Moesin activation controls bone resorption and tunneling nanotube-dependent osteoclast fusion

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Short title: Moesin inhibits osteoclastogenesis

ABSTRACT

Osteoclasts are multinucleated cells unique in their ability to resorb bone. Osteoclastogenesis involves several steps of actin-driven rearrangements that participate not only in the cell-cell fusion process, but also in the formation of the sealing zone, the adhesive structure determining the resorption area. Despite the importance of these actin cytoskeleton-based processes, their precise mechanisms of regulation are still poorly characterized. Here, we found that moesin, a member of the Ezrin/Radixin/Moesin (ERM) protein family, is activated during osteoclast maturation and plays an instrumental role for both osteoclast fusion and function. In mouse and human osteoclast precursors, moesin is negatively regulated to potentiate their ability to fuse and degrade bone. Accordingly, we demonstrated that moesin depletion decreases membrane-to-cortex attachment and enhances formation of tunneling nanotubes (TNTs), F-actin-containing intercellular bridges that we revealed to trigger osteoclast fusion. In addition, via a β3-integrin/RhoA/SLK pathway and independently of its role in fusion, moesin regulates the number and organization of sealing zones in mature osteoclast, and thus participates in the control of bone resorption. Supporting these findings, we found that moesin-deficient mice are osteopenic with a reduced density of trabecular bones and increased osteoclast abundance and activity. These findings provide a better understanding of the regulation of osteoclast biology, and open new opportunities to specifically target osteoclast activity in bone disease therapy.

INTRODUCTION

Bone undergoes continual remodeling that is necessary for skeleton growth, fracture healing and calcium homeostasis. It involves the sequential actions of bone-resorbing osteoclasts, bone-forming osteoblasts and osteocytes, which are tightly regulated in space and time [1–4]. Various conditions, including aging, infections, chronic inflammation and cancer, disturb the balance between bone resorption and formation resulting in bone diseases, the most common being osteoporosis [5–8]. This disease is characterized by increased osteoclast differentiation and activity leading to reduced bone density, alteration of bone structure and pain. Advancing our understanding of osteoclast biology may therefore help to devise novel treatments for osteoclast-related bone diseases [6,9].

Long-lived multinucleated osteoclasts are the exclusive bone-resorbing cells essential for bone homeostasis. They also have immune functions [10] . They differentiate through the concerted action of macrophage-colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) [11]. Postnatal maintenance of osteoclasts is mediated by acquisition of new nuclei from circulating blood cells that migrate towards bones and fuse with multinucleated osteoclasts in contact with the bone matrix [1,12–14]. Although *in vitro* studies suggest that the fate of osteoclasts is to die by apoptosis [11], multinucleated osteoclasts can also undergo fission producing smaller cells, called osteomorphs, that can fuse again to form new osteoclasts [13]. Mature osteoclasts contain up to around 20 nuclei *in vivo* [15] and control of osteoclast fusion appears crucial for bone resorption as the multinucleation degree and the osteoclast size are most often correlated with osteolysis efficiency [5,16,17]. Osteoclast fusion is a highly coordinated process that involves the migration of precursor cells towards one another, establishment of a fusion-competent status and initiation of cell-to-cell contacts, cytoskeletal reorganization, and finally fusion of their membranes to form multinucleated osteoclasts [18]. Upon attachment to bone, mature osteoclasts form an F-actin rich structure crucial for bone resorptive activity called the sealing zone. This bone-anchored adhesion structure demarcates the area of bone resorption from the rest of the environment and consists of a complex assembly of podosomes [19–23]. Each of these steps of osteoclastogenesis involves rearrangements of the actin cytoskeleton, but, the precise mechanism(s) and sequence of events still remain poorly understood [16,24].

Tunneling nanotubes (TNTs) are F-actin-containing intercellular bridges not touching the substrate and enabling the transfer between cells of a wide variety of cargos such as organelles, RNAs, pathogens and misfolded/aggregated proteins [25,26]. While osteoclasts can form TNTs [16,27–30], their characteristics and the molecular actors involved in their formation, stability or function are poorly defined [28–30].

Ezrin, Radixin and Moesin (ERM) proteins compose a family of proteins linking the actin cytoskeleton with the plasma membrane. Thereby, they regulate various fundamental cellular processes that involve the remodeling of the cell cortex such as cell division and cell migration [31–35]. Phosphorylation of a conserved threonine residue in their C-terminal actin-binding

domain activates them by stabilizing their open-active conformation, thereby favoring actin attachment to the plasma membrane. This phosphorylation is mediated by several kinases including the Rho kinase ROCK, the isoenzyme protein kinase C (PKC) and the Ste20-like l-kinase (SLK) [36]. ERM proteins are widely expressed in a developmental and tissue-specific manner, with distinct as well as overlapping distribution patterns and functions [33,37]. In leukocytes, ezrin and moesin are predominantly expressed [38,39], and they have unique or redundant functions in cell adhesion, activation and migration, as well as in the formation of the phagocytic cup and the immune synapse [36,39–41]. Moesin-deficient (*Msn*-/-) mice exhibit T, B and NK cell defects, underscoring an important role for moesin in lymphocyte homeostasis [38,41,42]. In the context of HIV-1 infection, ezrin, and to a lesser extent moesin, are involved in fusion-dependent virus entry and replication [43–45], and in the regulation of the virological synapse and the cell-to-cell fusion process [46,47].

Although different steps of osteoclastogenesis such as cell-cell fusion and sealing zone formation involve rearrangements of the actin cytoskeleton, until now, the role of cortex rigidity and ERM proteins in these processes has not been investigated. Here, we demonstrate that moesin is the major activated ERM during osteoclastogenesis and that its depletion promotes *(i)* the fusion of osteoclast precursors, which correlates with the efficiency of TNT formation and reduced membrane-to-cortex attachment, and *(ii)* the formation of the sealing zones in mature osteoclasts, and consequent bone degradation. In mature osteoclasts, ERM activation is dependent on the β3-integrin/RhoA/SLK pathway. Importantly and consistently with our in vitro results, we report that moesin-deficient mice exhibit an osteopenic phenotype.

RESULTS

TNTs are essential for early stages of the osteoclast fusion process

During early stages of osteoclastogenesis, osteoclast precursors form abundant TNT-like structures prior to cell fusion [16,26,30]. Here, to directly test the implication of TNTs in osteoclast fusion per se, we used two complementary osteoclast models that differentiate with different kinetics: (i) osteoclasts derived from human blood monocytes (hOCs) and (ii) murine osteoclasts (derived from an immortalized myeloid cell line, mOCs) (**Figure S1A**) [48,49]. In both models, F-actin staining showed the presence of podosomes (F-actin dots) but also of TNT-like structures at early stages (Day 3) of differentiation, whereas zipper-like F-actin structures, as described between osteoclasts [50,51], were more apparent during the later stages between adjoining multinucleated cells (Day 10 and 7 for hOCs and mOCs, respectively, **Figure 1A-B**). According to the shared definition of TNTs [25,26,30,52,53], we quantified TNTs as F-actin-positive structures that connect at least two cells and that do not adhere to the glass coverslip. Thick TNTs were classified based on their diameter (≥2 μm) and the presence of microtubules, versus thin TNTs, which were <2 μm and devoid of microtubules (**Figure S1B**), as previously defined [54]. We also noticed that thick TNTs were usually positioned higher with respect to the substrate than the thin ones (**Figure 1C** and **S1B)**. Both thin and thick TNTs were observed throughout the early stages of cell-cell fusion (**Figure 1C**, **S1B, movies 1&2**). As osteoclast maturation progressed, the percentage of cells forming thick TNTs decreased, whereas those forming thin TNTs was unchanged in both models (**Figure 1C**). Using live imaging experiments in hOCs (**Figure 1D**, **S1C** and **movies 3-5**), we showed that the contact of a cell emitting a TNT-like structure with its cell partner and fusion of their cytoplasms took place within 90 min. Thus, these results demonstrate that TNTs participate in the cell-cell fusion process and suggest that thick TNTs are preferentially required during the early stages of osteoclast formation.

