Year: 2013

Caprin-1, a novel Cyr61-interacting protein, promotes osteosarcoma tumor growth and lung metastasis in mice

Sabile, Adam A; Arlt, Matthias J E; Muff, Roman; Husmann, Knut; Hess, Daniel; Bertz, Josefine; Langsam, Bettina; Aemisegger, Caroline; Ziegler, Urs; Born, Walter; Fuchs, Bruno

Abstract: Osteosarcoma (OS) is the most common primary bone malignancy in children and adolescents. More than 30% of patients develop lung metastasis, which is the leading cause of mortality. Recently, the extracellular matrix protein Cyr61 has been recognized as a malignancy promoting protein in OS mouse model with prognostic potential in human OS. In this study, we aimed at the identification of novel Cyr61-interacting proteins. Here we report that Cyr61 associates with Caprin-1, and confocal microscopy showed that stable ectopic expression of Caprin-1 leads to the formation of stress granules containing Caprin-1 and Cyr61, confers resistance to cisplatin-induced apoptosis, and resulted in constitutive phosphorylation of Akt and ERK1/2. Importantly, ectopic expression of Caprin-1 dramatically enhanced primary tumor growth, remarkably increased lung metastatic load in a SCID intratibial OS mouse model, and decreased significantly ($p<0.0018$) the survival of the mice. Although Caprin-1 expression, evaluated with a tissue microarray including samples from 59 OS patients, failed to be an independent predictor for the patients' outcome in this limited cohort of patients, increased Caprin-1 expression indicated a tendency to shortened overall survival, and more strikingly, Cyr61/Caprin-1 co-expression was associated with worse survival than that observed for patients with tumors expressing either Cyr61 or Caprin-1 alone or none of these proteins. The findings imply that Caprin-1 may have a metastasis promoting role in OS and show that through resistance to apoptosis and via the activation of Akt and ERK1/2 pathways, Caprin-1 is significantly involved in the development of OS metastasis.

Caprin-1, a Novel Cyr61-Interacting Protein, Promotes Osteosarcoma Tumor Growth

and Lung Metastasis in Mice

Adam A Sabile,[1] Matthias JE Arlt,[1] Roman Muff,[1] Knut Husmann,[1] Daniel Hess,[2] Josefine Bertz,[1] Bettina Langsam,[1] Caroline Aemisegger,[3] Urs Ziegler,[3] Walter Born,[1] and Bruno Fuchs[1]

[1]Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Zurich, Switzerland

[2]Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland

[3]Center for Microscopy and Image Analysis, University of Zurich, Zurich, Switzerland

Address correspondence to: Dr. Adam Sabile, Laboratory of Orthopedic Research, Balgrist University Hospital, Forchstr. 340, 8008 Zurich, Switzerland

E-mail: research@balgrist.ch

1

ABSTRACT

Osteosarcoma (OS) is the most common primary bone malignancy in children and adolescents. More than 30% of patients develop lung metastasis, which is the leading cause of mortality. Recently, the extracellular matrix protein Cyr61 has been recognized as a malignancy promoting protein in OS mouse model with prognostic potential in human OS. In this study, we aimed at the identification of novel Cyr61-interacting proteins.

Here we report that Cyr61 associates with Caprin-1, and confocal microscopy showed that stable ectopic expression of Caprin-1 leads to the formation of stress granules containing Caprin-1 and Cyr61, confers resistance to cisplatin-induced apoptosis, and resulted in constitutive phosphorylation of Akt and ERK1/2. Importantly, ectopic expression of Caprin-1 dramatically enhanced primary tumor growth, remarkably increased lung metastatic load in a SCID intratibial OS mouse model, and decreased significantly (p<0.0018) the survival of the mice. Although Caprin-1 expression, evaluated with a tissue microarray including samples from 59 OS patients, failed to be an independent predictor for the patients' outcome in this limited cohort of patients, increased Caprin-1 expression indicated a tendency to shortened overall survival, and more strikingly, Cyr61/Caprin1 co-expression was associated with worse survival than that observed for patients with tumors expressing either Cyr61 or Caprin-1 alone or none of these proteins. The findings imply that Caprin-1 may have a metastasis promoting role in OS and show that through resistance to apoptosis and via the activation of Akt and ERK1/2 pathways, Caprin-1 is significantly involved in the development of OS metastasis.

1. Introduction

Osteosarcoma (OS) is the most common highly malignant primary bone tumor of children and young adults [1]. OS has a high propensity for lung metastasis which is the main cause of death of OS patients [2, 3]. The cellular and molecular events that initiate and propagate osteosarcomagenesis remain poorly understood [4, 5]. Thus, a more detailed understanding of the molecular pathophysiological mechanisms causing OS and metastasis is required for the development of more effective therapeutics and/or a more personalized therapy. Recently, our group and others found that the expression of Cyr61 in primary tumor tissue correlated with poor prognosis for the OS patients and that overexpression of Cyr61 in experimental OS in mice enhanced lung metastasis [6, 7]. Cyr61, a member of the CCN (CYR61/CTGF/NOV) family, is a multifunctional matricellular protein with regulatory functions in several processes involved in tumor biology, and for which aberrant expression was associated with a myriad of pathologies, including various cancers [8-12]. Cyr61 can either associate with the extracellular matrix and cell surfaces or localizes in the cytoplasm and nucleus [13].

In the present study, we aimed at the identification of novel Cyr61-interacting proteins. By combined Cyr61 immunoprecipitation and mass-spectrometric analysis, we identified Caprin-1 (cytoplasmic activation/proliferation-associated protein1) as a novel Cyr61-interacting protein. Caprin-1 is a member of a family of cytoplasmic proteins that is highly conserved in vertebrates and that tightly correlates with cell proliferation [14], and is required for normal progression through the G_1-S phase of the cell cycle [15, 16]. The amino acid sequence of Caprin-1, consisting of 709 amino acids, contains an RGG motif characteristic for RNA-binding proteins [14]. Indeed, Caprin-1 was found to accumulate in the leading edge of migrating cells and was shown to co-localize with the GTPase-activating protein Src homology 3 domain binding protein (G3BP-1) in cytoplasmic RNA granules associated with microtubules [17, 18]. Interestingly,

Caprin-1 associates with another form of RNA granule in cell lines, cytoplasmic stress granules (SG) which are cytoplasmic aggregates that regulate translation of subsets of mRNA following exposure to environmental stress. Caprin-1 has been shown to be a component of the SG in HeLa cells and ectopic expression of Caprin-1 protein alone is sufficient to induce SG formation and phosphorylation of the eukaryotic translation initiation factor 2α (eIF-2α) through Caprin-1's ability to bind mRNAs [17]. On the other hand, the expression of Caprin-1 was shown to be suppressed by either overexpression of wild-type p53 (wt-p53) [19] or by interaction of miR-16 with the 3′ UTR of Caprin-1 mRNA [20].

