A novel function of Junctional Adhesion Molecule-C in mediating melanoma cell metastasis

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Short title: JAM-C in melanoma metastasis

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Supplementary Methods

Reagents

Fibronectin, BSA and tamoxifen (4-OHT) were from Sigma (St. Louis, MO) and SDF-1alpha from PeproTech (Rocky Hill, NJ). For flow cytometry, FITC conjugated anti-mouse JAM-C from Abcam, Cambridge, MA was used. 7-AAD and FITC anti-rat-IgG2 were from BD Biosciences. APC conjugated anti-mouse CD31 (clone 390) and FITC anti-VE-Cadherin (clone BV13) were from eBioscience, whereas monoclonal antibody (mAb) Gi11 against human JAM-C was described (1). D-Luciferin Potassium Salt for *in vivo* imaging of luciferase activity was bought from Caliper Life Sciences (Hopkinton, MA). Tissue melanoma microarrays for immunohistochemistry were bought from US Biomax (Rockville, MD). Mouse VCAM-1-Fc, mouse JAM-C-Fc and human JAM-C-Fc were from R&D systems (Minneapolis, MN). Recombinant soluble mouse JAM-C (smJAM-C) was produced as a 6xHis-tagged protein in Schneider *Drosophila* S2 cells (Invitrogen) as previously described (2). Control 6xHis peptide was purchased from Covance Research Products.

Generation of JAM-C conditional knockout mice

Mouse JAM-C gene is located on chromosome 9 and has 9 exons. In order to design a knockout construct we chose a targeting strategy of deletion of exon 1 which contains an ATG start codon. A 760 bp fragment of the JAM-C gene containing exon 1 and 560 bp of the promoter region was flanked by loxP sites for tissue specific deletion with Cre recombinase, as well as 6.4 Kb 5' long and 3 Kb 3' short arms for homologous recombination. Targeting vector ploxPFRTPGKneoFRT (kindly provided by Dr. G. Schütz, DKFZ, Heidelberg, Germany) also contained the neomycin resistance cassette flanked by frt sites for Flp mediated deletion. Electroporated and G418 selected embryonic stem cell clones were analyzed by southern blot using 473

bp Xba I/Nde I fragment as 5' probe to distinguish wt (14639 bp) and recombined (6420 bp) DNA cut with Spe I. The targeted ES cells were injected into blastocysts and chimeric mice were crossed with C57BL/6 to select for germ line transmission. Positive mice were crossed with an flp-deleter mouse line, resulting in a wt/flox mouse.

In order to generate complete JAM-C null mice, JAM-C wt/flox mice were crossed with EIIa-Cre deleter mice (Jackson Laboratories), resulting in generation of heterozygous mice. JAM-C flox/flox mice were in a mixed 129:B6 background, whereas heterozygous (+/-) mice were kept in a mixed 129:B6 background as well as were backcrossed into the C57BL/6 background for 7 generations. By intercrossing JAM-C+/- mice, the percentages of JAM-C+/+, +/- and -/- mice at birth were 31%, 52% and 17%, respectively, i.e. JAM-C-deficient mice were born at almost Mendelian frequency. A high degree of pre-wean mortality of JAM-C-/- mice was observed. In particular, the numbers of JAM-C+/+, +/- and -/- mice at weaning (around postnatal day 22) were 27%, 67% and 6%, respectively. In addition, male KO mice surviving to adulthood were infertile similar to a previous report (3).

For conditional JAM-C inactivation in endothelial cells, constitutive VEC-Cre (Jackson Laboratories) (4) and inducible endothelial-SCL-Cre-ERT (Telethon Institute for Child Health Research West Perth, Australia) (5) deleters were used. Endothelial specific deletion was confirmed by isolation of primary endothelial cells from lungs and subsequent flow cytometry analysis.

PCR genotyping was performed using a 5'primer specific for the 5'loxP site insertion, 5'- GCTAGCCTGGTCTATTTAGCCT-3', and a 3'primer from the genomic region downstream of exon 1 5'-CCGGACCTGGAGTCGTG-3'. The product sizes for the floxed, wt and null JAM-C allele were 1.3 Kb, 1 Kb and 400 bp, respectively. The Cre transgene was detected using universal primers for Cre, a 5'primer 5'-

CCTGGAAAATGCTTCTGTCCGTTTG-3' and 3'primer 5'-ACGAACCTGGTCGAAATCAGTGCG-3' with a product size of 325 bp.