Moesin depletion increases the fusion capacities of osteoclasts

ERM proteins link the actin cytoskeleton to the plasma membrane and thereby regulate the formation of F-actin-based structures, we thus investigated the potential contribution(s) of ERM proteins during osteoclast fusion. First, we confirmed that all three proteins ERM were expressed throughout RANKL-induced osteoclast differentiation in both mOCs and hOCs, confirming previous observations [55,56] (**Figure S2A-B**). Interestingly, we observed a strong increase in ERM activation status, as measured by the level of ERM phosphorylation (P-ERM) (**Figure 2A-B**), which peaked at Day 5 (mouse)/Day 6 (human), and coincided with the appearance of multinucleated mature osteoclasts (see **Figure 1A-B)**. To evaluate the functions of ERM, we generated mouse cell lines individually depleted for ezrin, radixin or moesin, herein referred to as ezrin, radixin and moesin knock out (KO). In each individual ERM KO, we did not observe compensation from the other ERM proteins (**Figure S2C**). While no difference in the cell-cell fusion was observed in the absence of either ezrin or radixin compared to

controls (**Figure S2D**), deletion of moesin resulted in premature fusion of osteoclast precursors (**Figure 2C** and **movie 6**), leading to a significant increase in the fusion index, in the area occupied by osteoclasts, and in the number of nuclei per multinucleated cell (**Figure 2D-E and S2E**). We also observed a higher number of cells expressing the osteoclast maturation marker β3-integrin on their surface (**Figure S2F**). Consistent with the role of moesin in controlling osteoclast fusion, moesin was the main activated ERM in mOCs (**Figure 2F and S2C**). No significant difference was observed in the mRNA expression levels of osteoclast-marker genes in moesin KO compared with controls (**Figure S2G**), suggesting that the regulation of cell fusion by moesin is not an indirect alteration of osteoclast differentiation. Finally, we found that partial depletion of moesin by siRNA in human monocytes was also associated with a decline of P-ERM level (**Figure S2H-I**) and a significant increase in the fusion of hOCs (**Figure 2G-H**) in keeping with the findings obtained in mOCs. Together, these results imply that moesin functions to restrain cell-cell fusion during osteoclastogenesis.

ERM activation correlates with fusion extent both in osteoclasts and macrophages

To further investigate ERM activation in osteoclast fusion, we next synchronized this process using the hemifusion inhibitor lysophosphatidylcholine (LPC) that reversibly blocks membrane merging [57–59]. Accumulation of ready-to-fused mononuclear cells correlated with an increase in the level of P-ERM (**Figure S3A-C,** +LPC). Following the washout of the drug, which synchronized fusion events, the phosphorylation of ERM returned to baseline levels prior to treatment (**Figure S3A-C**, +/-LPC), suggesting that reduced level of ERM activation may promote osteoclast fusion.

We next explored P-ERM levels in different pathological settings known to exacerbate osteoclast fusion such as during inflammation and upon HIV-1 infection [7,60,61]. Murine osteoclasts derived from dendritic cells (DC-OCs) mimic osteoclasts in inflammatory conditions[62], whereby they differentiate into osteoclasts containing more nuclei compared to those derived from monocytes (MN-OCs)[61]. We found that ERM phosphorylation was significantly diminished in DC-OCs compared to their "classical" osteoclast counterparts (**Figure S3D**). Moreover, ERM phosphorylation was diminished in hOCs undergoing formation of HIV-1-positive giant syncytia (**Figure S3E-F**). Interestingly, the results were recapitulated when macrophage fusion was induced by HIV-1 infection leading to the formation of multinucleated giant cells [63,64] (**Figure S3G-H**), implying that the effect of reduced ERM activation is a general phenomenon of cell-cell fusion that extends beyond osteoclasts. Together, these data indicate that the level of moesin activation is strongly correlated with the extent of osteoclast and macrophage fusion in physiological and pathological contexts.

Enhanced fusion in moesin-depleted osteoclasts is associated with both increased TNT formation and reduced membrane-cortex attachment.

To explore the cellular mechanisms involved in the control of cell-cell fusion by moesin, we next monitored the subcellular localization of moesin and P-ERM (corresponding mainly to P-moesin) during osteoclast differentiation. In hOCs, moesin appeared associated to the plasma membrane, including at TNTs, zipper-like structures, podosome belts or sealing zones (**Figure S4-S5A** and **movie 7**). We also detected accumulation of P-ERM at the tips of a subset of TNTs (**Figure S5A**), leading us to characterize the impact of moesin depletion on the formation of TNTs. Interestingly, TNT formation was increased in the absence or after depletion of moesin in mOCs (**Figure 3A-B** and **S5B**) and hOCs (**Figure 3C-D** and **S5B**), respectively. In agreement with a specific role for thick TNTs (containing microtubules) in osteoclast fusion (**Figure 1**)**,** we found that only the number of cells forming thick TNTs, and not thin TNTs, was affected by moesin inhibition (**Figure 3B** and **3D**). Live imaging on 1:1 mixed cultures of Lifeact-Cherry control and Lifeact-GFP KO cells transfected showed that in the absence of moesin, cells form more TNT-like protrusions (**Figure S5C**).

ERM proteins regulate the physical properties of the membrane and the actomyosin cortex and control a plethora of cellular processes such as the formation of cell protrusions [65,66] and potentially TNTs [67]. As such, we asked whether the physical link between the actomyosin cortex and the plasma membrane (Membrane-to-Cortex Attachment, MCA) was affected by the absence of moesin in mOCs, using atomic force microscopy-based force spectroscopy [68]. Significantly lower forces were required to pull dynamic membrane tethers from moesin KO cells compared to controls (**Figure 3E-F**), corresponding to a 50% decrease in MCA after moesin depletion.

Thus, in osteoclast precursors, reduced levels of moesin are associated with a reduced attachment of the actin cytoskeleton to the plasma membrane, as well as increased fusion and ability to form TNTs. These data suggest that the depletion of the actin-membrane linker moesin could promote onset or stabilization of TNTs by decreasing MCA.

Moesin depletion boosts bone degradation and the formation of sealing zones

Because the bone degradative capacity of osteoclasts usually correlates with their multinucleation and size, we examined the effect of moesin depletion on bone resorption. Accordingly, we found that mOCs differentiated from moesin KO precursors exhibited a ~1.5-fold increase in bone resorption activity compared with control cells (**Figure 4A-B**). By performing mixed cultures of control-mcherry and KO moesin-GFP osteoclasts seeded on glass, we showed that the podosome belts (reminiscent of the sealing zones) were formed, for the majority, by moesin-depleted cells (**Figure S6A**). Of note, mixed color podosome belts were observed, consistent with the hypothesis that the fusion can occur between heterogeneous partners [69,70]. We then assessed the number and the architecture of the sealing zone, which is crucial for bone resorption [20,71]. In moesin KO mOCs seeded on bones, the total area covered by sealing zones was increased (**Figure 4C-D** upper panels, and **S6B**), corresponding to both an increase in the number and the surface covered by individual sealing

zones, without any change in their circularity. In addition, the width of the F-actin-rich region inside the sealing zone was increased (**Figure 4C-D**, lower panels). As expected from the effect of moesin depletion on cell fusion, the number of nuclei inside cells forming sealing zones was increased upon moesin KO (**Figure S6C**). Consistently, siRNA-mediated silencing of moesin in human monocytes recapitulated the effects of moesin KO on bone degradation and sealing zone formation (**Figure 4E-H**).

