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Cell clustering mediated by the adhesion protein PVRL4 is necessary for $\alpha 6\beta 4$ integrin-promoted ferroptosis resistance in matrix-detached cells

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Running title: PVRL4-mediated cell clustering influences ferroptosis

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Abbreviations: MDA, malondialdehyde; GPX4, glutathione peroxidase 4; ROS, reactive oxygen species; GSH, glutathione.

ABSTRACT

Ferroptosis is an iron-dependent form of programmed cell death characterized by the accumulation of lipid-targeting reactive oxygen species that kill cells by damaging their plasma membrane. The lipid-repair enzyme glutathione peroxidase 4 (GPX4) protects against this oxidative damage and enables cells to resist ferroptosis. Recent work has revealed that matrix-detached carcinoma cells can be susceptible to ferroptosis and that they can evade this fate through the signaling properties of the $\alpha 6\beta 4$ integrin, which sustains GPX4 expression. Although these findings on ferroptosis are provocative, they differ from those in previous studies indicating that matrix-detached cells are prone to apoptosis, via a process referred to as anoikis. In an effort to reconcile these discrepant findings, here we observed that matrix-detached epithelial and carcinoma cells cluster spontaneously via a mechanism that involves the cell adhesion protein PVRL4 (also known as Nectin-4). We found that this clustering process allows these cells to survive by stimulating a PVRL4/ $\alpha 6\beta 4$ /Src signaling axis that sustains GPX4 expression and buffers against lipid peroxidation. In the absence of $\alpha 6\beta 4$, PVRL4-mediated clustering induced an increase in lipid

peroxidation that was sufficient for triggering ferroptosis. When the clustering was inhibited, single cells did not exhibit a significant increase in lipid peroxidation in the absence of $\alpha 6\beta 4$, and they were more susceptible to apoptosis than to ferroptosis. These results indicate that ferroptosis induction depends on cell clustering in matrix-detached cells that lack $\alpha 6\beta 4$ and imply that the fate of matrix-detached cells can be determined by the state of their cell-cell interactions.

Ferroptosis is defined as an iron-dependent form of programmed cell death that is characterized by the accumulation of intracellular soluble and lipid reactive oxygen species (ROS) that damage the plasma membrane by peroxidation of polyunsaturated fatty acids (1). At a mechanistic level, ferroptosis is triggered by the loss of activity of the lipid repair enzyme glutathione peroxidase 4 (GPX4), which catalyzes the reduction of lipids and other peroxides and is a target of several ferroptosis inducers (2). The antiporter system X_C^- , which imports cystine into the cell in exchange for glutamate, also has a critical role in protecting against ferroptosis because cysteine, the monomeric form of cystine, is a building block for the antioxidant glutathione (GSH), which is a co-factor for GPX4 (3,4). Molecules that inhibit system x_c^- such as erastin

trigger ferroptosis and have proven to be useful for studying this process in detail (4,5).

Rapid progress is being made in unraveling the biochemical nature of ferroptosis and determining its role in specific biological and pathological processes. A central theme that is emerging from these studies is the essential role of GPX4 in enabling cells to resist ferroptosis, a role that is particularly important for more aggressive tumor cells (6). Less is known, however, about the cell biological context of the mechanisms used to either promote or evade ferroptosis. Recently, we discovered that ferroptosis is a component of cell death induced by the detachment of epithelial and carcinoma cells from matrix (7). We also observed that the $\alpha6\beta4$ integrin has the ability to confer ferroptosis resistance under these conditions. In other terms, matrix-detached cells undergo ferroptosis but only in the absence of $\alpha6\beta4$. The mechanism underlying this phenomenon involves the ability of $\alpha6\beta4$ to sustain GPX4 expression and activity in matrix-detached cells and buffer the increase in lipid peroxidation that occurs in these cells, which can trigger ferroptosis (7).