Isolation of total RNA and real time PCR analysis

Total RNA was isolated using the RNAeasy RNA extraction kit (Qiagen) and NucleoSpin RNAII (Macherey-Nagel), followed by DNAse I treatment with TURBO DNA-free kit (Ambion). For cDNA generation with ImProm-II reverse transcriptase and random primers (Promega) or RevertAidH (Fermentas), 100-250 ng of total RNA was used. The cDNA samples were diluted 1:10 with ddH2O and subjected to real-time PCR analysis using QuantiTech SYBR Green Detection System (Qiagen) or Fast start universal SYBR Green (Roche). The following primers were purchased from Qiagen: mJAM-C, Hprt; alternatively the following custom mouse primers were used: JAM-C: 5'-CGGCTGCGACTTCGACTGTA-3' (F1), and 5'-CACATTCCAGATCCTCAGGG -3' (R1); MT1-MMP: 5'- GCTTTACTGCCAGCGTTC-3'and 5'- CCCACTTATGGATGAAGCAAT-3'; MMP-9: 5'-ATGGAAACTCAAATGGTGCCC -3' and 5'-ACAGGGTTTGCCTTCTCCGTT-3'; ITGB1: 5'-AGAATCCCAGAGGCTCTCAA-3' and 5'-AGAAGCAGCTGTTGTGGTTG-3'; ITGA4: 5'-TGTAGGACACACCAGGCATT-3' and 5'-CCTCAGTGTTTCGTTTGGTG-3'; GAPDH: 5'-AACTTTGGCATTGTGGAAGG-3' and 5'-ACACATTGGGGGTAGGAACA-3'. Real time PCR was run on ABI Prism 7900HT Sequence Detection System and Step One Real time PCR system (Applied Biosystems).

Western blot analysis

B16 cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% Na-deoxycholate, 0.1% SDS, 25 mM NaF, 1 mM

sodium orthovanadate, and protease inhibitors) and 20-50 µg of protein was loaded and separated on 4-12% SDS gels (NUPAGE, Invitrogen), followed by 2 h transfer at 4ºC. Membranes were blocked with 5% skim milk in TBS-0.05% Tween-20, washed twice with TBS-0.05% Tween-20. Membranes were incubated with primary antibodies at 4ºC overnight. The following conditions were used: anti-mouse JAM-C (R&D) 1:2000 in 5% skim milk, anti-actin (Sigma) and anti-tubulin (Abcam) in 3%BSA in TBS-0.05%. Primary antibodies were washed intensively with TBS-0.05% Tween-20 and membranes were incubated with secondary HRP conjugated antibodies 1:1000 dilution in 5% skim milk for 45 min RT. After an intense wash with TBS-0.05% Tween-20, membranes were developed with ECL (Pierce). For detection of human JAM-C, 20 µg cell lysate was loaded on a 10% gel and after SemiDry transfer (50 min, room temperature), mAb against human JAM-C was used in 5% skim milk in TBS-0.05% Tween-20.

Immunohistochemistry of human lungs

We performed immunohistochemistry from melanoma lung metastases. Sections from 5 different lung metastases derived from lung excisions were provided by the Institute of Pathology of the University Dresden, Germany. Paraffin embedded tissue samples of patients were de-paraffinized with Roti-Histol and rehydrated using ethanol. Antigen retrieval was performed by heating the samples 3 x 5 minutes at 360W in Citrate buffer (pH 6) in a microwave oven. Endogenous peroxidase activity was blocked by 3% H_2O_2 (15 minutes). Slides ware pre-incubated with „Proteine block serum-free“ (Dako), then incubated with anti-JAM-C, followed by a biotinylated secondary antibody mix („Biotinylated Link Universal“, Dako). For signal-amplification, the slides were incubated with Streptavidin-HRP for 30 minutes. For the colour reaction, a DAB substrate Kit (LiquidDAB + Substrate Chromogen System,

Dako) was used. All sections were counterstained using Hematoxylin. Slides were covered with mounting medium and analyzed with a Nikon Optiphot 2 microscope.

References

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Supplementary Figures

Supplementary Figure 1

(A) Western blot analysis for human JAM-C and tubulin in IGR1 and SkMel28 human melanoma cells. (B) Immunohistochemistry staining for human JAM-C or control staining (omission of the primary antibody) in a human melanoma lung metastasis. Scale bar: 100µm.