To investigate whether moesin regulates bone degradation in addition to its role in osteoclast fusion, we uncoupled these two processes. To do so, we depleted moesin using siRNA in preformed mature hOCs (**Figure S7A**) and found no effect on the fusion index (**Figure S7B**). However, under these conditions, the level of expression of moesin and of P-ERM was reduced (**Figure S7C**) which coincided with an increase in bone degradation (**Figure S7D)**. Of note, the two main modes of bone resorption made by hOCs [72] were not differentially affected (**Figure S7E**). Finally, depletion of moesin in late stage of hOC differentiation also favored the formation of sealing zones and increased their thickness (**Figure S7G-H**). Thus, moesin inhibits osteolytic activity at two levels, (i) by controlling the fusion capacity of osteoclasts and (ii) by regulating sealing zone number and structure in a way that modulates the efficiency of the bone degradation machinery.

The RhoA/SLK axis acts downstream of β3-integrin to control ERM activation and sealing zone formation

Next, we explored by which signaling pathway moesin activation regulates the formation of the sealing zone in mature osteoclasts. Key regulators of actin dynamics known to regulate podosome belt and sealing zone dynamics include the small GTPases of the Rho family [73,74], RhoA, Rac1/2 and Cdc42 [9,19,75,76]. RhoGTPase-dependent signaling pathways are also known to regulate the ERM protein activation cycle in other cell types [35,77,78]. First, we tested whether pharmacological inhibition of RhoGTPases affect the activation status of ERM proteins in hOCs. For this, we used the exoenzyme C3 transferase (TATC3), NSC23766, and ML141 that target RhoA, Rac1/2 and Cdc42, respectively. Compared to the strong effects of calyculin A and staurosporine, used as positive and negative controls, respectively (**Figure S8A**), we observed a significant decrease in P-ERM levels only after TATC3 treatment (**Figure 5A and S8B**), suggesting that RhoA is the main small GTPase involved in ERM activation.

Two Ser/Thr kinases, SLK and ROCK, have been described to be activated by RhoA [79–81] and to directly phosphorylate ERM proteins [82–84]. In mature hOCs, treatment with Y-27632, which inhibits ROCK1 and ROCK2, did not impact the level of P- ERM (**Figure S8C**) while downregulation of SLK by siRNA resulted in a significant decrease in ERM activation (**Figure 5B**). Accordingly, the sealing zones in SLK-deficient osteoclasts are thicker than the controls (**Figure 5E-F**), mimicking the effect of moesin depletion (see **Figure 4**).

Finally, to determine the signal that could trigger RhoA/SLK-dependent ERM regulation, we tested the importance of αvβ3 integrin. Indeed, this marker of mature osteoclasts [85,86] mediates their ability to polarize, spread and degrade bone [9,87–89]. We showed that β3-integrin depletion (**Figure 5C**) affects the formation of the sealing zones confirming previous observations [9], with a decrease in their width (**Figure 5E-F**). Importantly, phosphorylation of ERM proteins was enhanced in β3-integrin-depleted cells (**Figure 5C**). Altogether, these results provide evidence that, in mature osteoclasts, ERM activation and sealing zone formation is under the control of the RhoA/SLK axis, downstream of the β3-integrin (**Figure 5D**).

Mice lacking moesin exhibit a low bone mass phenotype and increased osteoclast activity

Finally, to explore the physiological relevance of moesin to osteoclast and bone biology, we assessed moesin expression and function in long bones of mice. As shown by immunohistology experiments on serial sections of femur of wild type (WT*)* mice, we confirmed moesin expression in cathepsin K-positive osteoclasts lining bone surface (**Figure S9A**), in addition to other cells residing within the bone marrow. We next examined the bone phenotype of moesin global knockout mice (*Msn*-/-), that have been previously generated [41]. No overt difference in the size, weight or skeleton of matched littermates up to 40-weeks-of-age was observed (**Figure S9B**). Nonetheless, micro-computed tomographic (µCT) analysis of the distal femurs of 10-week-old male WT and *Msn*-/- mice revealed that the long bones of null mice exhibited trabecular bone loss (**Figure 6A**), as quantified by a significantly lower trabecular bone surface fraction (BS/TV), trabecular thickness and trabecular number associated with an increase in trabecular separation compared to WT mice (**Figure 6B**). Thus, moesin-deficient mice are osteopenic. However, in these mice, cortical parameters were not affected (**Figure S9C**). Histological analysis showed that, in addition to a reduction in bone density, the TRAP-positive signal of osteoclasts was significantly increased in bones of *Msn*-/- mice compared to WT (**Figure 6C-D**), showing that the deletion of moesin increases osteoclast number and activity in bones. These data demonstrate that moesin is involved in osteoclastogenesis *in vivo*.

DISCUSSION

Here, we show that ERM activation, specifically moesin activation, plays a negative regulatory role in osteoclast formation and bone resorption. First, we demonstrate that it acts during the early stages of osteoclastogenesis, by regulating cell-cell fusion via the formation and/or stabilization of TNTs. Second, in mature osteoclasts, activation of moesin, under the control of the β3-integrin/RhoA/SLK axis, regulates osteolysis by impacting the number and structure of the sealing zones. Related to osteoclast dysfunction *in vitro*, mice bearing total *moesin* deletion develop an osteopenic phenotype with increased osteoclast activity. This phenotype is observed in trabecular bones but not cortical ones that are less remodeled in adult mice. Given the intricate interplay between osteoclasts and other bone cells and the ubiquitous expression of moesin, we cannot exclude that moesin also exerts regulatory effects in other cells. However, the preferential effect of moesin on osteoclast activity over differentiation may make it a relevant candidate to control bone loss.

We first demonstrate that moesin activation acts as a novel regulatory mechanism that limits the extent of osteoclast fusion, preventing an excessive number of nuclei per osteoclast, and thus insuring optimal osteolytic activity. Consistent with this hypothesis, we demonstrate that the level of osteoclast fusion under pathological or drug-induced conditions is negatively correlated with the level of ERM activation; LPC-dependent fusion inhibition is associated with enhanced ERM activation while fusion increase in inflammatory context or upon HIV-1 infection is associated with decreased ERM phosphorylation. Moreover, macrophage fusion induced by HIV-1 infection also correlates with downregulation of P-ERM levels, suggesting that the inhibitory effect of ERM activation during cell fusion extends to other myeloid cell types. It would be interesting to know whether phosphorylation of ERM proteins serves as a general regulator of membrane fusion, for example in the formation of myofibers or syncytiotrophoblasts that do not necessarily involve TNT-like structures [16,90]. Depending on the cell type, ERMs have been identified as inhibitors or boosters of the cell fusion process, e.g. activated ezrin prevents the formation of HIV-1 induced T cell syncytia while it promotes trophoblast and myotube fusion [45,46,91–93]. Here, we propose that moesin activation acts as a novel regulatory mechanism that limits the extent of osteoclast fusion, preventing an excessive number of nuclei per osteoclast thus insuring optimal osteolytic activity.