In this study, we identified Caprin-1 as a Cyr61-interacting protein. We report that stable ectopic expression of Caprin-1 provoked co-accumulation with Cyr61 in SG, structures known to be involved in apoptosis protective processes. We further show that forced expression of Caprin-1 confers to the cells a more resistant phenotype to cisplatin-induced apoptosis, and leads to a constitutive activation of the Akt and ERK1/2 signaling pathways. Importantly, ectopic expression of Caprin-1 enhanced primary tumor growth, remarkably increased lung metastatic load in an intratibial OS model in SCID mice, and decreased significantly the survival of the mice. Our findings suggest that Caprin-1 may function as a tumor promoter in OS metastasis and elucidate a novel role of Caprin-1 in OS pathogenesis and metastasis.

2. Materials and Methods

2.1. Cell culture and transfection

Human SaOS-2 (HTB-85) and U2OS OS cells were cultured as previously described [7]. SaOS-2 and U2OS cells stably over-expressing Caprin-1 with an N-terminal Flag-tag (SaOS-2/Caprin-1 and U2OS/Caprin-1) were obtained by calcium phosphate precipitation-mediated transfection [21] with the plasmid pCMV-Tag4-Flag-Caprin-1 and subsequent selection for neomycin resistance with 500 μg/ml G418 (Invitrogen, Carlsbad, CA, USA). Control SaOS-2 or U2OS cells transfected with the pCMV-Tag4 empty vector (SaSO-2/EV or U2OS/EV cells) were obtained accordingly.

2.2. Purification of Cyr61-interacting proteins and mass spectrometry

For the identification of Cyr61-interacting proteins, lysates of SaOS-2 OS cells were prepared as described previously [7]. Supernatants were collected and incubated at 4°C overnight with Cyr61 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunocomplexes were adsorbed to protein A/G Plus-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The beads were washed 4 times with lysis buffer before the proteins were eluted with Laemmli buffer and separated by 6%-15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie (Coomassie G250, BioRad Laboratories, Hercules, CA, USA), selected protein bands were excised and digested with trypsin. The generated peptides were analyzed by nanoLC-MS/MS on a LCQ Deca XP (Thermo Scientific) and identified with MASCOT (Matrix Science) searching the data base UniProtKB/Swiss-Prot 2011_08.

2.3. Generation of Caprin-1 antibodies

The peptide NH2-CSHATEQRPQKEPID-CONH2 corresponding to amino acid sequence 452 to 465 of human Caprin-1, was chemically cross-linked to keyhole limpet hemocyanin (KLH). The conjugation product was used by Pineda Antikörper-Service (Berlin, Germany) for the immunization of three rabbits. Caprin-1 affinity-purified antibodies obtained from Pineda Antikörper-Service were screened by Western blot analysis of extracts of SaOS-2 and of SaOS-2/Caprin-1 cells.

2.4. Immunocytochemistry and confocal laser microscopy

Cells grown on glass coverslips were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 min and permeabilized with 0.2% Triton X-100. The fixed cells were treated with blocking solution (2% fetal bovine serum in PBS) and stained with mouse anti-Cyr61 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Caprin-1, or goat anti-TIA1 (Santa Cruz) in blocking solution (1:400) at room temperature for 1 h. Cells were washed 3 times with PBS, and stained with FITC-labeled donkey anti-mouse, Dylight 594-labeled donkey anti-rabbit, or Cy5-labeled donkey anti-goat secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., Baltimore, Pike West Grove, USA) at room temperature for 1 h. Nuclear DNA was stained with 0.2 µg/ml 4′,6′-diaminidino-2-phenylindole (DAPI, Molecular Probes Inc, Eugene, USA). Fluorescence imaging was performed with a confocal laser scanning microscope (SP5, Leica, Heerbrugg, Switzerland) equipped with a Plan-Apochromat 63 x NA 1.4 oil immersion objective.

2.5. Immunoprecipitation and immunoblotting

Proteins were extracted from cultured cell lines and immunoblotted as described previously [7]. For immunoprecipitation, 500 µg of lysate in lysis buffer were pre-cleared by incubation with

50 µl of protein A/G Plus-Agarose beads (Santa Cruz) at 4°C for 1 h and centrifugation at 5000 g for 2 min. 10 µg of Caprin-1 or Cyr61 antibodies or of control immunoglobulin G (IgG) were then added to the supernatants and the samples agitated at 4°C overnight. Subsequently, 50 µl of protein A/G Plus-Agarose beads were added and the samples incubated at 4°C for 90 min. After centrifugation at 5000 g for 2 min, pelleted beads were washed 4 times with lysis buffer and bound proteins were eluted by boiling in Laemmli buffer and subjected to SDS-PAGE. Proteins in immunoprecipitation, signaling and apoptosis experiments were then analyzed with antibodies to human Caprin-1, Cyr61 and GAPDH (Santa Cruz), Flag (Sigma, Buchs, Switzerland), phospho-Akt, Akt, phospho-ERK1/2 and ERK1/2, caspase 3, and PARP (Cell Signaling Technology, Danvers, MA, USA). The apoptosis-provoking chemotherapeutic drug Cisplatin was purchased from Sigma.

2.6. Intratibial human OS xenograft model in SCID mice

The mice were housed and the tumor cells inoculated as reported and in accordance with the guidelines of the ''Schweizer Bundesamt für Veterinärwesen'' and of the local authorities [7]. Briefly, 5 x 10^5 SaOS-2/EV or SaOS-2/Caprin-1 cells in 10 µl PBS were injected intramedullary into the left tibia of individual mice under X-ray control with a MX-20 DC Digital Radiography System (Faxitron X-Ray Corporation, Lincolnshire, IL, USA). The health status of the mice was monitored three times a week and primary tumor growth examined in 2-week intervals by X-ray and by calculating the tumor volume from measurements of the legs with a caliper. The equation length x $(width)^2/2$ of the tumor bearing tibia minus length x $(width)^2/2$ of the control tibia was used.

