Calretinin promotes invasiveness and EMT in malignant mesothelioma cells involving the activation of the FAK signaling pathway

SUPPLEMENTARY MATERIALS

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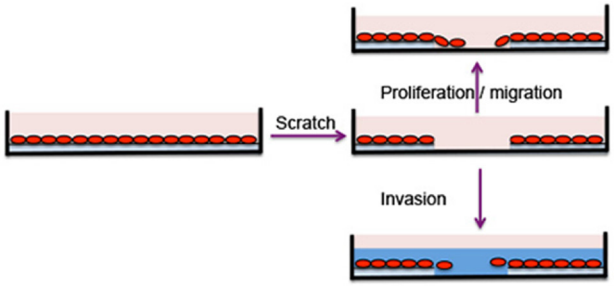
Transwell (Boyden chamber) invasion assay

The invasive ability of MM cells was additionally investigated using Fluoroblock transwell inserts (8-µm pore size; Corning, Chemie Brunschwig AG, Switzerland). The inserts were coated with 100 µl of Matrigel Basement Membrane Matrix at a final concentration of 200-300 µg/ml and incubated 2 h at 37°C for solidification. Un-coated inserts were used to assess migration. Briefly, serum-starved cells (SPC111 and SPC111-CR) were seeded in the upper chamber of the inserts (50,000 cells/insert) and medium-containing 10% FBS was added to each of the basal chambers. Plates were incubated for 20-22 h at 37°C in a 5% CO_2-humidified incubator. Then the medium was carefully removed from the apical chambers and non-migrating or non-invading cells on the upper surface of the filter were removed with cotton swabs. Cells that migrated or invaded to the underside of the membrane inserts were loaded with Calcein-AM (4 µg/ml; Invitrogen, Zug, Switzerland) in HBSS (Gibco) and incubated for 1 h. The fluorescence of migrating and invading cells was measured on a fluorescent plate reader at 517 nm. The relative fluorescent units (RFU) represent the percentage of invasion of the cells, and are calculated as the mean RFU of invading cells/mean of RFU of migrating cells. Experiments were performed in triplicates and an unpaired t-test was used to determine statistical significance of the results.

Scratch assay to measure proliferation/migration and invasion

MM cells (red) were grown on a thin layer of Matrigel (0.1 mg/ml; light blue). After the scratch, cells were grown in medium (pink) in the proliferation/migration assay. For the invasion assay, cells and the scratch region was covered by Matrigel (1 mg/ml; dark blue) and medium was added on top. In the invasion assay cells need to cross the Basement Membrane Matrix.



