification 2900x; Right panels: magnification 9300x. Note the presence of normal ER cisternae (arrows, top panels) and normal Golgi (arrows, bottom panels) in both mice. D) Western blot analysis of lysates of isolated bone marrow hematopoietic precursor cells from 8-week-old control (lane 1) and blood cell and osteoclast knockout (lane 2) mice. Note
the absence of GMAP-210 protein in the knockout mice. Re-probing of the western blot with an anti-actin antibody serves as a loading control. N=3, one representative result is shown.
Figure 9: *Ex vivo* inactivation of *Trip11* in primary chondrocyte pellet cultures. A) Western blot of cell lysates from 4-OH tamoxifen treated primary chondrocyte pellet cultures using antibodies against Perlecan (HSPG2) and GMAP-210. Lysates come from a control (lane 1) *Trip11*<sup>cko/cko</sup>;ROSA26<sup>mTmG</sup>/+, and from 2 different induced *Trip11* knockout (lanes 2 and 3) pellet cultures; (lane 2) Tg:CagCre/Esr1;*Trip11*<sup>cko/cko</sup>;ROSA26<sup>mTmG</sup>/+ and (lane 3) Tg:CagCre/Esr1;*Trip11*<sup>cko/-</sup>;ROSA26<sup>mTmG</sup>/+. Note the reduction in immunodetectable GMAP-210 and the increase in HSPG2 when *Trip11* was inactivated. Re-probing of the western blot with an anti-actin antibody serves as a loading control. B) Alcian blue stained sections through 4-OH tamoxifen treated control (Tg:CagCre/Esr1;*Trip11*<sup>cko/+-</sup>;ROSA26<sup>mTmG</sup>/+) and *Trip11* inactivated (Tg:CagCre/Esr1;*Trip11*<sup>cko/-</sup>;ROSA26<sup>mTmG</sup>/+) primary chondrocyte pellet cultures. Both control and *Trip11* inactivated cultures produce abundant extracellular matrix. C) Representative electron microscopy images of 4-OH tamoxifen treated control and *Trip11* inactivated primary chondrocyte
pellet cultures. **Top panels**: 4800x. Note both chondrocytes have enlarged endoplasmic reticulum (ER) cisternae. **Bottom panels**: 9300x. Note the Golgi apparatus (white arrows) is enlarged and more disorganized and in the *Trip11* inactivated chondrocytes. **D** Abundance of GMAP-210 and COL10A1 in lysates from 4-OH tamoxifen treated control (red) and *Trip11* inactivated (yellow) primary chondrocyte pellet cultures as determined by tandem tag mass spectroscopy. T-Test: student’s t-test adjusted p-value. TMT-RA: Tandem mass tag relative abundance. **E** Graph depicting extracellular matrix proteins whose intracellular abundance increased or decreased in 4-OH tamoxifen treated *Trip11* inactivated (yellow; Tg: *CagCre/Esr1;Trip11cko/cko;ROSA26mTmG/+*) versus control (red; (*Trip11cko/cko;ROSA26mTmG/+*) primary chondrocyte pellet cultures as determined by tandem tag mass spectroscopy. (CHADL: Chondro-adherin like; HSPG2: Perlecan; COL9A2: Type 9 collagen α2 chain; ACAN: Aggrecan; MATN4: Matrillin-4; NID2: Nidogen-2; NID1: Nidogen-1; DCN: Decorin)
**Supplementary Information**