Supplementary Figure 2

(A) Western blot analysis for mouse JAM-C and actin in B16 cells transfected with control siRNA (control), pooled siRNA targeting JAM-C (pool) or with individual JAM-C-targeting siRNAs si-09, si-10, si-11, si-12. (B) B16 melanoma cell transmigration through murine endothelial (b.End.3) cells. Prior to the transmigration experiment, B16 cells were not transfected (untreated) or transfected with control siRNA or siRNAs 11 or 12 targeting JAM-C. Data are shown as number of transmigrated B16 cells. The mean+/-S.E.M. (n=3) is shown. *, $p<0.05$.

Supplementary Figure 3

CHO cells were transfected with empty vector, with wildtype JAM-C (64-66 RIE) or mutant JAM-C isoforms E66A (64-66 RIA) and E66R (64-66 RIR). Successful expression of JAM-C or JAM-C mutants was monitored by western blot analysis. Alpha-tubulin served as loading control.

Supplementary Figure 4

(A, B) Murine endothelial (b.End.3) cells were grown to confluence. Murine B16 melanoma cells were allowed to adhere to the b.End.3 cells. (A) Prior to the adhesion experiment, B16 cells were transfected with control siRNA or siRNA targeting JAM-C.

B16 cell adhesion is shown as % of control. Adhesion of B16 cells transfected with control siRNA is set as the 100% control. (B) Prior to the adhesion assay, b.End.3 cells were transfected with control siRNA or siRNA targeting JAM-C. B16 cell adhesion is shown as % of control. Adhesion of B16 cells to b.End.3 cells transfected with control siRNA is set as the 100% control. In (A), (B) the mean+/-S.E.M. (n=3) is shown. (C) The adhesion of human SkMel28 cells to human dermal microvascular endothelial cells was studied in the absence (-) or presence of Fc or human Fc-JAM-C. Mean+/-SEM from a typical experiment is shown; similar results were observed in three separate experiments. SkMel28 cell adhesion is shown as % of control. Adhesion of SkMel28 cells to endothelial cells in the absence (-) of inhibitors is set as the 100% control. Fc-JAM-C did not affect SkMel28 cell adhesion to human dermal endothelial cells. (D) The transmigration of murine B16 melanoma cells expressing CXCR4 through murine endothelial (b.End.3) towards SDF-1alpha added to the lower well was studied in the presence of mouse Fc-JAM-C or control Fc-protein. The mean+/-S.E.M. (n=3) is shown. *, $p<0.05$.

Supplementary Figure 5

Real-time PCR analysis for the message of ITGB1, ITGA4, MT1-MMP and MMP-9 in B16 cells transfected with control siRNA (filled bars) or JAM-C targeting siRNA (open bars). JAM-C knockdown did not significantly affect the expression of these factors in B16 cells. ns, not significant.

Supplementary Figure 6

For genetic inactivation of JAM-C gene deletion of exon 1 was used. Gene targeting vector consisted of 5'-arm, exon 1 flanked with loxP sites, neomycin resistance (NEO) gene flanked with frt sites and 3'-arm. Flip deleter mice were used for deletion

of NEO and different Cre deleters were used for complete (EIIA-Cre) or conditional inactivation of JAM-C expression (VEC-Cre, Scl-Cre-ERT). (B) Western blot analysis for JAM-C protein expression in lung tissues from JAM-C +/+ (wt), -/- (ko), (+/-) (het) and floxed (fl/fl) mice. Actin was used as loading control.

Supplementary figure 7

Luciferase-expressing B16 cells were injected i.v. into the tail veins of littermate JAM-C-sufficient (WT) and complete JAM-C-deficient (JAM-C-/-) mice that were in a mixed 129:B6 background. After 21 days, animals were sacrificed, and the lungs were subjected to analysis for metastasis by luciferase assay. Luciferase activity was detected as a measure of metastasis. The data are shown as mean+/-S.E.M (n= 8-9 mice) and are expressed as percent of control; the luciferase activity (relative light units; RLU) of WT mice represents the 100% control. *, $p<0.05$.

Supplementary figure 8

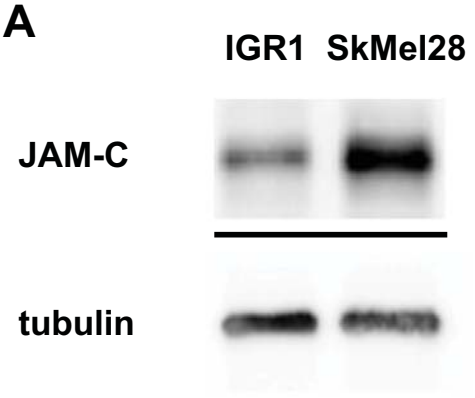
Mice were evaluated for metastasis by *in vivo* imaging of luciferase activity after intraperitoneal injection of D-luciferin using a pitch-black chamber with a cooled CCD camera (Xenogen). Representative images from a WT and a JAM-C-/- mouse are shown.