2.7. Histology of lung metastases

On the day of sacrifice, the lungs of the mice were prepared, fixed and embedded into paraffin as described [7]. Ten 5 μm sections were collected at 200 μm intervals from each lung and mounted on SuperFrost®Plus slides (Menzel GmbH & Co KG, Braunschweig, Germany) and then processed for hematoxylin-eosin (HE) staining according to standard protocols. The total area of all metastatic lesions recognized on an individual section was determined with a MATLAB (v2009b, Mathworks, MA) custom made program. Images were first converted into binary images. Total lung tissue area was determined with a morphological close operation to remove small gaps in between tissue and highlighted in red. Metastatic lesions (target area) were identified with a morphological open operation to remove areas of normal lung tissue and were highlighted in green. To assess the proliferation index in vivo, we used immunoreactive Ki67 in primary tumor and lung tissue sections as a marker of proliferation. Ki67 was immunostained with rabbit polyclonal Ki67 antibody (Abcam, Cambridge, UK), and the immunostaining was visualized with peroxidase-conjugated secondary antibodies (Vector Laboratories Inc., Burlingeme, CA, USA), Vectastain® Elite ABC and a substrate-chromogen system (Dako, Baar, Switzerland).

2.8. Human OS tissue microarray, Caprin-1 immunostaining and Kaplan-Meier analysis

Primary osteosarcoma biopsies of 59 patients and normal bone tissue specimens were collected between June 1990 and December 2005 according to the regulations of the Swiss ethics committee. Clinical information on the patients included is summarized in Table 1. The tissue microarray (TMA) was arranged and 4.5 µm tissue sections were processed as previously described [7, 22], stained with Caprin-1 or Cyr61 antibodies (1:400) and counterstained with

haematoxylin. The grading of the TMA was performed based on both, the intensity and the percentage of stained area. The intensity of the staining was evaluated by eye by two independent investigators and the percentage of stained areas was calculated with a custom MATLAB program. Immunostained (brown) and non-stained (blue) areas were determined by color deconvolution [23]. The percentage of Caprin-1 or Cyr61 expressing tissue area was defined as brown area (number of brown pixels) divided by the total tissue area (number of blue and brown pixels) x 100. Kaplan-Meier analysis was used to correlate Caprin-1 or/and Cyr61 expression with overall survival of the patients.

2.9. Statistical analysis

Results for the in vivo experiments are presented as mean ± standard error of the mean (SEM), and quantification results of the Western blots are presented as the mean ± standard deviation (SD). Differences between means were analyzed for significance by unpaired t-test with GraphPad Prism® 5.01 software. The Kaplan–Meier analysis was statistically analysed with the log-rank test. $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Identification of Caprin-1 as a novel Cyr61-interacting protein

In search of novel Cyr61-interacting proteins, Cyr61 was immunoprecipitated from SaOS-2 OS cell extracts with affinity purified Cyr61 polyclonal antibodies. Subsequent mass spectrometric analysis of co-precipitated proteins identified among others Caprin-1, a well conserved cytoplasmic protein that is involved in the regulation of cell proliferation (Fig. 1A). As described in the Materials and Methods section, we generated affinity-purified Caprin-1 antibodies in rabbits. On Western blots of proteins extracted from SaOS-2 and SaOS-2/EV cells, Caprin-1 antibodies recognized a single prominent protein with the expected molecular weight of 116 kDa (Fig. 1B, lanes 1 and 3). The specificity of Caprin-1 antibodies was further confirmed by Western blot analysis of protein extracts of SaOS-2/Caprin-1 cells overexpressing Flag-tagged Caprin-1. Caprin-1 antibodies recognized Flag-tagged Caprin-1, also recognized by Flag antibodies, and endogenous Caprin-1, which on 6% SDS-PAGE, migrated slightly faster than Flag-tagged Caprin-1 and was also present in extracts of control non-transfected SaOS-2 cells and in SaOS-2/EV cells (Fig. 1B). The Caprin-1 antibodies were also suitable for immunocytochemistry and localized endogenous Caprin-1 in the cytoplasm of SaOS-2 cells as expected (Fig. 1C) [14, 17].

3.2. Interaction and co-localization in stress granules of Caprin-1 and Cyr61

The interaction of Caprin-1 and Cyr61 was further confirmed by co-immunoprecipitation of the two proteins in both directions using SaOS-2 cell extracts. As shown before, immunoprecipitates obtained with Cyr61 antibodies contained Cyr61 and Caprin-1, but, importantly, Caprin-1 antibodies precipitated Caprin-1 together with Cyr61 (Fig. 2A). In control precipitates of SaOS-2 cell extracts with rabbit IgG, Caprin-1 and Cyr61 remained undetectable.

Consistent with a stable interaction between Caprin-1 and Cyr61 in living cells, immunofluorescent staining of the two proteins in SaOS-2/Caprin-1 cells combined with confocal microscopy co-localized the two proteins in cytoplasmic granule-like structures (Fig. 2B). Interestingly, earlier studies in HeLa cells overexpressing Caprin-1 showed accumulation of the protein in cytoplasmic stress granules (SG), characterized by immunostaining with specific antibodies to the well-established SG marker T-cell restricted antigen 1 (TIA1), which is normally localized in the nucleus [17, 24]. Therefore, we hypothesize that the cytoplasmic granule-like structures in Fig. 2B might be SG. Interestingly, only cells that highly expressed Caprin-1 showed stress granule formation and are co-stained with TIA1 (Fig. 2C, arrow), whereas cells expressing low levels of Caprin-1 showed uniform nuclear TIA1-staining with no signs of stress granules (Fig. 2C, arrow head). These results indicate that overexpression of Caprin-1 in OS cells indeed provoked the formation of stress granules. Furthermore, triple immunostaining of Cyr61, Caprin-1 and TIA1 demonstrated Caprin-1 and Cyr61 co-localized within the SG (Fig. 2D). Taken together, this immunocytochemical analysis demonstrated that overexpression of Caprin-1 in SaOS-2 cells leads to the formation of SG and that the interaction of overexpressed Caprin-1 with endogenous Cyr61 results in co-accumulation of the two proteins in SG.