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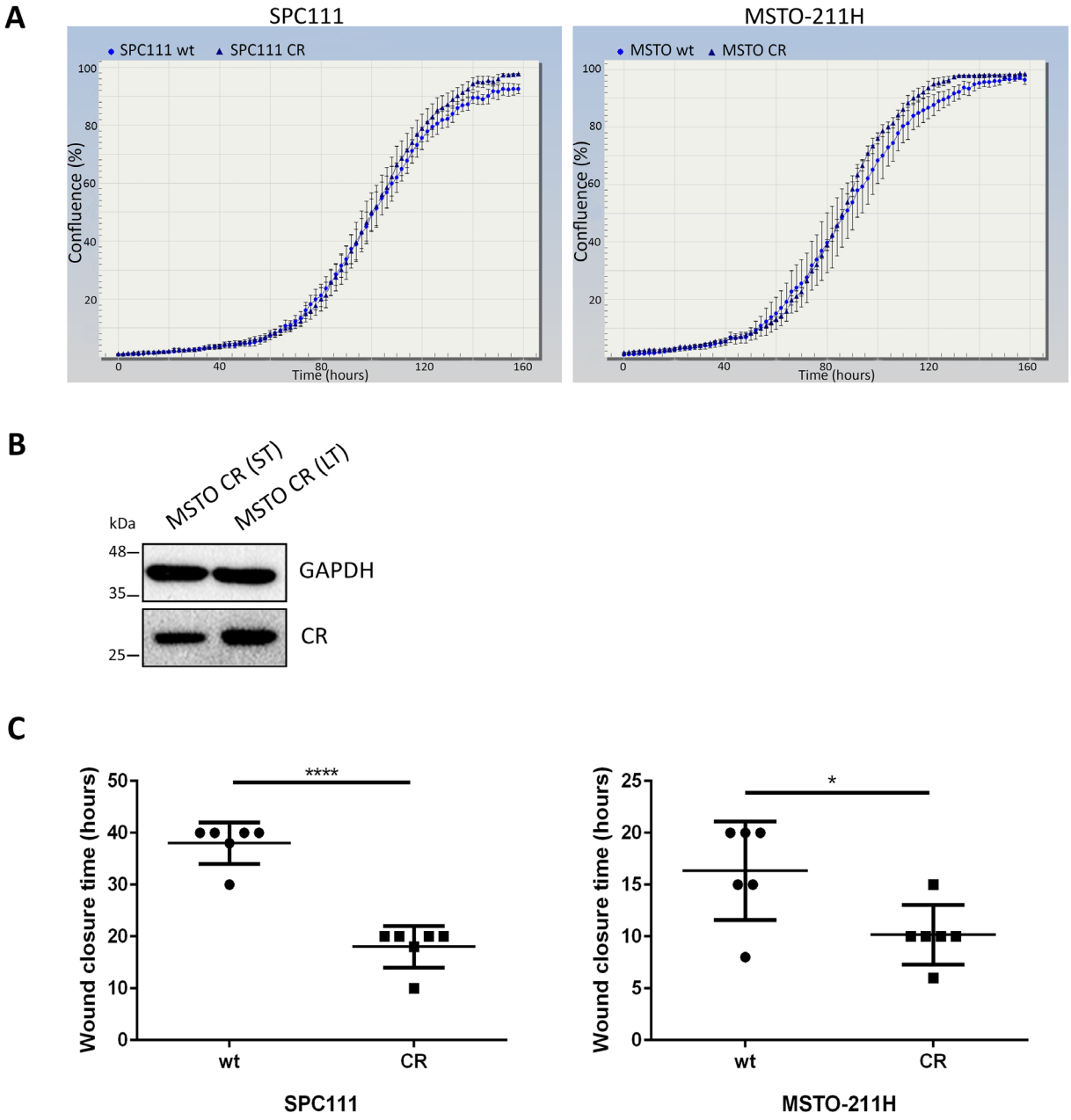
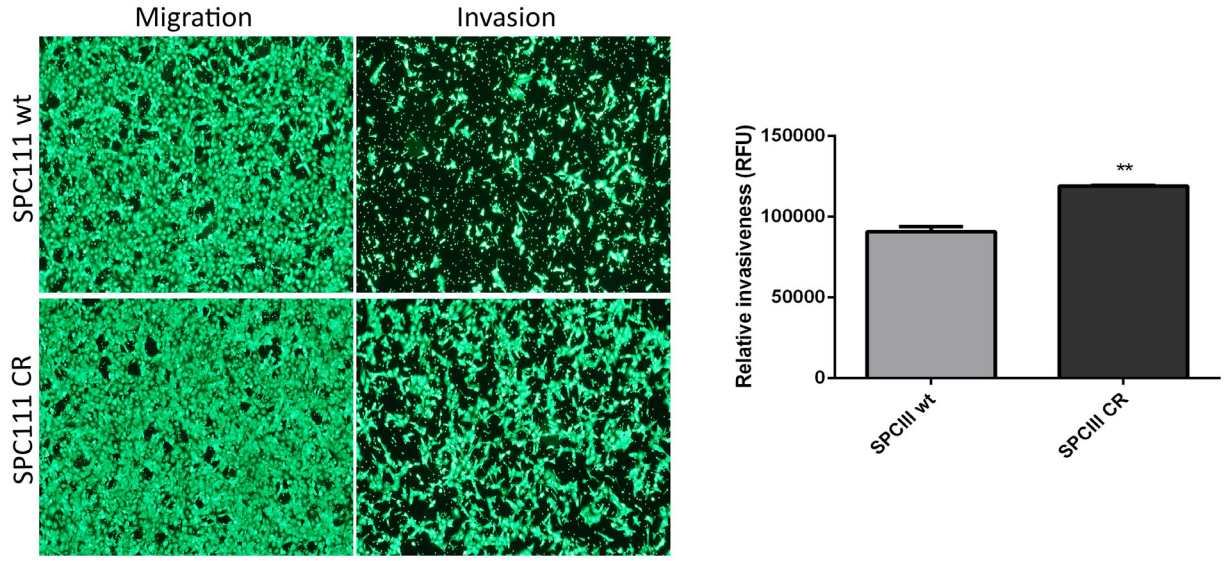
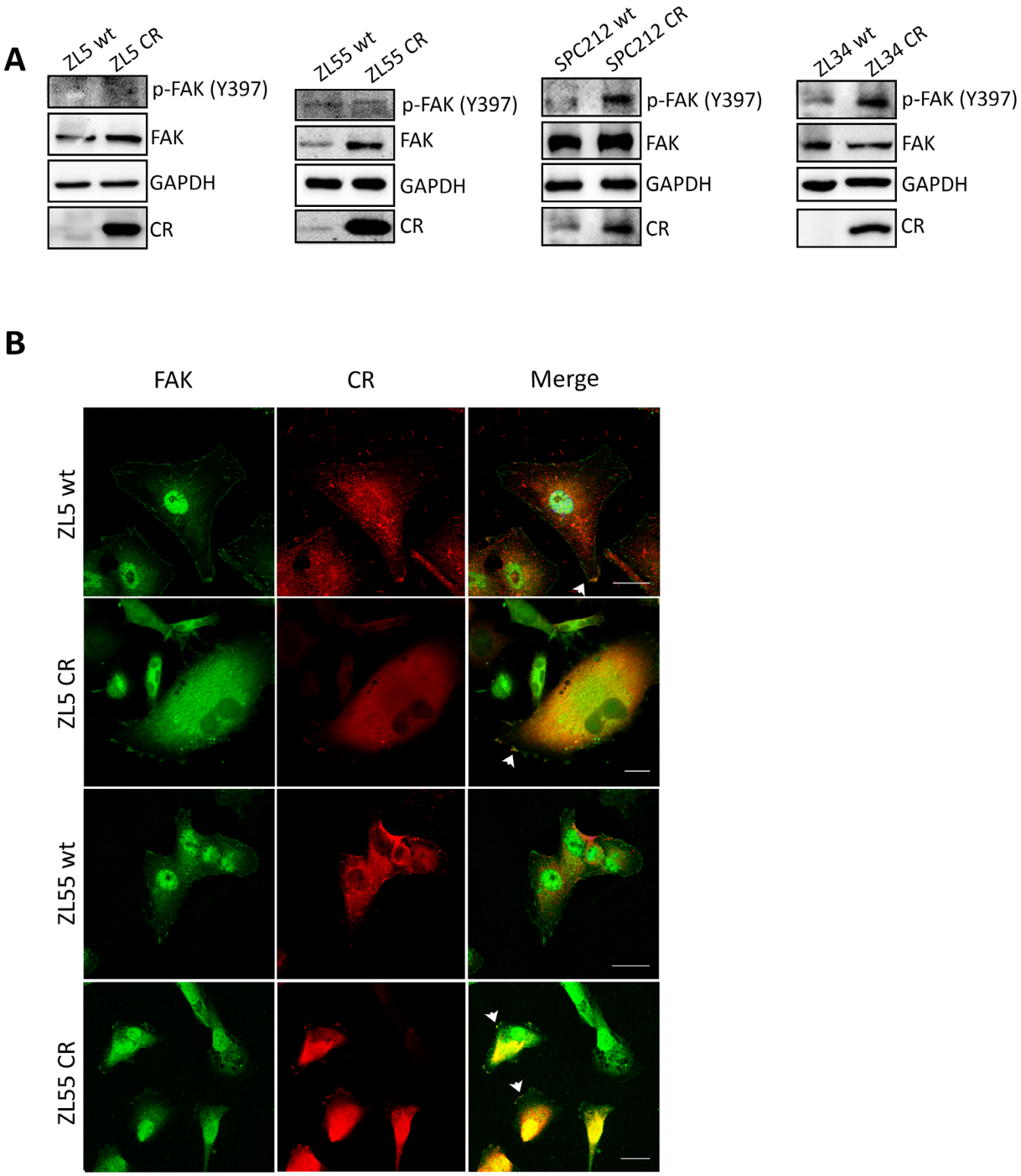
2. Brown MC, Perrotta JA, Turner CE. Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. J Cell Biol. 1996; 135:1109–23. https://doi.org/10.1083/jcb.135.4.1109