We then propose that moesin acts to control fusion in osteoclasts by limiting the number of thick TNTs. First, our live cell imaging studies provide definitive proof that TNTs play a critical role in osteoclast fusion, as previously suggested [16,28–30,94]. Consistently, a peak in the number of cells emitting TNTs precedes the fusion process. Second, our data strongly suggest that moesin activation controls TNT number during osteoclastogenesis. This novel function of moesin is consistent with the well-known role of ERM proteins in the formation of actin-rich protrusions such as filopodia and microvilli [65,95–97]. Indeed, P-ERM is localized over the entire surface of TNTs where we found cell fusion to occur. How TNT formation contributes to the fusion process remains speculative. A simple explanation could be that the more TNTs are present, the more membrane surface is available for fusion events to occur. Another tempting hypothesis is that TNTs serve as membrane platforms to bridge connections between distal

cells, not necessarily the closest, but probably between ideal fusion-competent partners [98]. Third, we highlight the exclusive involvement of thick TNTs, which allow the microtubule-dependent transport of material between two connected cells [26], in moesin-dependent osteoclast fusion. Thick TNT formation decreases during osteoclast maturation and increases in moesin-depleted cells, showing a correlation between the fusion extent and the number of this subtype of TNTs. This is in coherent with intercellular transports of molecules essential to the fusion process, such as phospholipids and the DC-STAMP protein, which occurs through TNTs in osteoclast precursors [28].

Consistent with the role of ERM proteins in connecting the plasma membrane to the cortical actin network in many cell types [99,100], depletion of moesin in osteoclast precursors strongly decreases membrane-to-cortex attachment while the number of TNT-forming cells and the fusion index increase. Thus, the release of actin from the membrane seems to be a favorable condition for the fusion process, either indirectly by supporting TNT onset, or directly by contributing to membrane fusion. As an example of a direct contribution, low membrane tension induced by myosin IIA reduction allows preosteoclast fusion [101]. From our results, we propose that during the early steps of osteoclastogenesis, a low ERM activity promotes the formation of actin-protrusions favorable to the fusion, then, and as soon as the proper number of nuclei per osteoclast is reached, moesin is activated and counteracts the fusion process.

We show that moesin acts at a second step during osteoclastogenesis. In addition to regulating osteoclast fusion, moesin also influences the number and architecture of the sealing zones. These structures are composed of a dense network of podosomes organized in clusters [19,21] and as defined in macrophages, each individual podosome can exert a protrusion force on the substrate that is correlated to the F-actin content [102]. Thus, the increased width of the podosome-rich zone observed upon moesin depletion may favor more efficient sealing of osteoclasts to the bone and therefore increase the concentration of bone-degradative molecules in the resorption area [19,86]. Consequently, the exacerbation of bone resorption observed after moesin deficiency could result not only from the increase in the number and surface area of the sealing zones, but also from the ability of osteoclasts to adhere to the bone. Very few data described a role for ERM proteins in podosome organization. Phosphorylation of moesin appears important for podosome rosette formation in Src-transformed fibroblasts [103] while in pancreatic cancer cells, ezrin regulates podosome organization but independently of its activation [104]. As mentioned previously, ERM proteins crosslink the actin cytoskeleton to several transmembrane proteins including CD44 [105,106], which might participate in the organization of the sealing zone. Indeed, in addition to its role in cell fusion [16], CD44 localizes to podosome cores and participates in podosome belt patterning in osteoclasts [107].

In mature osteoclasts, we showed that ERM activation depends on the RhoA/SLK axis. Such a mechanism of ERM regulation has already been described in several contexts, including the cell rounding at mitotic entry of dividing cells or the formation of the apical domain of

epithelial cells [35,97]. Moreover, we identified β3-integrin as an upstream regulator of this pathway. This marker of mature osteoclasts [85,86] mediates their ability to polarize, spread and degrade bone [9,87–89]. We propose that activation of β3-integrin limits the phosphorylation of moesin through the inhibition of the RhoA/SLK axis and in this way controls the number/architecture of the sealing zones. Moesin could be a new effector of the β3/Rho pathway, acting as a complementary regulatory mechanism to those already described [9,89].

In conclusion, in addition to the well-characterized role of ERM proteins in cell polarization and migration, this study provides evidence for a new role of these proteins in osteoclast formation and function, including in vivo, by controlling fusion events and osteolytic activity. In osteoclasts, moesin is a key actin-structure regulator, regulating both actin-protrusive TNTs and podosome organization in the sealing zones. Targeting this protein or its regulatory pathway may present an opportunity to modulate the activity of osteoclasts without affecting their viability or differentiation, and thus may represent a potential target for the treatment of osteoclast-related bone diseases.

METHODS

Materials and methods, chemical and antibodies, cell culture and transfection, HIV-1 infection, immunoblotting, qPCR, immunofluorescence, live imaging and microscopy, including scanning and atomic force microscopy, flow cytometry, bone resorption assays, histological and µCT analyses of mice bones, and statistical analysis are described in supplemental information, supplemental Methods.

Human monocytes were provided by Etablissement Français du Sang, Toulouse, France, under contract 21/PLER/TOU/IPBS01/2013–0042. *Msn* -/- mice were housed under barrier conditions in the Children's Hospital of Philadelphia animal facility, in accordance with protocols approved by the Institutional Animal Care and Use Committee.

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AUTHORSHIP CONTRIBUTION

OD performed all the experiments, with the help of PV, TS, MP, JH, RM, RG, GA and MBN. OD, BRM and CV designed the experiments and analyzed the results. MB participated in MCA experiments. PV generated the KO cells. MJ collected the bones. AM performed histological analysis. CJP and JDB assisted with microcomputed tomography experiments. BRM and CV supervised the project. RP, FL, VLC, SB and JKB co-supervised the project. IMP, NJP, ADM, FL, SC, CBW, RP and JKB obtained funding. BRM and CV wrote the manuscript with input from the other authors.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors have declared that no conflict of interest exists.

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FIGURE LEGENDS

Figure 1: Tunneling nanotubes (TNTs) participate in the fusion of osteoclast precursors. A. Human monocytes isolated from blood were differentiated into osteoclasts (hOC) and analyzed at day 3, 6 and 10. Representative super-resolution microscopy images: F-actin (phalloidin, white) and nuclei (DAPI, cyan). Scale bar, 20 µm. **B.** Same experiment as in A with osteoclasts derived from the murine *HoxB8* immortalized cell line (mOC) at day 3, 5 and 7. White arrowheads show TNTs and pink arrowheads show zipper-like structures. **C.** Left panels: super-resolution microscopy images of TNTs with colored-coded Z-stack of F-actin (phalloidin) staining of 3 day-hOC or 3 day-mOC from 0 µm (substrate, dark blue) to 15 µm (yellow). Scale bar, 20 µm. See also **Movies 1&2**. Right panels: quantification of the percentage of cells forming thick and thin TNTs in hOCs and mOCs after immunofluorescence analysis (see methods), from one representative differentiation out of 3. n>250 cells per condition, means ± SEM are shown. **D.** Brightfield confocal images from a time-lapse movie of hOCs fusing from a TNT (hour:min). See **Movie 3**. Dashed green and red lines delineate the nuclei before cell fusion and dashed orange lines after fusion. Arrowhead shows a TNT-like protrusion. Scale bar, 10 µm.