3.3. Overexpression of Caprin-1 decreases the sensitivity of OS cells to cisplatin-evoked apoptosis

SG are subcellular structures that are involved in cell death protective processes. Cisplatin is one of the most commonly used chemotherapeutic drugs for the treatment of OS. However, resistance to cisplatin is a major cause of treatment failure in human OS [25]. Here, we

investigated the postulated anti-apoptotic activity of Caprin-1 by comparing the apoptotic potency of cisplatin in SaOS-2/EV and SaOS-2/Caprin-1 OS cells treated with increasing concentrations of the drug (0, 0.7, 3.5, 7 µg/ml) for 48 h. As readouts of cisplatin-evoked apoptosis, we examined on Western blots the cleavage of procaspase-3 and of PARP, a substrate of caspase-3, in extracts of non-treated and treated cells. Caspase-3 activation in SaOS-2/EV cells treated with the indicated concentrations of cisplatin resulted in a decrease in detectable levels of procaspase-3 (Fig. 3A). In SaOS-2/Caprin-1 cells, however, procaspase-3 levels were indistinguishable from those in non-treated control cells (Fig. 3A). The cleavage of PARP in SaOS-2/EV cells in response to cisplatin treatment was in perfect agreement with that observed for capsase-3 activation. In SaOS-2/Caprin-1 cells, on the other hand, cisplatin-evoked PARP cleavage was significantly less compared to that observed in SaOS-2/EV cells (Fig. 3B). Similarly, treatment of U2OS/EV cells with 3.5 µg/ml cisplatin resulted in a significant cleavage of both procaspase-3 and of PARP, whereas U2OS/Caprin-1, much like SaOS-2/Caprin-1 cells, are more resistant to cisplatin (Fig. 3C and 3D). Interestingly, pre-incubation of the cells with the pancaspase inhibitor Z-VAD prior to cisplatin treatment significantly inhibited the decrease in procaspase-3 and PARP cleavage (Fig. 3C and 3D) demonstrating that the observed cisplatin-evoked apoptosis in OS cells is mainly caspase-dependent. Taken together, these results provide solid evidence for an apoptosis-protective activity of overexpressed Caprin-1 in two OS cell lines, SaOS-2 and U2OS.

3.4. Caprin-1 overexpression activates Akt and ERK1/2 in OS cells

Caprin-1 is involved in the regulation of cell proliferation [14, 15]. The MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) and PI3K/Akt (lipid kinase phosphoinositide-3-kinase) signaling pathways are well known to play an important role

in transduction of signals to the cell nucleus, where they influence the expression of genes that regulate important cellular processes such as cell proliferation, apoptosis and survival. We therefore investigated the status of the activation of Akt and ERK1/2 in two OS cell lines SaOS-2 and U2OS. We found that overexpression of Caprin-1 in both OS cell lines increases significantly the levels of phosphorylated Akt and ERK1/2 (Fig. 4). These in vitro results thus indicate that, in SaOS-2 and U2OS cells, Caprin-1 activates the Akt and ERK1/2 signaling pathways and thereby suggest that Caprin-1 might have malignancy-enhancing properties in OS.

3.5. Caprin-1 promotes intratibial primary tumor growth and lung metastasis in mice

In order to investigate a potential malignancy promoting role of Caprin-1 in OS, as suggested by its reported regulatory activities in cell proliferation [14, 15], and the constitutive activation of Akt and ERK1/2 shown above, we compared the growth and metastasis of primary intratibial OS tumors derived from SaOS-2/Caprin-1 and SaOS-2/EV cells in SCID mice. All mice injected with SaOS-2/Caprin-1 cells developed primary tumors that became visible on X-ray within 28 days after tumor cell injection (Fig. 5A, bottom panels). In all mice injected with SaOS-2/EV control cells, on the other hand, primary tumors remained radiologically undetectable until day 64 after tumor cell injection (Fig. 5A, top panels), but all these mice developed visible tumors between day 64 and day 90 post tumor cell inoculation. The tumors in all mice showed comparable osteolytic and osteoblastic lesions in the bone and the surrounding soft tissue (Fig. 5A). A difference in volume between the tumor-bearing and the control leg in mice injected with SaOS-2/Caprin-1 cells was first observed 21 days after the injection. In control mice injected with SaOS-2/EV cells, measurable leg swelling was not observed before day 64 after tumor cell inoculation. These findings were consistent with the observed tumor progression monitored by X-ray. It is important to note that the mice injected with SaOS-2/Caprin-1 cells became moribund

13

and had to be sacrificed before the control mice even developed measurable or radiologically visible primary tumors (Fig. 5B). Accordingly, Kaplan-Meier survival curves plotted for the mice in the two groups indicated a significantly (p<0.0018) shorter mean survival of the SaOS-2/Caprin-1 cell injected mice compared to SaOS-2/EV cell injected control animals (Fig. 5C). Interestingly, at sacrifice the mean size of primary tumors in the SaOS-2/Caprin-1 cell-injected mice was smaller to that in the control animals, suggesting that the mice with SaOS-2/Caprin-1 cell-derived tumors suffered from severe metastasis. This was confirmed by histological examination of HE stained sections of the lungs. Although all SaOS-2/Caprin-1 cell injected mice were sacrificed at least 60 days before the first mouse in the control group had to be sacrificed, they showed more and larger metastatic foci in their lungs than the control mice (data not shown).

To confirm this observation, we performed a second study, an end-point study in which all mice of both SaOS-2/Caprin-1 and control groups, were sacrificed on the same day, at the time when the first animals in the SaOS-2/Caprin-1 group became moribund. In contrast to control mice with SaOS-2/EV cell-derived tumors, which showed metastatic lung lesions less than 1 mm in diameter (Fig. 6A, left panel), the mice with SaOS-2/Caprin-1 cell-derived tumors presented large lung metastases (Fig. 6A, right panel) and, as a consequence, the mean number of metastatic lesions per section was not representative for the metastatic load. Consequently, the metastatic load was defined as the percentage of metastatic tissue per lung tissue section estimated with a MATLAB program as described under Materials and Methods. In mice with SaOS-2/EV cell-derived tumors the mean area of metastatic tissue amounted to approximately 5% of the total area (Fig. 6A, a-b), whereas lung sections of SaOS-2/Caprin-1 cell injected mice showed 44% metastatic tissue (Fig. 6A, c-d).