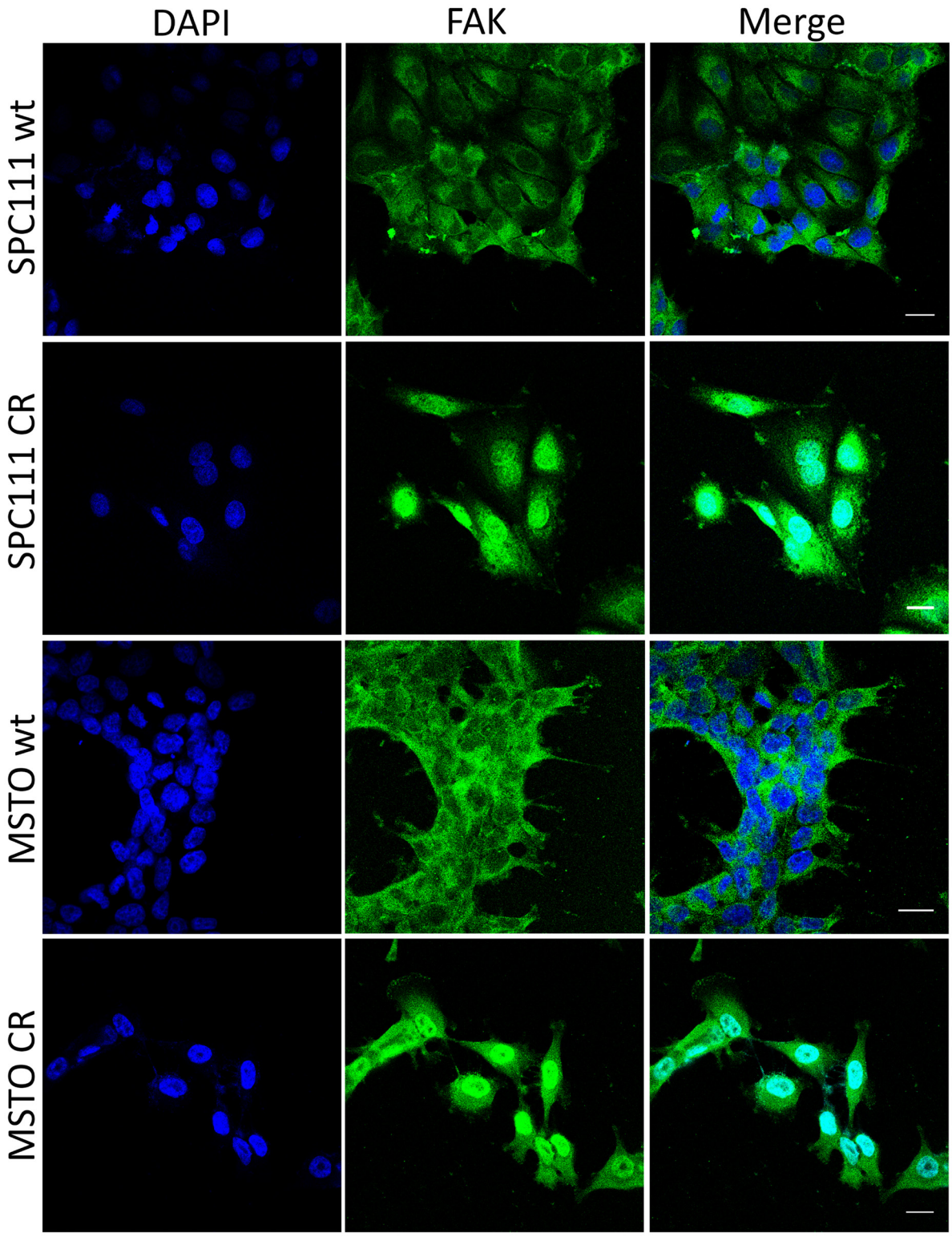
Supplementary Figure 1: Comparison of growth curves, CR protein levels and wound closure times of wt and CR-overexpressing MM cells. (A) No differences are observed in the growth curves between wt and CR-expressing cells (SPC111, left graph; MSTO-211H, right graph) at passages >5. **(B)** CR protein levels show no significant changes between short-term (ST <10) and long-term (LT >30) passages in MSTO-CR cells. A slight trend towards higher CR levels at later passages was observed. **(C)** Wound closure times were quantified in CR-overexpressing and wt cells. SPC111-CR cells showed an average wound closure time of 18 h, while wt cells showed an average time of 38 h ($^{****}p \leq 0.0001$). The corresponding values in MSTO-211H-CR and wt cells were 10 h and 16 h, respectively ($^{*}p \leq 0.05$) (n = 6 independent experiments).