Figure 2: Moesin knock-out increases the fusion capacities of murine and human osteoclasts. A. Western blot analysis of activated ERM (P-ERM) expression level during mOC differentiation (day 3, 5 and 7); actin was used as loading control and quantification of P-ERM level was normalized to actin. Each circle represents an independent experiment, means ± SD are shown, n=5. **B.** Same experiment as in A during hOC differentiation (day 3, 6 and 10). Each circle represents a single donor, means ± SD are shown, n=9. (A-B) Predicted molecular weight are indicated. n.s. not significant. **C.** Reprensentative brightfield microscopy images from a time-lapse movie of control (CTL) and Moesin knock-out mOC (Moesin KO) at day 4 of differentiation. Black arrowheads point to multinucleated giant osteoclasts. See **Movie 6.** Scale bar, 100 µm. **D-E.** Microscopy analysis of cell fusion in control (CTL) and Moesin KO mOC. (D) Representative microscopy images: F-actin (phalloidin, white) and nuclei (DAPI, cyan). Scale bar, 200 µm. (E) Quantification of fusion index (n=6 independent experiments, 4 images/experiment, 1000 nuclei/image,) and nuclei number per multinucleated osteoclast (160- 250 cells/condition, n=3). **F.** Representative Western blot analysis of P-ERM expression level in control (CTL), ezrin KO, radixin KO and moesin KO mOC; actin was used as loading control, n=2. Predicted molecular weight are indicated. **G-H.** Microscopy analysis of hOC fusion after treatment with non-targeting siRNA (siCTL) or siRNA targeting Moesin (siMoesin). (G) Representative microscopy images: F-actin (phalloidin, white) and nuclei (DAPI, cyan). Scale bar, 100 µm. (H) Quantification of fusion index (each circle represents a single donor, n=8) and nuclei number per multinucleated osteoclast (one representative experiment, 100-200 cells/condition, n=8).

Figure 3: Moesin depletion enhances the formation of tunneling nanotubes (TNTs) and reduces membrane-cortex attachment. A-D. Effect of moesin depletion on TNT formation in mOCs (A-B) and hOCs (C-D). (A, C) Representative scanning electron microscopy images of mOCs (day 3) CTL *versus* Moesin KO (A) and hOCs (day 3) treated with non-targeting siRNA (siCTL) or targeting moesin (siMoesin) (C). White arrowheads show TNTs. (A) A giant mOC is colored in purple. Scale bar, 50 µm (A) and 20 µm (C). (B, D) Quantification of the percentage of cells forming thick and thin TNTs after immunofluorescence analysis in mOCs (B, n= 3 independent experiments) and hOCs (D, n=4 donors) (see Fig. S1B and methods), n>250 cells per conditions, means ± SD are shown. **E-F.** Analysis of force by atomic force spectroscopy operated in dynamic tether pulling mode. (E) Force-velocity curve from dynamic tether pulling on CTL and moesin KO (MKO) mOCs. Data points are mean tether force ± SEM at 2, 5, 10 and 30 µm/sec pulling velocity. At least 17 cells per condition were analyzed in 4 independent experiments. (F) Mean and standard deviation of the MCA parameter Alpha obtained from fitting the Brochard-Wyart model (see Methods for details).

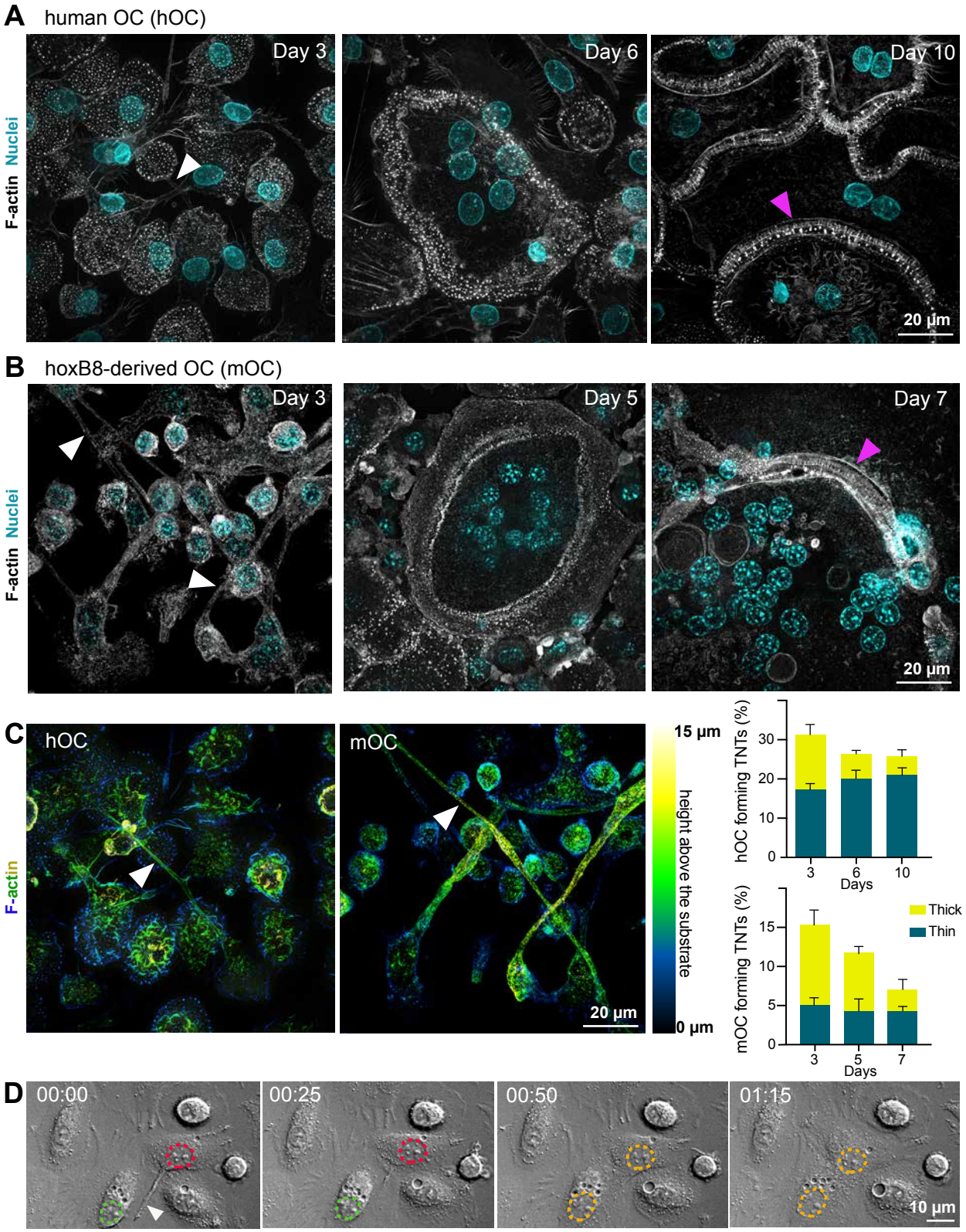
Figure 4: Moesin depletion boosts bone degradation in both murine and human osteoclasts. A-D. Effect of moesin KO on bone degradation (A-B) and sealing zone (SZ) formation (C-D) in mOCs. (A-B) mOC control (CTL) *versus* moesin KO (MKO) were cultured for 7 days on bone slices; after cell removal, bone was stained with toluidine blue. (A) Representative images of bone degradation, eroded bone surfaces are delineated by dashed white lines. Scale bar, 50 µm. (B) Quantification of bone eroded surface (%) using semi-automatic quantification (n=3 independent experiments, 2 or 3/bone slices/condition, means ± SD are shown). (C-D) mOC control (CTL) versus moesin KO (MKO) were cultured for 5 days on glass coverslips, detached and then seeded for additional 2 days on bone slices. (C) Representative microscopy images of sealing zones visualized by F-actin staining (phalloidin, white in upper panels and colored-coded intensity in lower panels). Scale bars, 20 and 5 µm. (D) Quantification of the number of sealing zones per bone surface (n=3 independent experiments, 2 bone slices/condition, means ± SD are shown) and of sealing zone thickness (n=3 independent experiments, 15-20 SZ/condition, 3 locations/SZ). **E-H.** Effect of moesin depletion on bone degradation (E-F) and sealing zone formation (G-H) in hOCs. 6 day-differentiated hOCs on glass coverslips treated at day 0 with siCTL or siMoesin (siM) were detached and seeded for additional 24h on bone slices. (E) Same legend as in A. Scale bar, 100 µm. (F) Same legend as in B (n=4 donors, 2 bone slices/condition, SD are shown). (G) Same legend as in C. (H) Same legend as in D. Quantification of the number of sealing zones (n=4 donors, 2 bone slices/condition) and of sealing zone thickness (n=3 donors, 15-20 SZ/condition, 3 locations/SZ).