Furthermore, we assessed the proliferation index by Ki67 immunostaining of sections of primary tumors in mouse legs as well as sections of the lungs. Ki67 staining of the primary

14

tumors showed no significant difference in the percentage of proliferating cells in vivo (Fig. 6B, upper panels). Similarly, Ki67 staining of the lung metastatic nodules showed no significant difference in the percentage of proliferating cells (Fig. 6B, lower panels). Interestingly, beside the metastatic nodules, the healthy lung tissue of mice injected with SaOS-2/Caprin-1 cells appears to contain significantly more proliferative cells in comparison to the healthy lung tissue in control mice (Fig. 6B, lower panels). Altogether, the results of these in vivo experiments in mice confirmed the postulated more malignant phenotype of SaOS-2/Caprin-1 compared to SaOS-2/EV cells that consequently accounted for the observed shorter survival of the SaOS-2/Caprin-1 compared to the control SaOS-2/EV tumor bearing mice.

3.6. Expression of Caprin-1 in human OS tissues

Based on our in vivo results, we hypothesized that the expression of Caprin-1 in human OS primary tumors might affect the aggressiveness of the disease and, consequently, the patient's survival. The expression of Caprin-1 was therefore investigated immunohistochemically in OS primary tumor tissue on a tissue microarray (TMA). Representative immunohistochemical staining is shown in Fig. 7A. A Kaplan-Meier survival analysis demonstrated that, irrespective of metastatic or local disease, 43 patients with immunohistochemically detectable expression of Caprin-1 in tumor tissue had a shorter overall survival of 128 ± 12 months (mean $\pm$ SE) than 16 patients with non-detectable Caprin-1 expression (mean overall survival of 173 ± 18 months). However, the difference in overall survival was not statistically significant (p=0.2056) (Fig. 7B). A second Kaplan-Meier survival analysis that, in addition to Caprin-1 expression, distinguished between patients with local or with metastatic disease revealed the shortest survival time of 53 ± 12 months for 16 patients with metastases and positive expression of Caprin-1 (Fig. 7C). Interestingly, 6 patients with metastatic disease, but non-detectable Caprin-1 expression, survived

15

considerably longer (mean survival time of 116 ± 32 months), but the overall survival was again not significantly different from that of the patients with Caprin-1 expressing tumors (p=0.124). Thus, despite the relatively small number of patients examined, our findings implicate that Caprin-1 might be associated with worse outcome of OS patients. Strikingly, a Kaplan-Meier survival analysis combining robust expression of both Caprin-1 and Cyr61 demonstrated that the overall survival of the patients with Caprin-1 and Cyr61 co-expressing tumours was significantly ($p < 0.05$) shorter than that of patients who expressed in their tumours one or the other or none of the two proteins (Fig. 7D). Taken together, abundant expression of Caprin-1 in primary tumors of OS patients might at least in part contribute to its malignancy.

4. Discussion

Recent studies identified Cyr61 as a novel malignancy-enhancing molecule in experimental OS in mice and as an immunohistochemical marker in human OS tissue with prognostic potential for poor prognosis [6, 7]. In the present study, we undertook a proteomic approach, e.g. immunoprecipitation of Cyr61 combined with mass spectrometry, to identify proteins that, through interaction with Cyr61, may co-operate with the malignancy-promoting protein during tumor development and metastasis in OS. We identified Caprin-1 as a novel Cyr61-interacting protein and demonstrated that Caprin-1, similar to Cyr61 [7], has malignancy promoting properties in experimental intratibial OS in SCID mice when overexpressed in the inherently low metastatic human SaOS-2 OS cell line.

First, we confirmed the Cyr61/Caprin-1 interaction in intact cells and investigated cellular responses to Caprin-1 overexpression in vitro. In an effort to find subcellular structures and locations where Cyr61 and Caprin-1 interact, confocal imaging revealed predominant co-localization of Cyr61 and Caprin-1 in SG, identified by TIA1 staining, a specific marker for these cellular structures. Interestingly, SG formation in response to overexpression of Caprin-1 has been reported [17, 26] and selective binding of Caprin-1 to mRNA encoding c-Myc and cyclin D2 in SG was observed [17]. SG are multi-molecular aggregates of stalled translation pre-initiation complexes that prevent the accumulation of misfolded proteins [27]. SG and their components have been implicated in the pathogenesis of several diseases including cancer [24, 28]. SG arise in response to cell stress and are considered as subcellular structures that are involved in cell death protective processes. This was proposed based on the observation that stress-exposed cells with impaired SG assembly showed poor survival [29-31]. In cells with irreversible damage caused by stress, however, SG appeared to direct these cells to apoptosis [24,

32]. Along these lines, SG were shown to also contain apoptosis regulatory factors in addition to RNA-protein complexes [24, 32].

These previously reported findings together with our new results describing a diminished cisplatin-provoked apoptotic response of cells overexpressing Caprin-1 compared to control cells, point to a hitherto unknown apoptosis-protective function of Caprin-1. It is conceivable that the co-accumulation of Caprin-1 and Cyr61 in SG reflects one apoptosis-protective activity of Caprin-1. Among other cellular roles, Cyr61 is known to have apoptosis and senescence promoting functions through interaction with integrin $\alpha_6\beta_1$ and heparan sulfate proteoglycans [33]. Thus, it is tempting to speculate that Caprin-1, reported to be a component of SG, may sequester some proteins, including Cyr61, to SG and thereby transiently neutralize their activity. A similar mechanism of protein-inactivation by subcellular granular deposition has been reported for TNF-α receptor associated factor 2 (TRAF2). TRAF2 is sequestered by eIF4GI into heat shock-induced SG. This impairs TNF-α-mediated activation of NF-κB, which is a key transcriptional regulator of inflammatory responses and apoptosis [34].

Gene-targeting experiments, using homologous recombination to disrupt caprin-1 alleles, showed that Caprin-1 is needed for normal progression through the G_1-S phase of the cell cycle [15], but the corresponding molecular mechanisms employed by Caprin-1 are largely unknown. Down-regulation of Caprin-1 by miR-16 has been considered as a mechanism that may contribute to prolongation of the G_1 phase of the cell cycle [20]. The present study, on the other hand, provides evidence for a regulatory function of Caprin-1 in Akt and ERK1/2 phosphorylation, known to mediate signals that regulate multiple cellular processes including cell proliferation. Interestingly, overexpression of Caprin-1 in two OS cell lines led to elevated levels of phosphorylated Akt and ERK1/2 consistent with a constitutive activation of both signaling.

Surprisingly, however, proliferation rates of Caprin-1-overexpressing and of control SaOS-2/EV cells in vitro were not significantly different (not shown).