Supplementary Figure 2: Invasion assay measured with Fluoroblock transwell inserts in SPC111 cells. SPC111-CR cells showed a significant enhanced invasive phenotype compared to the corresponding SPC111 wt cells ($^{**}p \leq 0.01$; n = 3 independent experiments).

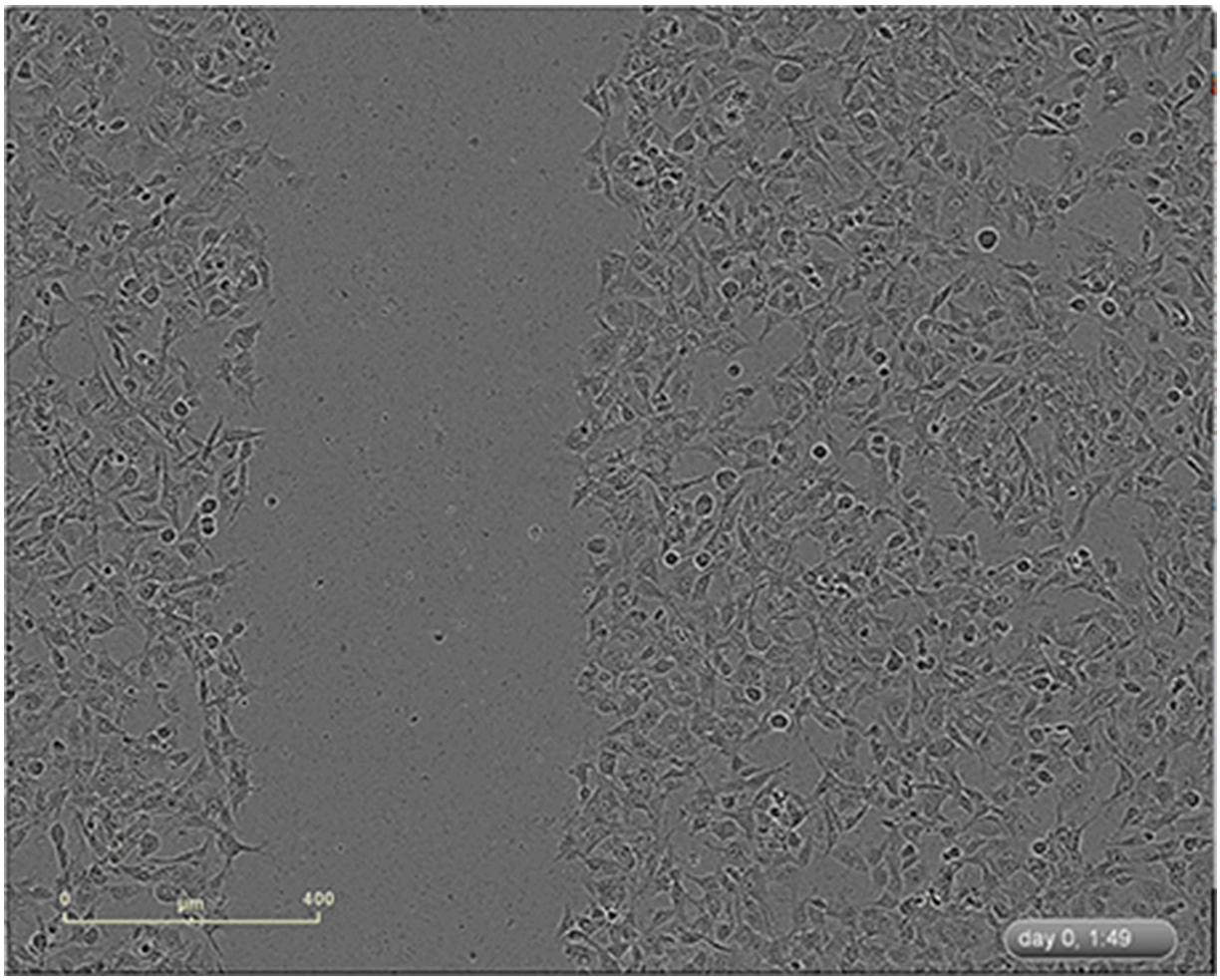


Supplementary Figure 3: FAK protein levels and immunofluorescence analyses in other CR-overexpressing MM cell lines. (A) CR overexpression led to an up-regulation of FAK levels in the cell lines ZL5 and ZL55. In addition, p-FAK (Tyr^{397}) levels were increased in ZL34-CR and SPC212-CR cells. GAPDH was used as loading control. **(B)** Representative confocal images from fixed cells of FAK (green) and CR (red) in wt and CR-overexpressing cells from epithelioid origin, ZL5 and ZL55. FAK levels were clearly increased in CR-expressing cells when compared to the corresponding wt cells. In addition, both CR-overexpressing cell lines showed CR co-localization with FAK (represented by the yellow color in the merged image) at the leading edge of the cells forming punctate-like patterns along the plasma membrane (arrowheads); while in wt cells this co-localization was rare. Scale bars: 20 μm in images ZL5 CR, ZL55 CR; 10 μm in images ZL5 wt, ZL55 wt. For more details of basal CR protein levels in the different MM cell lines, see [1].