Figure 5: The Rho/SLK axis downstream of β3-integrin controls ERM activation and sealing zone formation. A. RhoA inhibition reduces ERM activation. 6 day-hOCs were treated or not

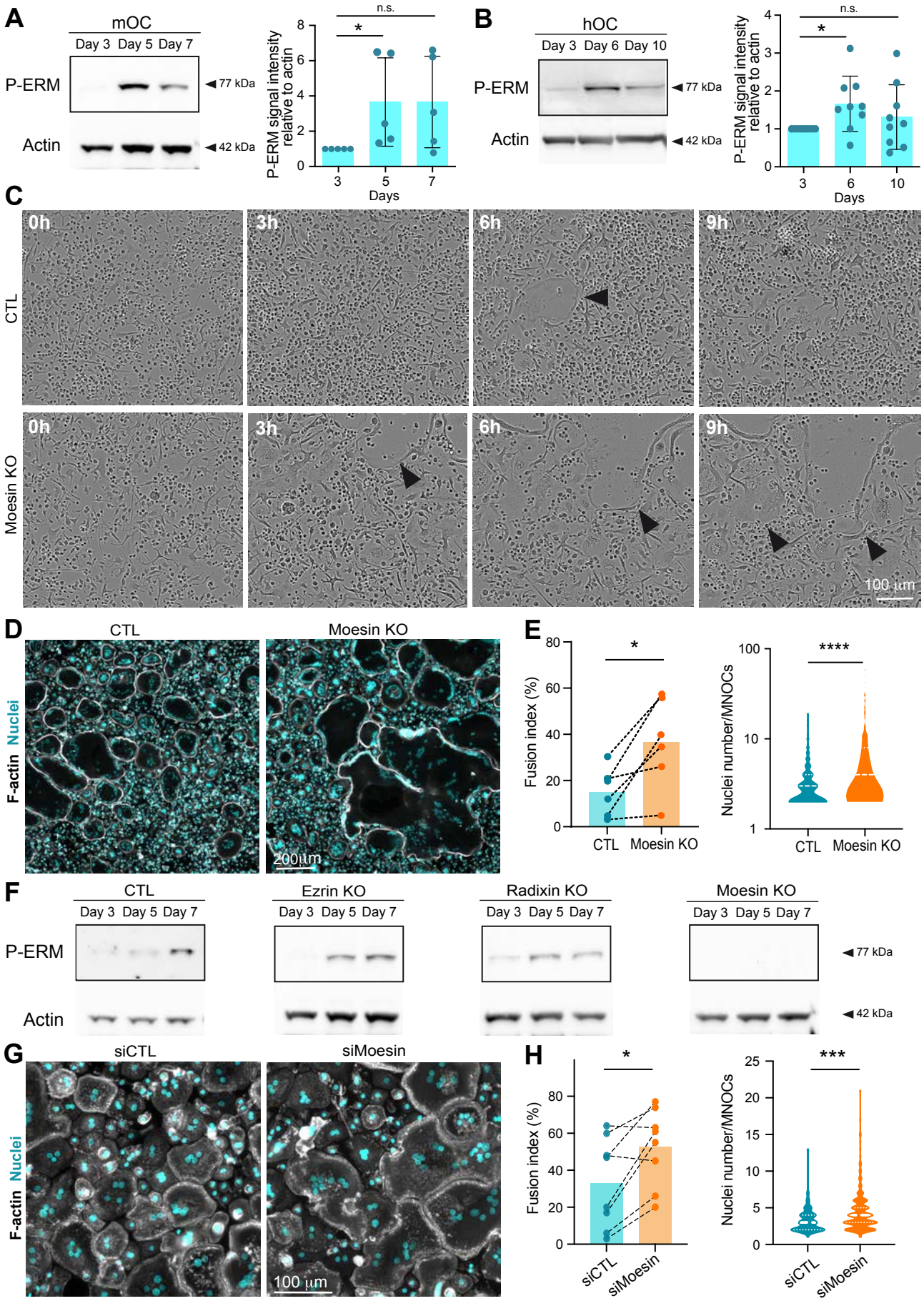
(CTL) with TatC3, targeting the RhoGTPases RhoA. Representative Western blot analysis (left) and quantification of P-ERM signal normalized to actin (right). Each circle represents a single donor (n=6, means ± SD are shown). **B.** SLK suppression reduces ERM activation. hOCs were treated with non-targeting siRNA (siCTL) or siRNA targeting SLK kinase (siSLK). Representative Western blot analysis (left) and quantification of P-ERM signal normalized to actin (right). Each circle represents a single donor (n=7, means ± SD are shown). **C.** β3-integrin suppression favors ERM activation. hOCs were treated with non-targeting siRNA (siCTL) or siRNA targeting β3-integrin (siβ3-integrin). Representative Western blot analysis (left) and quantification of P-ERM signal normalized to actin (right). Each circle represents a single donor (n=7, SD are shown). (A-C) Predicted molecular weight are indicated. **D.** Schematics showing the proposed Rho/SLK axis downstream of β3-integrin for ERM activation**. E-F.** Effect of SLK and β3-integrin depletion on the formation of sealing zones in hOCs. (E) Representative super-resolution microscopy images of sealing zones visualized by colored-coded intensity of F-actin (phalloidin, Scale bar, 5 µm) and (F) quantification of sealing zone thickness (n=2 donors, 15-20 cells/condition and 3 locations/SZ).

Figure 6: Moesin deficiency translates in bone defects and high osteoclast activity in vivo. A. Representative microcomputed tomography images of trabecular section of distal femurs from WT mice and *Msn*$^{-/-}$ mice. **B.** Histograms indicate means ± SD of trabecular bone volume per total volume (BV/TV), trabecular thickness (Tb.Th), number (Tb.N) and separation(Tb.Sp) . Animal groups were composed of 6 mice of each genotype, and 11 femora were analyzed in total for *Msn*$^{-/-}$ mice and 9 femora for the WT mice group. n.s. not significant. **C.** Histological analysis of osteoclasts using TRAP staining (osteoclast in purple) and fast green (bone is green) on femurs from WT mice and Msn-/- mice. Scale bars: 50 µm. **D.** Histogram indicates the mean ± SD of bone surface (upper panel) and TRAP+ surface per bone surface (lower panel) for each condition. N ≥ 2-3 sections chosen among the most median part of 4 mice for each genotype. Each circle represents a single bone section.