Despite these observations in vitro, constitutively elevated levels of phospho-Akt and -ERK1/2 in Caprin-1 overexpressing OS cells might enhance their aggressiveness in vivo. This hypothesis was confirmed by the results of our study in the intratibial xenograft OS model in SCID mice, in which SaOS-2/Caprin-1 cells showed a strikingly enhanced malignant phenotype compared to control SaOS-2/EV cells. This was reflected by a remarkably faster growth of the primary intratibial tumors and of the metastatic lesions in the lungs and, consequently, by a significantly shorter survival of the mice injected with SaOS-2/Caprin-1 cells compared to the animals that received control SaOS-2/EV cells. Although the indistinguishable proliferation rates of the SaOS-2/Caprin-1 and of the SaOS-2/EV cells in vitro remain unexplained, the results of the animal studies are consistent with a growth promoting effect of Caprin-1 overexpression in SaOS-2 cells in both the bone and lung tissue environment, likely mediated by activation of Akt and ERK1/2 pathways. The comparative histological analysis of the lungs of mice injected with SaOS-2/Caprin-1 cells and of animals injected with SaOS-2/EV cells suggested that the observed massive increase in lung metastatic load upon Caprin-1 overexpression was mainly caused by a faster and more invasive growth of the cells in lung tissue rather than by an increase in the number of cells metastasizing from the primary tumors. These observations are different from our findings in mice with Cyr61 overexpressing SaOS-2 cell-derived intratibial tumors and lung metastases [7]. These mice showed not only larger lung lesions, but also a significantly higher density of lung metastases than mice bearing SaOS-2/EV cell-derived primary tumors. Thus, SaOS-2/Caprin-1 and SaOS-2/Cyr61 cells exhibit a slightly different phenotype when developing to metastatic lesions in the lungs of SCID mice, but both proteins enhanced the aggressiveness of SaOS-2 cells. Strikingly, a Kaplan-Meier survival analysis correlating Caprin-1 and Cyr61

immunostaining of OS tissues with the survival of the patients, showed that Caprin1/Cyr61 co-expression was associated with significantly worse survival of these patients compared to the patients with tumors that expressed only one or none of the two proteins. These results are in good agreement with our findings in the experimental OS model in SCID mice and suggest indeed an in vivo cooperation between Caprin-1 and Cyr61 that enhances OS malignancy. Collectively, the proposed apoptosis-protective activities of Caprin-1 overexpression together with the constitutive activation of Akt and ERK1/2 signaling are consistent with the observed malignancy-promoting properties of Caprin-1 protein in SaOS-2 OS cells in vivo.

5. Conclusion

This study identifies Caprin-1 as a novel Cyr61-interacting protein and elucidates a novel function of Caprin-1. Forced expression of Caprin-1 in the human SaOS-2 and U2OS OS cell lines provoked co-accumulation with Cyr61 in stress granules, known to be involved in apoptosis protective processes. Importantly, we found that SaOS-2 and U2OS cells overexpressing Caprin-1 are more resistant to cisplatin-induced apoptosis. Furthermore, Caprin-1 overexpression constitutively activated Akt and ERK1/2 signaling in both OS cell lines. These observations at the cellular level are consistent with the strikingly enhanced malignant phenotype of SaOS-2 OS cells overexpressing Caprin-1 compared to control cells in an orthotopic OS mouse model. Finally, our immunostaining analysis of Caprin1/Cyr61 co-expression in human OS tissues suggests an in vivo cooperation between Caprin-1 and Cyr61. From this and previous studies, which focused on Cyr61 [6, 7], we conclude that both Caprin-1 and Cyr61, at elevated expression levels in human OS, have related and additive, but distinct OS malignancy-enhancing properties in addition to largely unknown interaction-dependent functions that are currently under investigation.

Conflict of interest statement

All authors state that they have no conflicts of interest.

Acknowledgements

References

[1] G. Ottaviani, N. Jaffe, The epidemiology of osteosarcoma, Cancer Treat Res. 152 (2009) 3-13.

[2] B. Fuchs, D.J. Pritchard, Etiology of osteosarcoma, Clin Orthop Relat Res. (2002) 40-52.

[3] N. Marina, M. Gebhardt, L. Teot, R. Gorlick, Biology and therapeutic advances for pediatric osteosarcoma, The oncologist 9 (2004) 422-441.

[4] R. Gorlick, Current concepts on the molecular biology of osteosarcoma, Cancer Treat Res. 152 (2009) 467-478.

[5] K.B. Jones, Osteosarcomagenesis: modeling cancer initiation in the mouse, Sarcoma (2011) 694136.

[6] O. Fromigue, Z. Hamidouche, P. Vaudin, F. Lecanda, A. Patino, P. Barbry, B. Mari, P.J. Marie, Cyr61 downregulation reduces osteosarcoma cell invasion, migration and metastases, J Bone Miner Res. 26 (2011) 1533-42.

[7] A.A. Sabile, M.J. Arlt, R. Muff, B. Bode, B. Langsam, J. Bertz, T. Jentzsch, G.J. Puskas, W. Born, B. Fuchs, Cyr61 expression in Osteosarcoma indicates poor prognosis and promotes intratibial growth and lung metastasis in mice, J Bone Miner Res. 27 (2012) 58–67.

[8] L.F. Lau, S.C. Lam, The CCN family of angiogenic regulators: the integrin connection, Exp Cell Res. 248 (1999) 44-57.

[9] C.C. Chen, L.F. Lau, Functions and mechanisms of action of CCN matricellular proteins, Int J Biochem Cell Biol. 41 (2009) 771-783.

[10] A. Dhar, A. Ray, The CCN family proteins in carcinogenesis, Exp Oncol. 32 (2010) 2-9.

[11] L. Kular, J. Pakradouni, P. Kitabgi, M. Laurent, C. Martinerie, The CCN family: a new class of inflammation modulators?, Biochimie 93 (2011) 377-388.

[12] L.F. Lau, CCN1/CYR61: the very model of a modern matricellular protein, Cell Mol Life Sci. 68 (2011) 3149-3163.

[13] I. Tamura, J. Rosenbloom, E. Macarak, B. Chaqour, Regulation of Cyr61 gene expression by mechanical stretch through multiple signaling pathways, Am J Physiol Cell Physiol. 281 (2001) C1524-1532.

[14] B. Grill, G.M. Wilson, K.X. Zhang, B. Wang, R. Doyonnas, M. Quadroni, J.W. Schrader, Activation/division of lymphocytes results in increased levels of cytoplasmic activation/proliferation-associated protein-1: prototype of a new family of proteins, J Immunol. 172 (2004) 2389-2400.