Supplementary figure 9

WT mice receiving i.v. administration of B16 melanoma cells were treated with either soluble mouse JAM-C or control peptide and 21 days after B16 injection, mice were sacrificed, lungs were removed and sections were stained with hematoxylin and eosin. Soluble JAM-C treatment decreased substantially melanoma lung metastasis.

Langer et al., supplementary Fig 1

A



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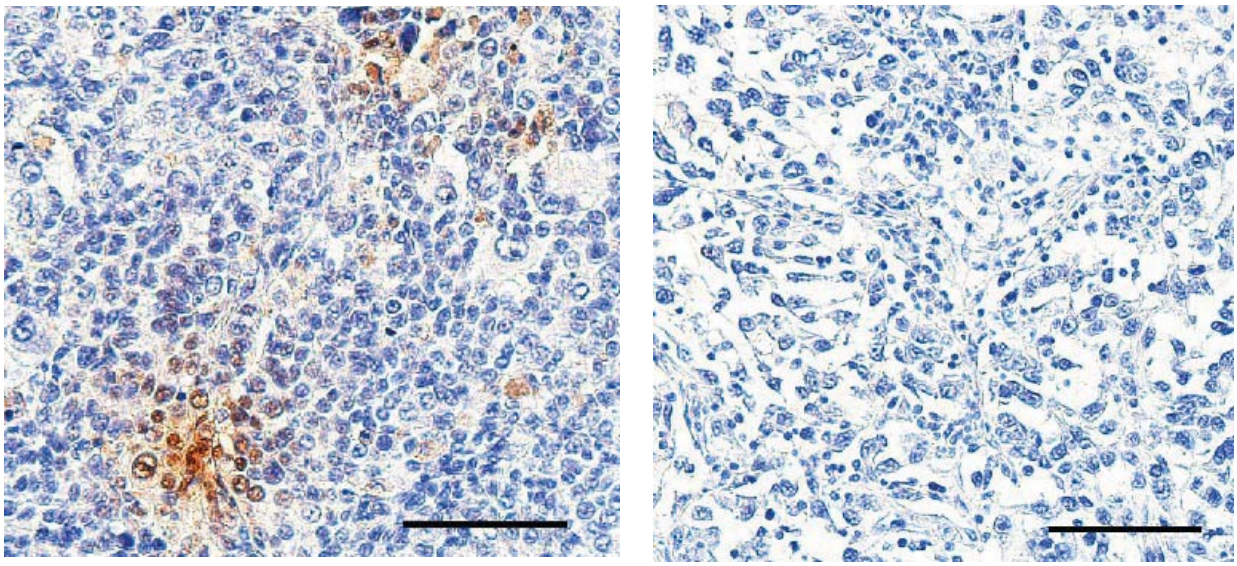
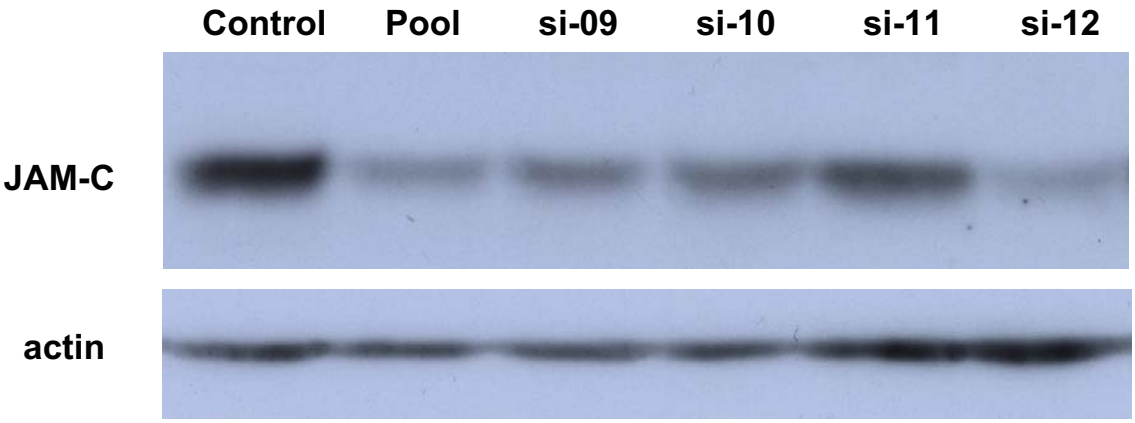