Our findings on ferroptosis are provocative, but they differ from prior studies concluding that matrix-detached cells are prone to apoptosis, a process referred to as anoikis, unless survival mechanisms are enabled (8) (9). In an effort to reconcile these discrepant findings, we observed that matrix-detached epithelial and carcinoma cells cluster spontaneously by a mechanism that involves PVRL4 (also known as Nectin-4). If this clustering is inhibited, single cells are more susceptible to apoptosis in the absence of $\alpha6\beta4$. Based on these observations, we explored the possibility of a functional connection between cell clustering and ferroptosis. Indeed, the data we report reveal that ferroptosis is dependent on cell clustering in matrix-detached cells that lack $\alpha6\beta4$.

RESULTS

Matrix-detached cells cluster spontaneously by a mechanism that is independent of $\alpha6\beta4$: Our interest in understanding the distinction between ferroptosis and apoptosis in matrix-detached carcinoma cells was piqued by the observation that immortalized mammary epithelial (MCF10-A) and breast carcinoma cells (SUM-159 and Hs578t)

cluster spontaneously upon detachment (**Figure 1A and B**). These clusters could be dissociated into single cells with either 0.5% methylcellulose or 2mM EDTA (**Figure 1A and B**). Interestingly, we did not observe significant differences in cluster number upon depletion of $\alpha6\beta4$ using CRISPR/Cas9 to knock-down the $\beta4$ subunit using two guide RNAs that target different regions of the first exon of ITGB4 (referred to as $\beta4$ -3 and $\beta4$ -4) (**Figure 1B, C**), indicating that this integrin does not contribute to clustering.

Clustering of matrix-detached cells influences the relative levels of ferroptosis and apoptosis in the absence of $\alpha6\beta4$:

The observations described above prompted us to assess the role of cell clustering in ferroptosis that is triggered by the loss of $\alpha6\beta4$. For this purpose, we assayed cell viability 24 hrs after matrix detachment as a function of $\alpha6\beta4$ expression in clustered cells or single cells (MCF10-A, SUM-159 and Hs578t) that had been generated using either methylcellulose or EDTA. These cells, which express $\alpha6\beta4$, remained viable 24hrs after matrix-detachment regardless of their clustering status (**Figure 2A-C**). In response to loss of $\alpha6\beta4$ by CRISPR/Cas9-mediated knock-down of the $\beta4$ subunit, however, a dramatic decrease in viability was seen in all conditions (**Figure 2A-C**), confirming the importance of the $\alpha6\beta4$ integrin in maintaining survival in response to stress. It is important to note that CRISPR-mediated depletion of the $\beta4$ subunit in these cells does affect expression of the $\alpha6$ integrin subunit and they express the $\alpha6\beta1$ integrin (7). Specificity is also evidenced by our previous finding that expression of $\beta4$ construct that cannot be targeted by these CRISPRs in $\beta4$ -depleted cells rescued their viability in ECM-detached conditions (7). Moreover, the $\alpha3\beta1$ integrin does not promote ferroptosis resistance in these cells (7).

To determine whether the nature of cell death differed between clustered and single detached cells in the absence of $\alpha6\beta4$, we compared the ability of ferrostatin-1, ZVAD-fmk or both inhibitors to rescue the viability of detached, $\alpha6\beta4$ -depleted cells in the presence or absence of either methylcellulose or EDTA. In the absence of methylcellulose or EDTA either inhibitor alone yielded a partial rescue of viability

and the use of both inhibitors resulted in a complete rescue (**Figure 2A**), indicating that ferroptosis and apoptosis contribute to the death of these cells. Interestingly, though, ferrostatin-1 was unable to rescue viability in the presence of methylcellulose or EDTA (single cells) but ZVAD-fmk did rescue viability almost completely (**Figures 2B, C**). These results indicate that cell clustering pre-disposes cells to ferroptosis in the absence of $\alpha6\beta4$, and that disruption of these clusters results in apoptosis. Moreover, the finding that $\alpha6\beta4$ -depleted cells are prone to both ferroptosis and apoptosis in the absence of EDTA and methylcellulose probably reflects the fact this population is comprised of a mixture of clustered and single cells (**Figure 1A**).