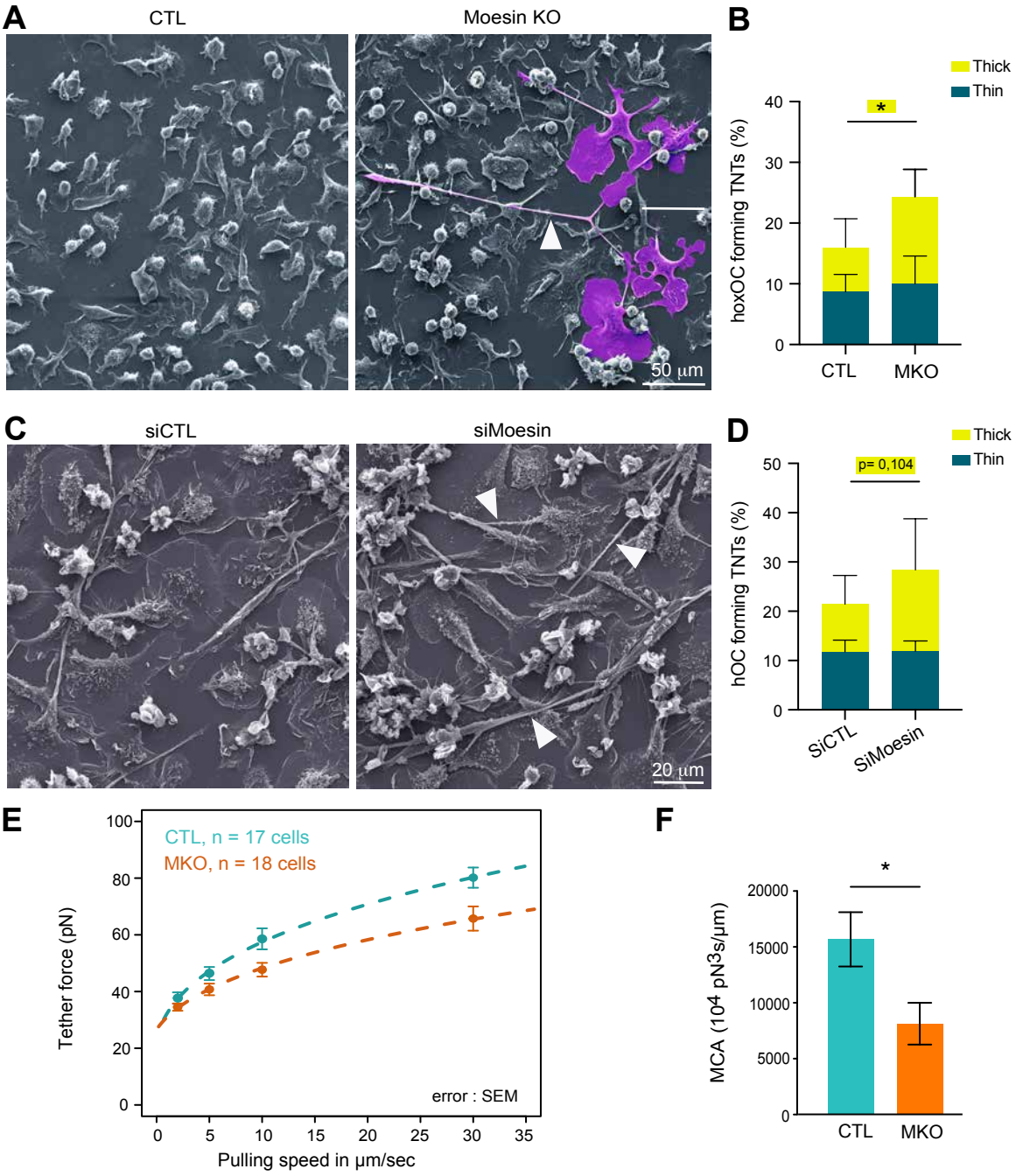
Dufrançais, et al. Figure 1



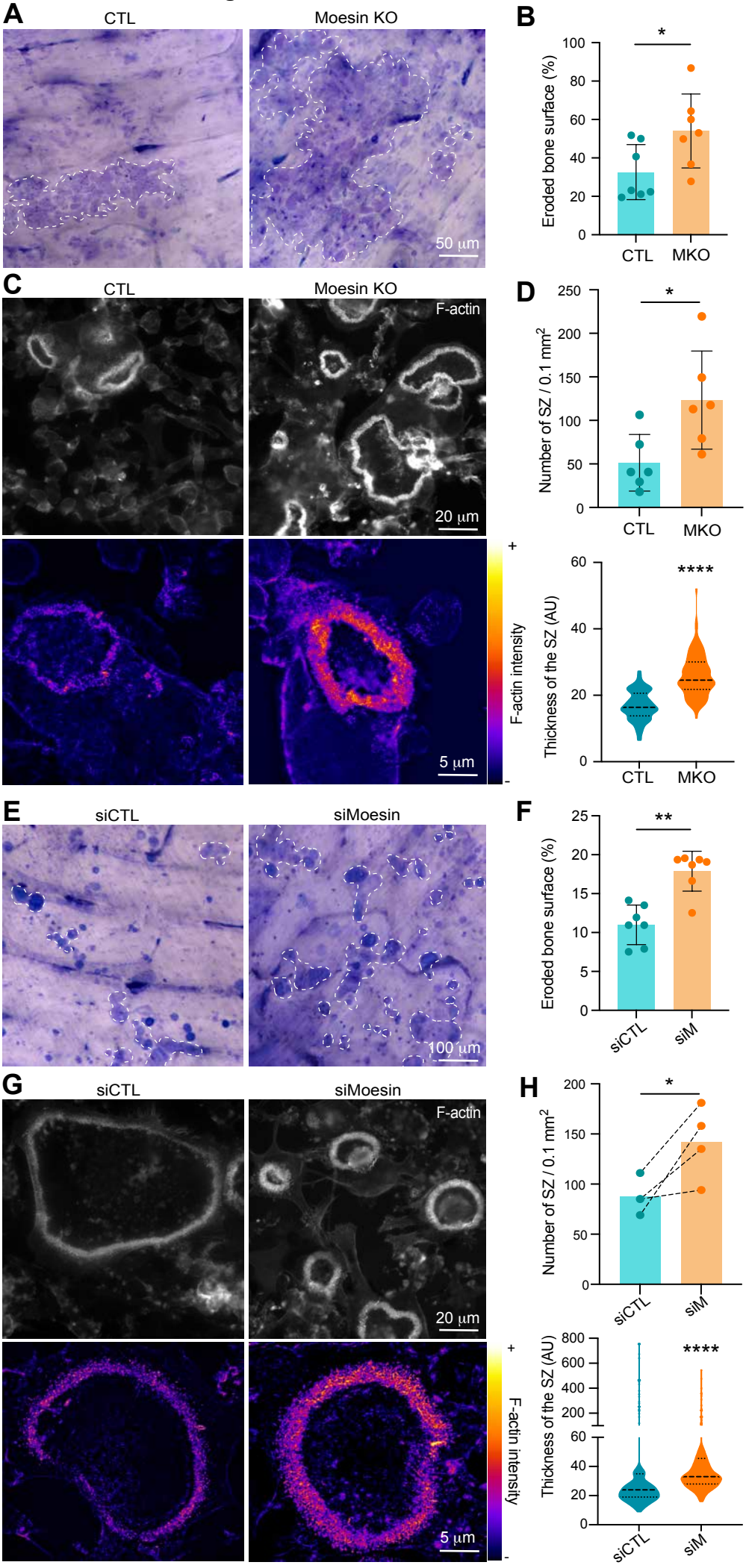
Dufrançais, et al. Figure 2



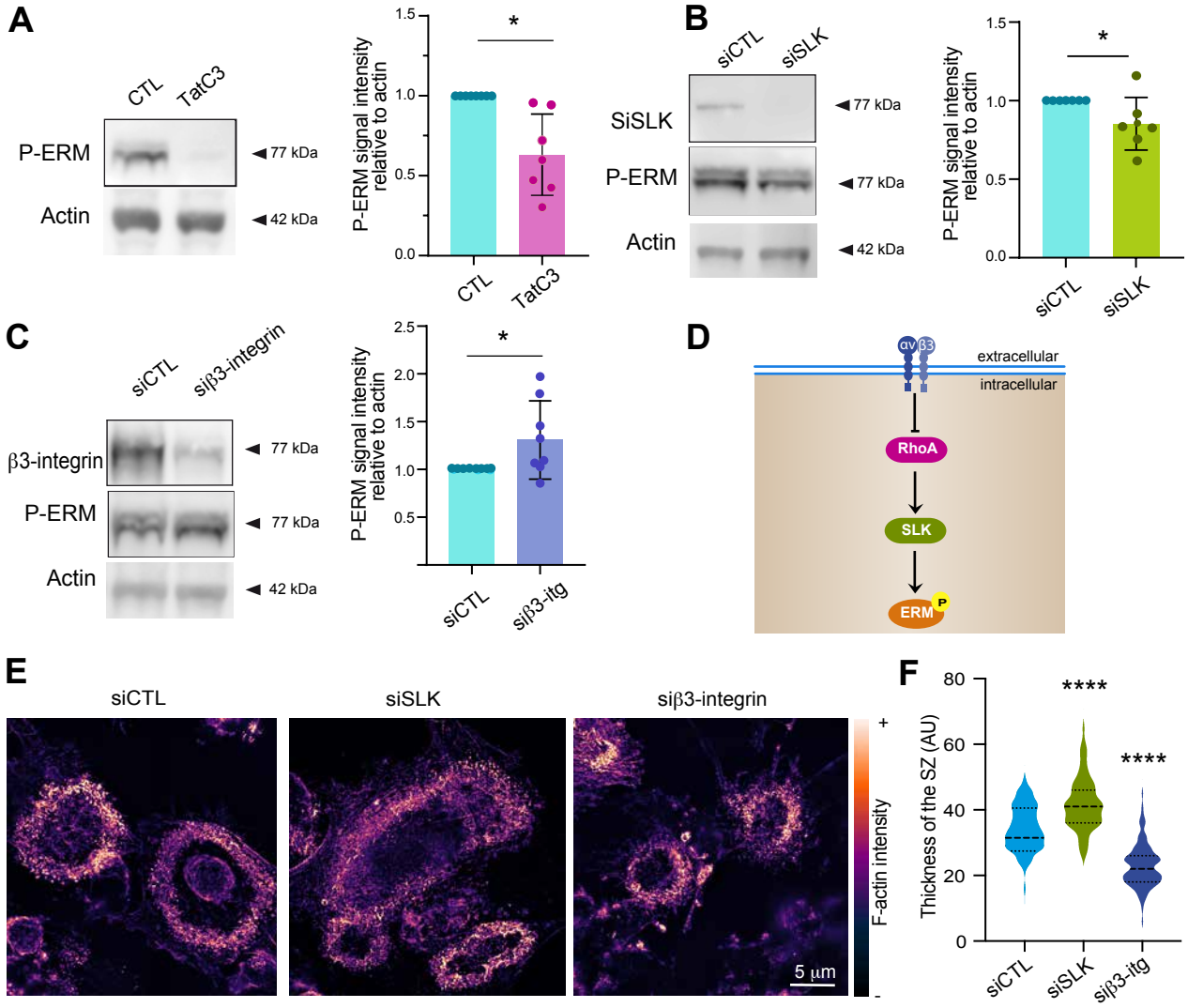