JAM-C

control

Langer et al., supplementary Fig 2

A



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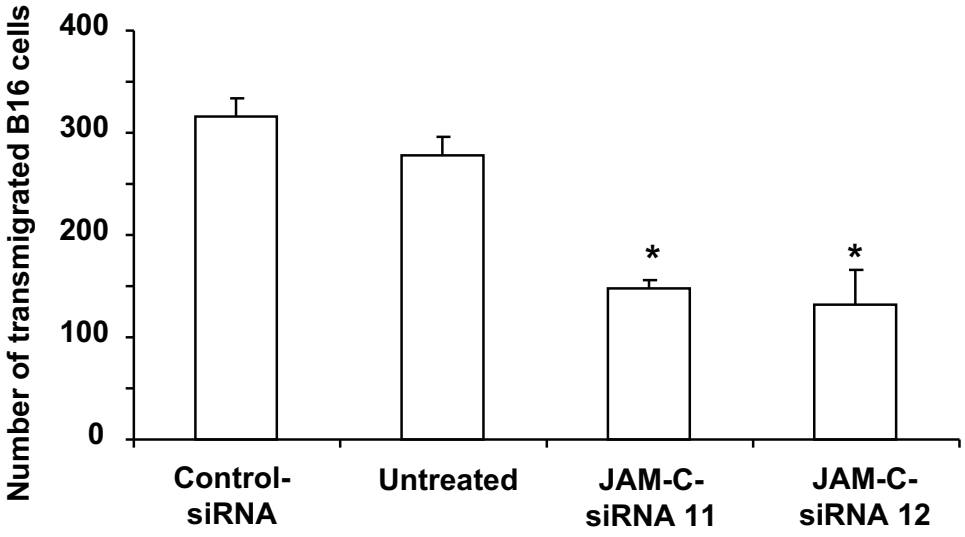
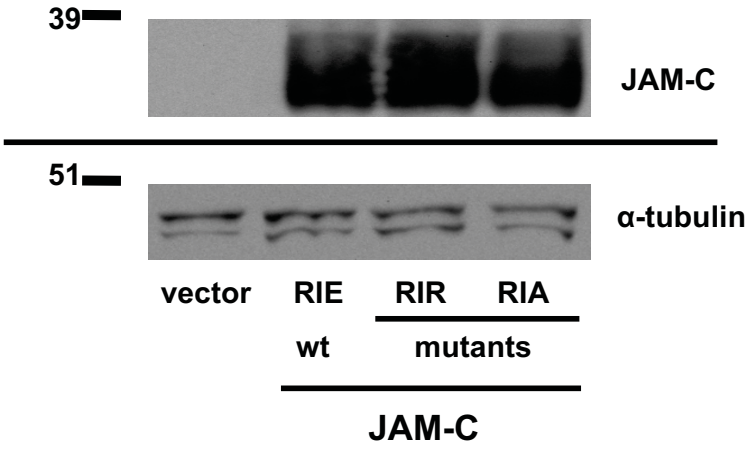