Lipid peroxidation is significantly higher in clustered, detached cells compared to single cells in the absence of $\alpha6\beta4$: To understand why clustered but not single cells are prone to ferroptosis in the absence of $\alpha6\beta4$, we quantified lipid peroxidation, which is the root cause of ferroptosis, using the malondialdehyde (MDA) assay. Indeed, depletion of $\alpha6\beta4$ resulted in a marked increase in lipid peroxidation in clustered but not single cells (**Figure 3A**). This finding suggests that the clustering of detached cells has the potential to increase lipid peroxidation to levels that are sufficient to trigger ferroptosis and that $\alpha6\beta4$ mitigates these effects on clustered cells. The observation that detached single cells do not exhibit this increase in lipid peroxidation is consistent with our finding that these cells are not susceptible to ferroptosis.

The above results suggest that $\alpha6\beta4$ function differs between detached clustered and single cells, and that this integrin facilitates a mechanism to evade ferroptosis in detached, clustered cells. Based on our previous work (7), we focused on the ability of $\alpha6\beta4$ to induce GPX4 expression and activity in these distinct populations. We observed that GPX4 mRNA (**Figure 3B**) and protein (**Figure 3C**) expression increased significantly upon matrix-detachment of clustered cells (2 hours), but that this increase was less evident in single cells. The increase in GPX4 expression is dependent on $\alpha6\beta4$, consistent with our previous results (7). Moreover, the viability of the clustered, $\alpha6\beta4$ -depleted cells was rescued by

either the lipophilic antioxidant α -tocopherol (**Figure 3D**) or exogenous expression of GPX4 (**Figure 3E**). In contrast, neither α -tocopherol nor GPX4 re-expression in the $\alpha6\beta4$ -depleted, single cells rescued viability. These data infer that $\alpha6\beta4$ signaling differs between clustered and single, matrix-detached cells. Given that the induction of GPX4 expression upon matrix detachment is dependent on $\alpha6\beta4$ /Src signaling (7), we assessed Src activation in detached clustered and single cells as a function of $\alpha6\beta4$ expression. Matrix-detachment caused an increase in activated Src as assayed by Y418 immunoblotting in clustered cells that was dependent on $\alpha6\beta4$ expression and not detected in either EDTA or methylcellulose-treated cells (**Figure 3F**).

PVRL4 mediates clustering of matrix-detached cells and ferroptosis resistance: An important issue that arose from our data is the mechanism by which matrix-detached cells cluster and the contribution of this mechanism to ferroptosis resistance. The possibility that this clustering is mediated by E-cadherin is negated by the fact that two of the cell lines we studied (SUM-159 and Hs578t) have a mesenchymal phenotype and express little, if any, E-cadherin. We confirmed this assumption by treating matrix-detached cells with a function-blocking E-cadherin Ab (DECMA-1) and observing no effect on clustering (**Figure 4A**). Interestingly, however, DECMA-1 had no effect on the percent of MCF10-A cells in clusters, although it did cause a significant decrease in the number of large (10+ cells) clusters (**Figure 4B**). In contrast, treatment of adherent MCF10-A cells with DECMA-1 disrupted cell-cell adhesion (**Figure 4C**). For this reason, we focused on the cell surface receptor PVRL4 (also referred to as nectin-4) because it has been shown to mediate the clustering of matrix-detached carcinoma cells by interacting *in trans* with PVRL1 (10). Importantly, PVRL4 also maintains the survival of these cells by interacting with $\alpha6\beta4$ *in cis* and enabling Src activation (10). These seminal studies, however, did not consider a potential role for PVRL4 in ferroptosis resistance. For this reason, we initially examined the ability of a function-blocking PVRL4 Ab to disrupt the clustering of matrix-detached cells and found that this Ab was as effective as either EDTA or

methylcellulose in disrupting clusters into single cells (**Figure 4A**). Depletion of $\beta 4$ did not significantly affect PVRL4 expression in $\beta 4$ -depleted cells (**Figure 4D**).