### Figure A

**BV/TV**

Student's t-test: 0.2697

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### Figure B

**BV/TV**

Student's t-test: 0.9769

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Supplemental Figure 1: Absence of GMAP-210 in osteoblasts or osteoclasts does not affect bone formation. 1A) Trabecular BV/TV μCT measurements of the tibia of male control (4 Tg:Bglap-Cre;Trip11^{cko/+};ROSA26^{mTmG/+}) and osteoblast knockout (3 Tg:Bglap-Cre;Trip11^{cko/-};ROSA26^{mTmG/+}) mice. 1B) Trabecular BV/TV μCT measurements of the tibia of male control (three Trip11^{cko/-};ROSA26^{mTmG/+} and two Tg:Vav1-Cre;Trip11^{cko/+};ROSA26^{mTmG/+}) and hematopoietic knockout (Tg:Vav1-Cre;Trip11^{cko/-};ROSA26^{mTmG/+}) mice. Note the absence of a significant difference in BV/TV for both knockout models.
**Supplemental Figure 2: Absence of GMAP-210 in lymphocytes does not interfere with IgG secretion.** 0.1 ul of serum, extracted from 8-week old mice of the indicated genotypes, was separated on a 3-8% Tris-Acetate SDS-PAGE gel and stained with Coomassie blue. Note that there is no difference in staining intensity of the IgG heavy chain band between control (1, 2, 4, 5 and 6) and mutant (3, 7 and 8) mice. (litter 1: samples 1, 2 and 3; litter 2: samples 4, 5, 6, 7 and 8).
Supplemental Figure 3: Specific inactivation of Trip11 in the exocrine acinar cells of the pancreas does not result in swelling of ER cisternae. A) Western blot analysis of lysates generated from the pancreas of 8-week old control (1 and 2: Tg:Mist1-CreER;Trip11^{cko/+};ROSA26^{mTmG/ +}) and acinar cell knockout (3: Tg:Mist1-CreER;Trip11^{cko/-};ROSA26^{mTmG/ +}) mice, 1 week after tamoxifen treatment. Note the reduction in GMAP-210 protein levels in the acinar cell knockout. B) Transmission electron microscopy picture of ER cisternae of acinar cells from 12 week-old control (Tg:Mist1-CreER;Trip11^{cko/+};ROSA26^{mTmG/ +}) and acinar cell knockout (Tg:Mist1-CreER;Trip11^{cko/-};ROSA26^{mTmG/ +}) mice, 1 month after their tamoxifen treatment. Magnification 6800x. Note the absence of swelling of ER cisternae in control and mutant mice. N=3, one representative result is shown.
Supplemental Figure 4: Ex vivo inactivation of Trip11 in primary chondrocyte pellet cultures.

A) Accumulation of COL9A2 in GMAP-210 depleted chondrocytes. Western blot of cell lysates from 4-OH tamoxifen treated primary chondrocyte pellet cultures using antibodies against COL9A1 and actin (loading control). Lysates were generated from two separate pellet culture experiments. Lanes 1 and 3: control pellet cultures (Trip11<sup>cko/cko;ROSA26<sup>mTmG/+</sup></sup>); Lanes 2 and 4: 4-OH-tamoxifen induced Trip11 knockout pellet cultures (Tg:CagCre/Esr1;Trip11<sup>cko/-;ROSA26<sup>mTmG/+</sup></sup>). Note the increase in immuno-detectable COL9A2 in the Trip11 inactivated samples.

B) Absence of GMAP-210 does not result in the intracellular accumulation of most extracellular matrix proteins.

Abundance of non-significantly affected extracellular matrix proteins in the lysates of 4-OH tamoxifen treated control (Trip11<sup>cko/cko;ROSA26<sup>mTmG/+</sup></sup>) (red) and mutant (Tg:CagCre/Esr1;Trip11<sup>cko/-;ROSA26<sup>mTmG/+</sup></sup>) (yellow) chondrocyte pellet cultures as determined by tandem tag mass spectroscopy. T-Test: student’s t-test adjusted p-value. B/H: Benjamini-Hochberg adjusted p-value. TMT-RA: Tandem mass tag relative abundance. (COL2A1: Type 2 collagen α1 chain; COMP: Cartilage oligomeric matrix protein; FMOD: Fibromodulin; BGN: Biglycan; HPLN1: Cartilage link protein; COL9A1: Type 9 collagen α1 chain; ASPN: Asporin; LAMB1-2: Laminin beta 1-2; MATN3; Matrilin 3; COL1A1: Type 1 collagen α1 chain)
Supplemental Figure 5: Schematic representation of the method used to generate the *Trip11* conditional (*Trip11*<sup>cko</sup>) and knockout (*Trip11*) alleles. The *Trip11*<sup>cko</sup> targeting vector contained the following features from 5′ to 3′: a 5000 bp 5′ homology arm; a 5′ LoxP site located 672 bp upstream from the start of the ATG containing first exon of *Trip11*; a 2661 DNA fragment (-671 to +1989, 0 = start of transcription) containing the first exon; a FRT flanked neomycin positive selection cassette; a 3′ LoxP site located 1989 bp downstream of the start of transcription; a 3000 bp 3′ homology arm and a Thymidine Kinase (TK) negative selection cassette. Homologous recombination in ES cells generated the *Trip11*<sup>cko-neo</sup> allele. After generation of chimeric mice and germline transmission of the *Trip11*<sup>cko-neo</sup> allele, the conditional (*Trip11*<sup>cko</sup>) allele was generated by the removing the neo cassette using FLPeR mice. Finally a knockout allele (*Trip11<sup>-</sup>*) was generated using Ella-Cre mice. Arrows indicate locations of PCR primers used for genotyping the different alleles.
Supplemental Table 1: All LC-MS/MS/MS identified and quantified proteins and signal-to-noise values for the quantified TMT channels.

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Supplemental Table 2: LC-MS/MS/MS identified and quantified proteins with significantly changed protein abundances between the Trip11 knockout and control cells.

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