[15] B. Wang, M.D. David, J.W. Schrader, Absence of caprin-1 results in defects in cellular proliferation, J Immunol. 175 (2005) 4274-4282.

[16] L. Drougat, S.O. Stichelen, M. Mortuaire, F. Foulquier, A.S. Lacoste, J.C. Michalski, T. Lefebvre, A.S. Vercoutter-Edouart, Characterization of O-GlcNAc cycling and proteomic identification of differentially O-GlcNAcylated proteins during G1/S transition, Biochimica et Biophysica Acta. 1820 (2012) 1839-1848.

[17] S. Solomon, Y. Xu, B. Wang, M.D. David, P. Schubert, D. Kennedy, J.W. Schrader, Distinct structural features of caprin-1 mediate its interaction with G3BP-1 and its induction of phosphorylation of eukaryotic translation initiation factor 2alpha, entry to cytoplasmic stress granules, and selective interaction with a subset of mRNAs, Mol Cell Biol. 27 (2007) 2324-2342.

[18] E. Kolobova, A. Efimov, I. Kaverina, A.K. Rishi, J.W. Schrader, A.J. Ham, M.C. Larocca, J.R. Goldenring, Microtubule-dependent association of AKAP350A and CCAR1 with RNA stress granules, Exp Cell Res. 315 (2009) 542-555.

[19] M. Saffari, O.S. Dinehkabodi, S.H. Ghaffari, M.H. Modarressi, F. Mansouri, M. Heidari, Identification of novel p53 target genes by cDNA AFLP in glioblastoma cells, Cancer letters 273 (2009) 316-322.

[20] T. Kaddar, J.P. Rouault, W.W. Chien, A. Chebel, M. Gadoux, G. Salles, M. Ffrench, J.P. Magaud, Two new miR-16 targets: caprin-1 and HMGA1, proteins implicated in cell proliferation, Biol Cell 101 (2009) 511-524.

[21] F.L. Graham, A.J. van der Eb, A new technique for the assay of infectivity of human adenovirus 5 DNA, Virology 52 (1973) 456-467.

[22] J. Kononen, L. Bubendorf, A. Kallioniemi, M. Barlund, P. Schraml, S. Leighton, J. Torhorst, M.J. Mihatsch, G. Sauter, O.P. Kallioniemi, Tissue microarrays for high-throughput molecular profiling of tumor specimens, Nat Med, 4 (1998) 844-847.

[23] A.C. Ruifrok, D.A. Johnston, Quantification of histochemical staining by color deconvolution, Analytical and quantitative cytology and histology / the International Academy of Cytology [and] American Society of Cytology, 23 (2001) 291-299.

[24] K. Arimoto, H. Fukuda, S. Imajoh-Ohmi, H. Saito, M. Takekawa, Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways, Nat Cell Biol 10 (2008) 1324-1332.

[25] A.J. Chou, R. Gorlick, Chemotherapy resistance in osteosarcoma: current challenges and future directions, Expert Rev Anticancer Ther. 6 (2006) 1075-1085.

[26] E.R. Towers, J.J. Kelly, R. Sud, J.E. Gale, S.J. Dawson, Caprin-1 is a target of the deafness gene Pou4f3 and is recruited to stress granules in cochlear hair cells in response to ototoxic damage, J Cell Sci. 124 (2011) 1145-1155.

[27] P. Anderson, N. Kedersha, RNA granules, The Journal of cell biology 172 (2006) 803-808.

[28] B.J. Moeller, Y. Cao, C.Y. Li, M.W. Dewhirst, Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules, Cancer Cell 5 (2004) 429-441.

[29] A. Baguet, S. Degot, N. Cougot, E. Bertrand, M.P. Chenard, C. Wendling, P. Kessler, H. Le Hir, M.C. Rio, C. Tomasetto, The exon-junction-complex-component metastatic lymph node 51 functions in stress-granule assembly, J Cell Sci. 120 (2007) 2774-2784.

[30] S. Kwon, Y. Zhang, P. Matthias, The deacetylase HDAC6 is a novel critical component of stress granules involved in the stress response, Genes Dev. 21 (2007) 3381-3394.

[31] T.S. Eisinger-Mathason, J. Andrade, A.L. Groehler, D.E. Clark, T.L. Muratore-Schroeder, L. Pasic, J.A. Smith, J. Shabanowitz, D.F. Hunt, I.G. Macara, D.A. Lannigan, Codependent functions of RSK2 and the apoptosis-promoting factor TIA-1 in stress granule assembly and cell survival, Mol Cell 31 (2008) 722-736.

[32] J.R. Buchan, R. Parker, Eukaryotic stress granules: the ins and outs of translation, Mol Cell 36 (2009) 932-941.

[33] C.C. Chen, J.L. Young, R.I. Monzon, N. Chen, V. Todorovic, L.F. Lau, Cytotoxicity of TNFalpha is regulated by integrin-mediated matrix signaling, Embo J 26 (2007) 1257-1267.

[34] W.J. Kim, S.H. Back, V. Kim, I. Ryu, S.K. Jang, Sequestration of TRAF2 into stress granules interrupts tumor necrosis factor signaling under stress conditions, Molecular and cellular biology 25 (2005) 2450-2462.

Legends to figures

Fig. 1. Identification of Caprin-1 as a novel Cyr61-interacting protein. (A) SDS-PAGE of proteins immunoprecipitated from SaOS-2 cell extracts with control rabbit IgG (lane 1) or rabbit antibodies to Cyr61 (lane 2). MW: molecular weight markers. Cyr61 (predicted size: 39 kDa) and co-precipitated Caprin-1 (predicted size: 116 kDa), identified by mass spectrometry, were recognized in lane 2 and non-detectable control IgG (lane 1). (B) Validation of custom-made Caprin-1 affinity-purified rabbit antibodies (Caprin-1 antibodies). 6% SDS-PAGE and Western blot analysis of whole-cell extracts of SaOS-2 (lane 1), of SaOS-2/Caprin-1 and of control SaSO-2/EV OS cells (lane 3) with Caprin-1 antibodies (top panel) or with antibodies to Flag (middle panel) or to the protein loading control GAPDH (bottom panel), revealed indicated protein components of the predicted size. (C) Cytoplasmic localization of Caprin-1 with Caprin-1 antibodies in SaOS-2 cells with DAPI-stained nuclei in blue (scale bar, 50 μm).