Langer et al., supplementary Fig 3



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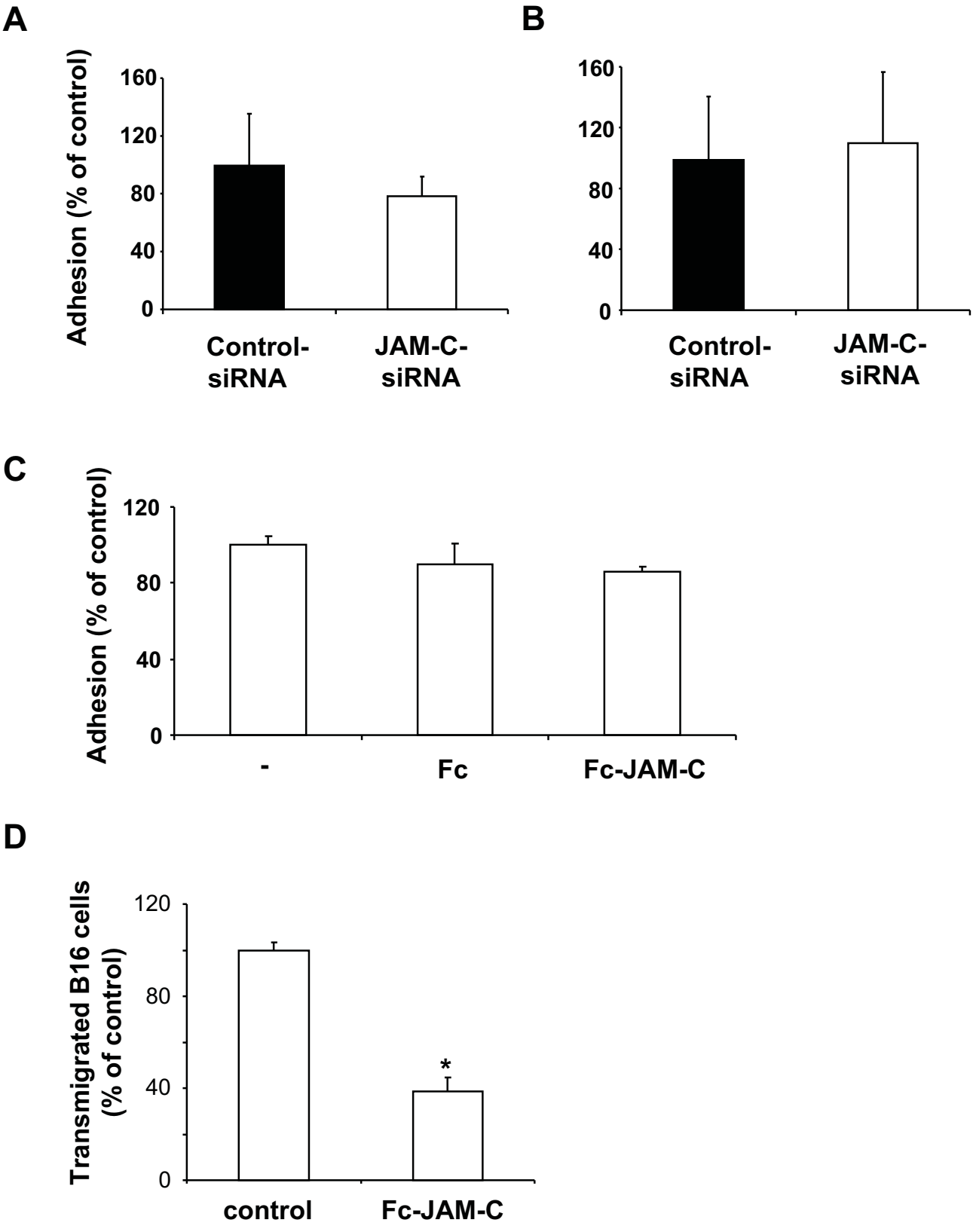

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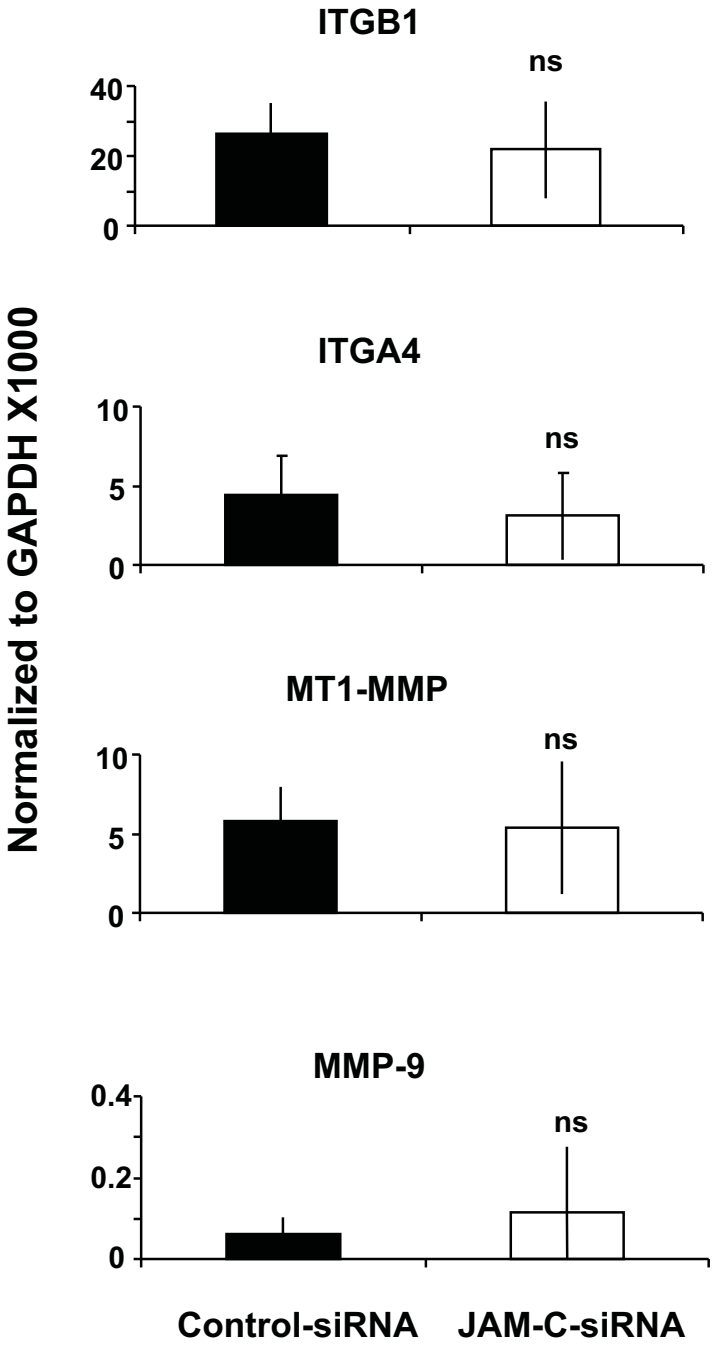