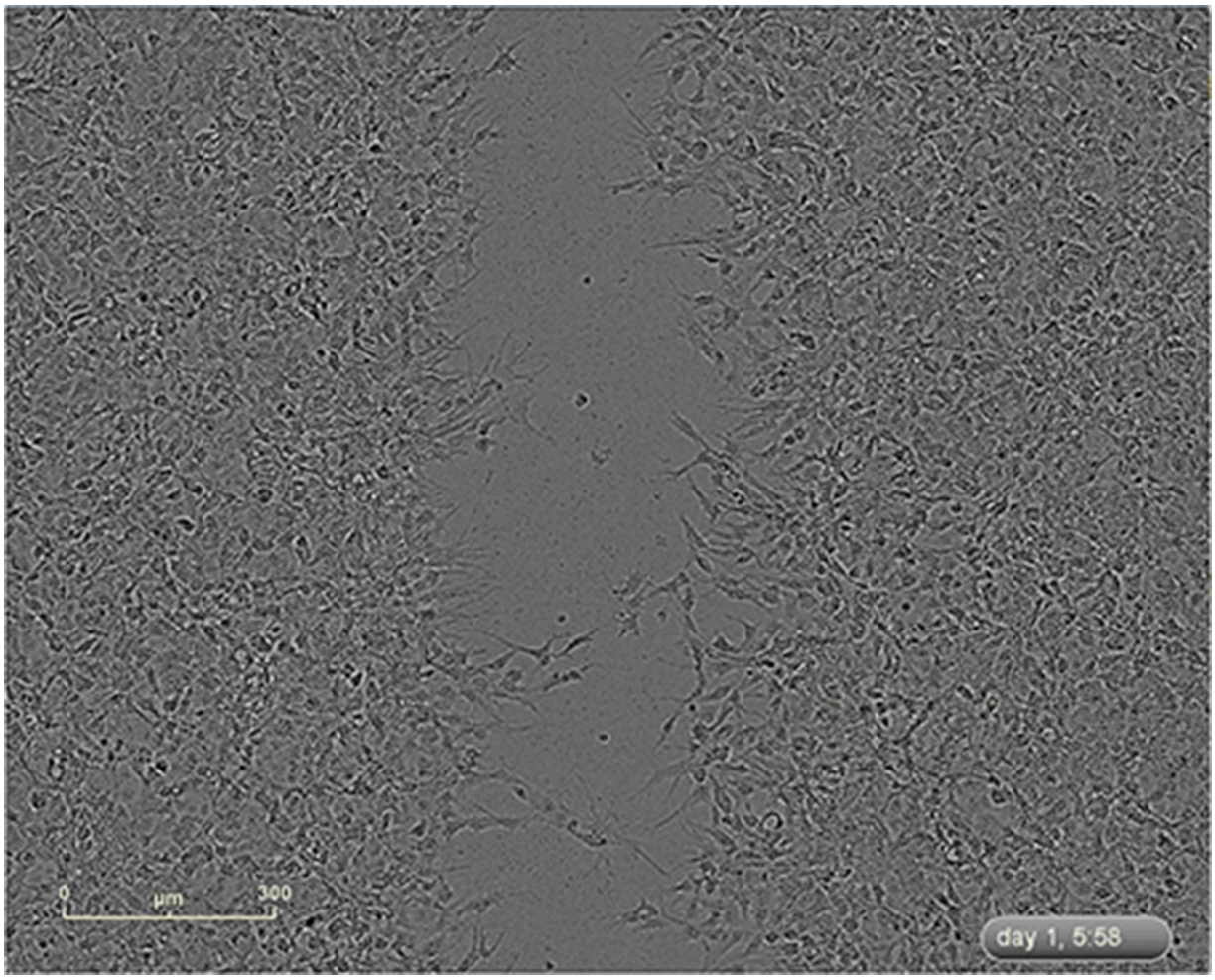
Supplementary Figure 4: FAK immunofluorescence and DAPI staining of SPC111 and MSTO-211H wt and CR-overexpressing cells. Representative confocal fluorescence images from fixed cells stained for FAK (green) and with DAPI (blue). In both CR-overexpressing cell lines FAK showed a strong nuclear staining that co-localized with DAPI (evidenced by the light blue (cyan) color of the in the merged image). Scale bar: 20 μm.