Fig. 2. Caprin-1 and Cyr61 co-immunoprecipitation and co-localization in stress granules of SaOS-2/Caprin-1 cells. (A) Co-immunoprecipitation of Cyr61 and Caprin-1 with antibodies to Cyr61 or to Caprin-1 from extracts of SaOS-2 cells visualized on Western blots with antibodies to Caprin-1 (top panel) or to Cyr61 (bottom panel). Total cell extract (lane 1), control immunoprecipitation (IP) with rabbit IgG (lane 2), or with Cyr61 (lane 3) or with Caprin-1 antibodies (lane 4). (B) Immunofluorescent staining of Cyr61 (green), of Caprin-1 (red) and nuclear staining with DAPI (blue) in SaOS-2/Caprin-1 cells co-localized Caprin-1 and Cyr61 (yellow) in cytoplasmic granule-like structures, as shown by confocal microscopy in overview images (top panels; scale bar, 20 μm) and in high magnification images of the boxed area (bottom panel; scale bar, 5 μm). (C) SaOS-2/Caprin-1 cells with low levels of exogenous Caprin-1 (arrow heads), unlike cells with high levels of exogenous Caprin-1 (arrow), lacked stress granules as shown by uniform cytoplasmic TIA1 immunostaining (red) visualized by confocal microscopy (scale bar, 10μm). (D) Immunofluorescent staining of Cyr61 (green) and Caprin-1 (red) in stress granules identified by TIA1 (grey) and visualized by confocal microscopy in overview images (top panels; scale bar, 40 μm) and at high resolution of the boxed area (bottom panels; scale bar 5 μm). Co-localization in the merged images on the far right in white.

Fig. 3. Overexpression of Caprin-1 in OS cells inhibits apoptosis. (A) Representative Western blot analysis of procaspase-3 and PARP cleavage in SaOS-2/EV cells (lanes 1) and in SaOS-2/Caprin-1 cells (lanes 2) in response to treatment for 48 h with indicated doses of cisplatin. Total cell extracts were analyzed with antibodies to procaspase 3, to PARP and to GAPDH used as a protein loading control. (B) Quantitative analysis of PARP cleavage of three independent Western blot experiments. The data are shown as the mean ± SD of cleaved PARP normalized to the uncleaved PARP. (C) SaOS-2/EV (lanes 1) or SaOS-2/Caprin-1 (lanes 2) cells or (D) U2OS/EV (lanes 1) or U2OS/Caprin-1 (lanes 2) cells were either treated with the vehicle DMSO alone as a control, or with 3.5 μM cisplatin, or pre-treated for 2 h with 100 μM of the general caspase inhibitor Z-VAD-FMK prior to cisplatin treatment. Cell lysates were then subjected to immunoblot analysis for procaspase-3, PARP and GAPDH (upper panels). A quantitative analysis of PARP cleavage is shown in the lower panels. The results are shown as the mean ± SD of cleaved PARP normalized to the uncleaved PARP from four independent Western blot experiments, *P<0.05, **P<0.01, ***P<0.001.

Fig. 4. Overexpression of Caprin-1 in OS cells activates Akt and ERK1/2. Representative Western blot of extracts of SaOS-2/EV (lane 1), SaOS-2/Caprin-1 (lane 2), U2OS/EV (lane 3) and U2OS/Caprin-1 (lane 4) OS cells, analyzed with antibodies to phospho-Ser^{473}-Akt (p-Akt) and Akt (A), or with antibodies to phospho-ERK1/2 (p-ERK1/2), ERK1/2, and GAPDH (B) indicating constitutive phosphorylation of Akt and ERK1/2 in Caprin-1 overexpressing cells. Respective quantitative analysis of Western blots is indicated in the right panels. Bars are mean ± SD of p-Akt and p-ERK1/2 normalized to total Akt and total ERK1/2 respectively in individual protein extracts from three independent experiments, *P<0.05, **P<0.01.

Fig. 5. Overexpression of Caprin-1 in SaOS-2 OS cells promotes intratibial primary tumor growth and consequently shortens animal survival. (A) Representative X-ray images of tumor-bearing hind limbs of mice injected with SaOS-2/EV cells (EV, top panels) or with SaOS-2/Caprin-1 cells (Caprin-1, bottom panels). The images show primary tumor appearance on indicated days after tumor cell injection. † = all mice injected with SaOS-2/Caprin-1 cells had to be sacrificed before the indicated days. (B) Intratibial primary tumor growth over time in mice injected with SaOS2/EV cells (blue) or with SaOS-2/Caprin-1 cells (red). (C) Kaplan-Meier

survival analysis indicating significantly (p<0.0018) shorter overall survival of mice injected with SaOS-2/Caprin-1 cells (red) compared to animals with SaOS2/EV cell-derived tumors (blue).

*: Due to restrictions for animal experiments imposed by the local ethical authorities, we injected SaOS-2/Caprin-1 and SaOS-2/EV cells in SCID mice at the same time as we injected SaOS-2/Cyr61 cells [7], allowing us to use the SaOS-2/EV injected mice as a control for both Cyr61 and Caprin-1 experiments.

Fig. 6. Overexpression of Caprin-1 in SaOS-2 OS cells promotes lung metastasis in SCID mice. (A) Representative images of HE stained lung sections of mice injected with SaOS2/EV cells (left panel) or with SaOS-2/Caprin-1 cells (right panel). Metastatic lesions appearing as dense HE stained areas were labeled in green (a, c) and healthy lung tissue in red (b, d) for quantitative analysis of respective areas with the MATLAB program. (B) Representative images of Ki67 immunostained sections of primary tumor tissue in hind limbs (upper panels) and Ki67-stained sections of lungs (lower panels) of mice injected with SaOS-2/EV cells (left panels) or with SaOS-2/Caprin-1 cells (right panels).

Fig. 7. Kaplan-Meier analysis correlating immunohistochemical staining of Caprin-1 in human OS tissues in a tissue microarray with overall survival of the patients. (A) Representative images of tumor tissue microarray sections showing entire spots (upper panels) and higher magnification (lower panel) with non-detectable (left), moderate (middle) and intense (right) Caprin-1 immunostaining. (B) Overall survival of OS patients with non-detectable (Caprin-1 neg) or detectable (Caprin-1 pos) immunostaining of tumor tissue. (C) Overall survival of patients without (Mets neg) or with (Mets pos) metastases and Caprin-1 neg or Caprin-1 pos tumors. (D) Overall survival of patients with tumors expressing both Caprin-1 and Cyr61 (Caprin-1 & Cyr 61 pos) compared to the survival of all other patients.