Langer et al., supplementary Fig 6

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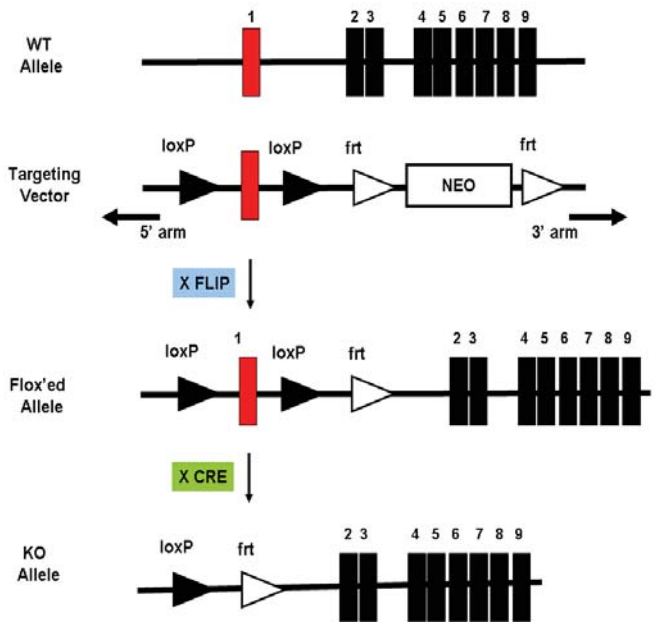
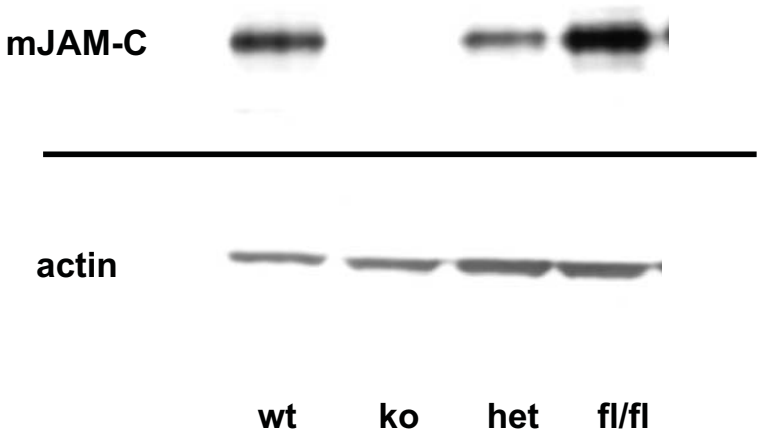