Supplementary Figure 5: Sequence comparison of the LD2 domain of paxillin and the F-helix of EF-hand 5 of calretinin. The conserved "LD domain" residues are boxed in yellow. The previously identified FAT-domain of FAK interacting with paxillin consists of an aspartate (D; red) and the residues LLLQ all aligned on the same side of an alpha helix (for details, see [2]). Note the arrangement of the essential amino acids (red and green) is almost identical in CR; the only difference is a change from asparagine (Q) to glutamine (N) in the sequence of CR.



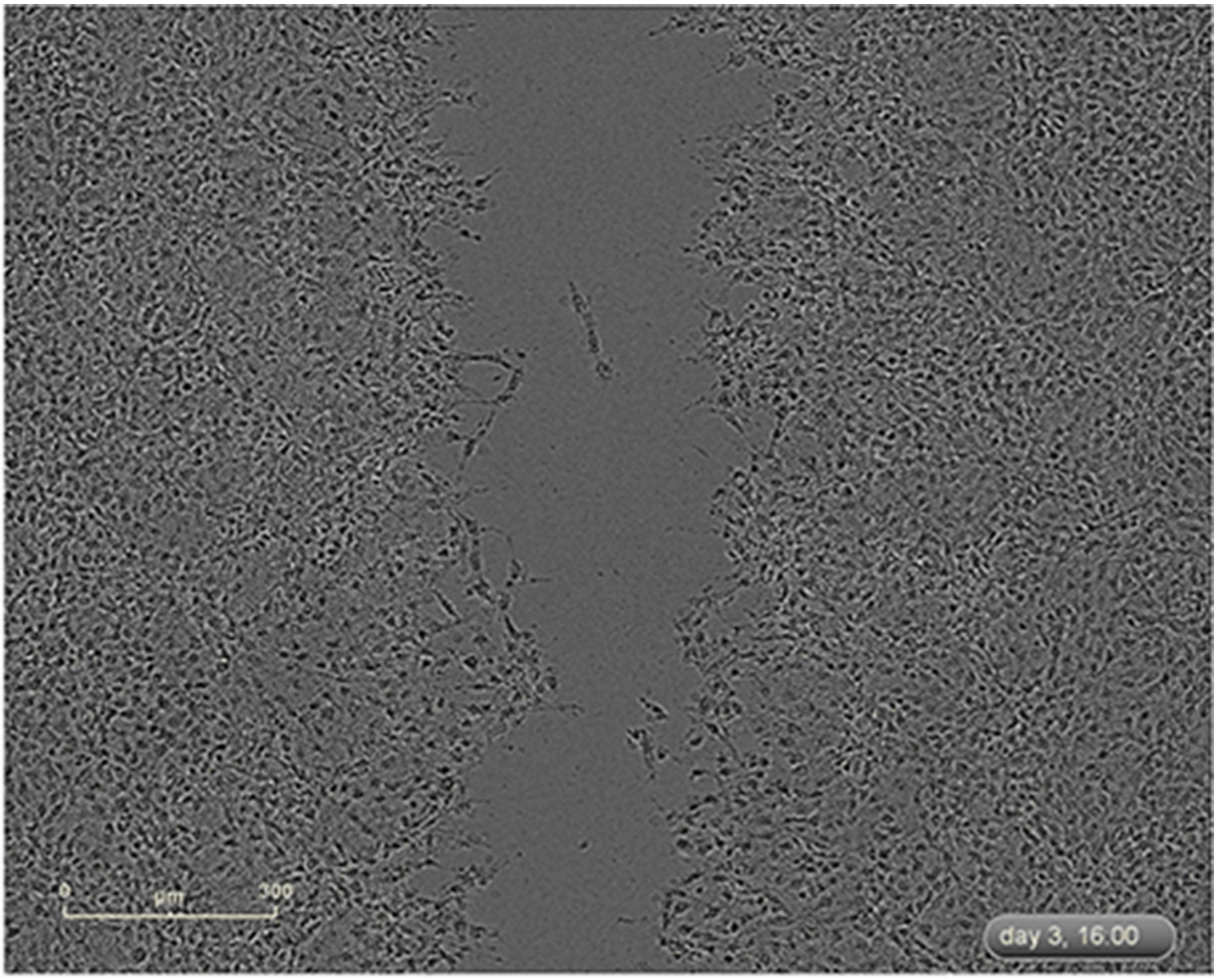
Supplementary Movie 1: Time-lapse video of invasion assay using MSTO-211H-CR cells.