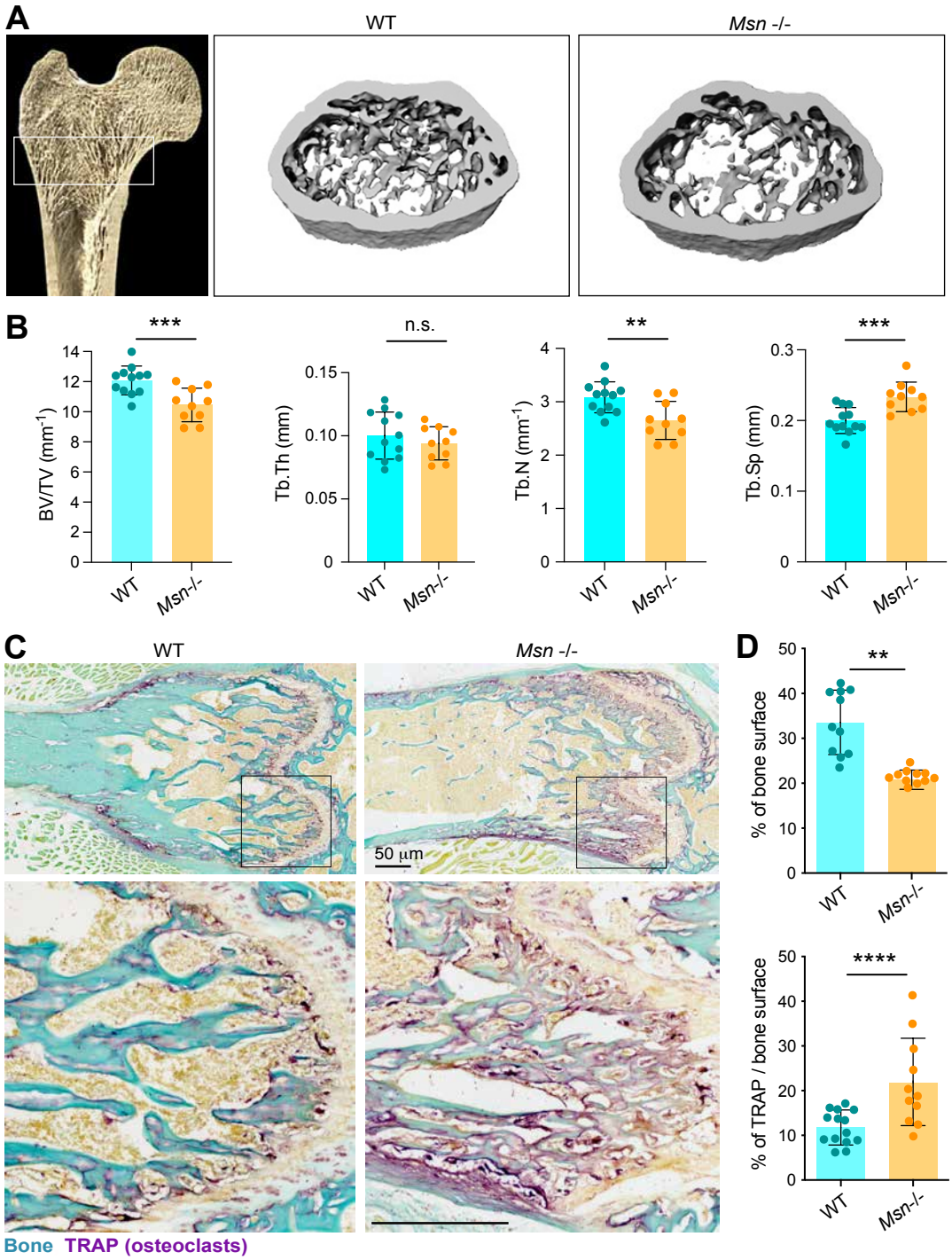
Dufrançais, et al. Figure 3



Dufrançais, et al. Figure 4



Dufrançais, et al. Figure 5



Dufrançais, et al. Figure 6