B

LUNG LYSATES



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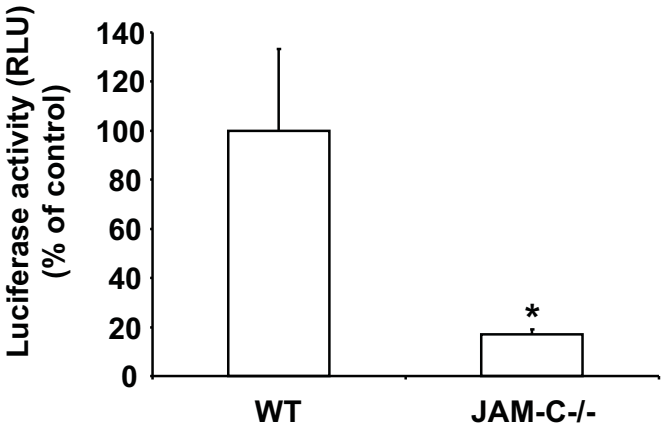

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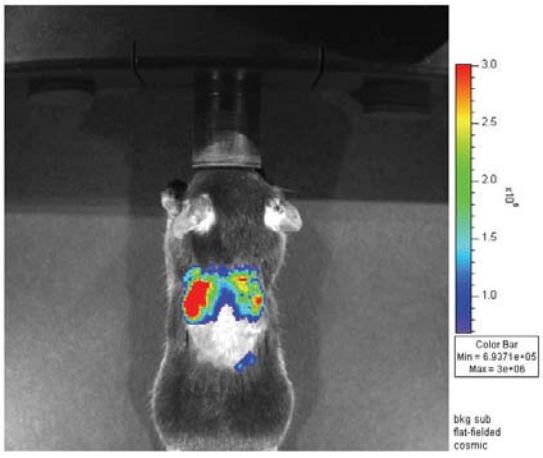
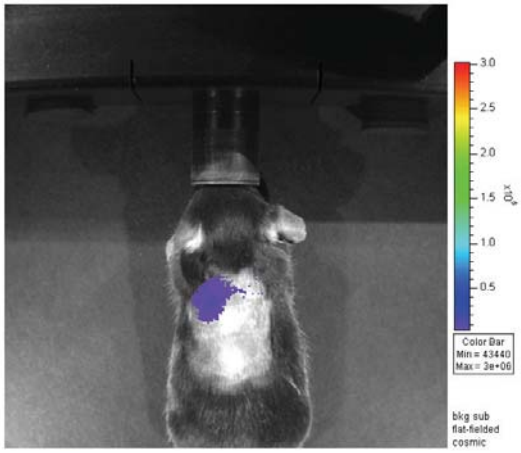


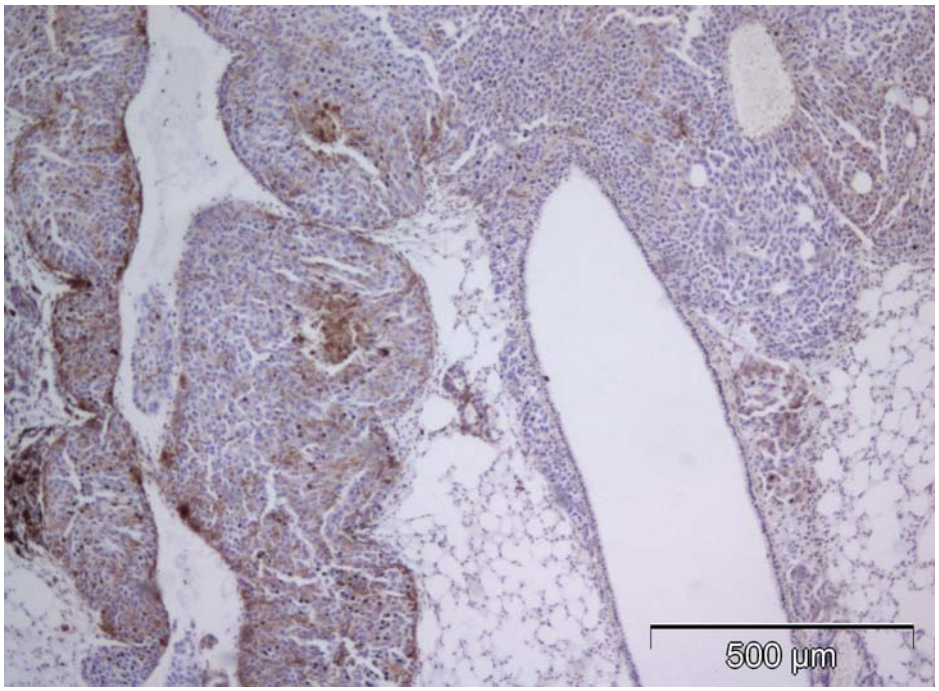
WT



JAM-C -/-

Langer et al., supplementary Fig 9

control



smJAM-C