See Supplementary File 1



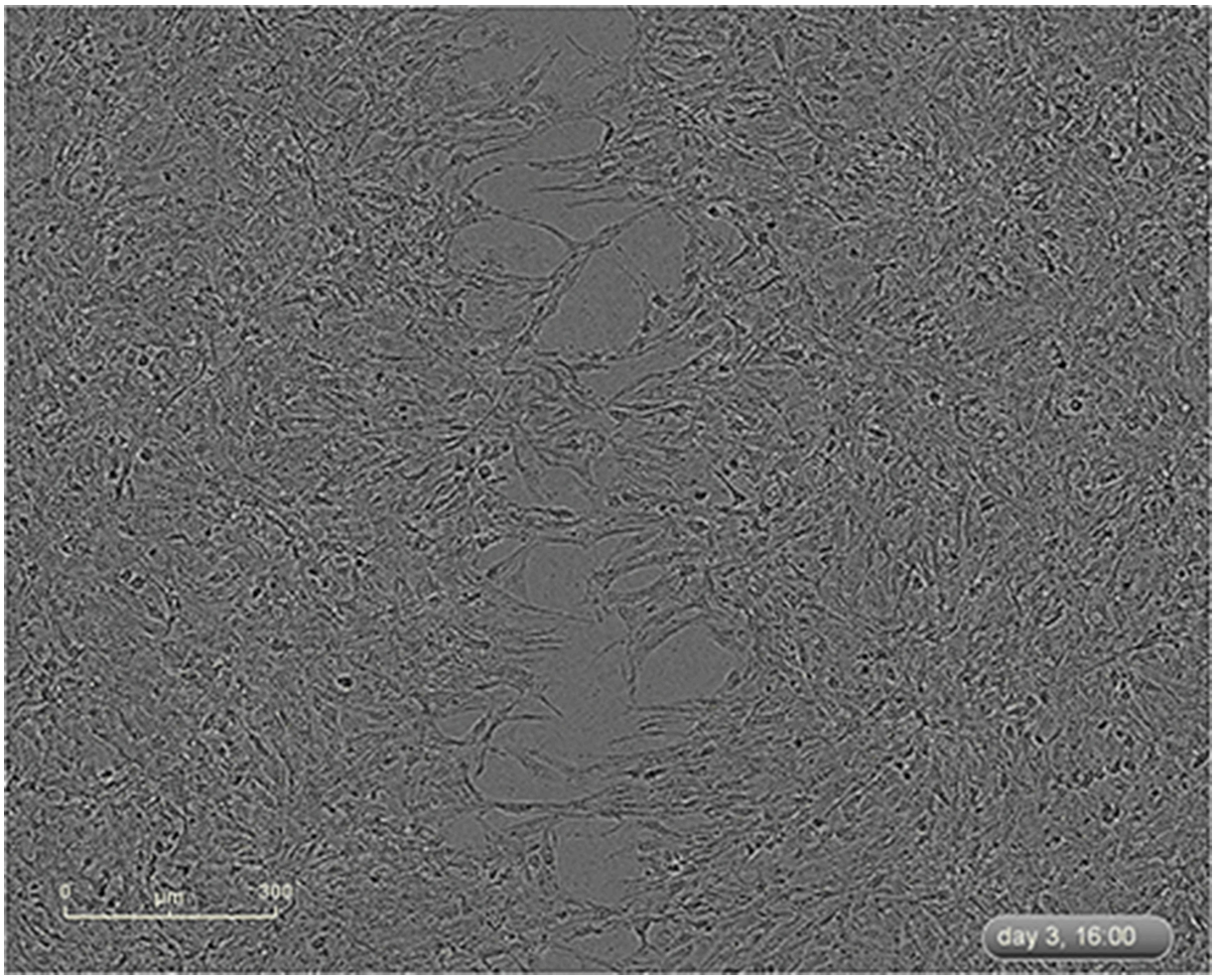
Supplementary Movie 2: Time-lapse video of invasion assay using MSTO-211H wt cells.

See Supplementary File 2



Supplementary Movie 3: Time-lapse video of invasion assay using SPC111 wt cells.

See Supplementary File 3



Supplementary Movie 4: Time-lapse video of invasion assay using SPC111-CR cells.

See Supplementary File 4