The above finding prompted us to assess the contribution of PVRL4 to the cell death that occurs upon matrix-detachment. Matrix-detached cells that express $\alpha 6\beta 4$ exhibited no significant loss of viability in response to treatment with the PVRL4 function-blocking antibody compared with IgG control (**Figure 5A**), supporting our conclusion that this integrin promotes the survival of clustered and single, matrix-detached cells. As we reported previously (7), matrix-detached cells that lack $\alpha 6\beta 4$ exhibit a dramatic loss of viability that is rescued by ferrostatin-1. In marked contrast, $\alpha 6\beta 4$ -depleted cells treated with the PVRL4 function-blocking antibody exhibited a significant loss of viability that was rescued by ZVAD-fmk and not ferrostatin-1 (**Figure 5B**). This result indicates that disruption of PVRL4-mediated clustering of $\alpha 6\beta 4$ -depleted cells renders them sensitive to apoptosis as opposed to ferroptosis, and that PVRL4 is necessary to execute ferroptosis in this population of cells.

Given that $\alpha 6\beta 4$ -mediated Src signaling maintains the survival of matrix-detached cells (7), we examined the impact of the PVRL4 function-blocking antibody on Src activation in matrix-detached cells. Indeed, disruption of cell clustering by this antibody resulted in a significant decrease in Src activation compared to control cells as assessed by phospho-Src (Y418) immunoblotting (**Figure 5C**). A causal role for Src activation in ferroptosis resistance is indicated by the observation Src inhibition using PP2 decreased the viability of clustered, matrix-detached cells significantly, and that this loss of viability was rescued by ferrostatin-1 but not ZVAD-fmk (**Figure 5D**). Together, these results indicate that PVRL4-mediated clustering is necessary for $\alpha 6\beta 4$ -mediated Src activation.

DISCUSSION

The results of this study reveal an intimate relationship between the clustering of matrix-detached carcinoma cells and ferroptosis. We conclude from our data that matrix-detached carcinoma cells cluster spontaneously and that this clustering triggers an increase in lipid peroxidation

that is sufficient to induce ferroptosis. Ferroptosis can be evaded under these conditions if these cells express the $\alpha 6\beta 4$ integrin, which functions to buffer lipid peroxidation by sustaining GPX4 expression. In marked contrast, dissociation of clustered, detached cells into single cells results in apoptosis in the absence of $\alpha 6\beta 4$. An important and novel inference from these data is that the fate of matrix-detached cells can be determined by the state of their cell-cell interactions.

Our findings build upon the seminal study by Elledge and colleagues that identified PVRL4 in a gain of function screen for genes that promote the survival of matrix-detached carcinoma cells (10). Not only did they implicate PVRL4 in the clustering of matrix-detached carcinoma cells and anoikis resistance, they also demonstrated that PVRL4 enables $\alpha 6\beta 4$ -mediated Src activation and the importance of this pathway in the survival of these detached cells. Our work substantiates these findings and extends them to ferroptosis resistance. Indeed, we demonstrate that the PVRL4/ $\alpha 6\beta 4$ /Src axis allows matrix-detached cells to resist ferroptosis by sustaining GPX4 expression and minimizing lipid peroxidation, establishing a novel role for PVRL4 in ferroptosis resistance mechanisms. Interestingly, however, we also show that PVRL4 can promote ferroptosis in the absence of $\alpha 6\beta 4$ by mediating cell clustering and contributing, either directly or indirectly, to increased lipid peroxidation. In other terms, this observation implies that the process of cell clustering mediated by PVRL4 stimulates an increase in lipid peroxidation by a mechanism to be determined, and that this scenario does not occur in single cells, which do not engage PVRL4-mediated adhesion.

Previous studies have noted the role of cell ‘aggregation’ in promoting apoptosis resistance in matrix-detached squamous carcinoma and ErbB2-expressing MCF10-A mammary epithelial cells (11) (12). These studies focused on cells that expressed E-cadherin and they implicated E-cadherin as the mediator of cell aggregation and apoptosis resistance. For this reason, our finding that PVRL4 mediates the clustering of matrix-detached MCF10-A cells is intriguing and raises the need to investigate the relationship between E-cadherin- and PVRL4-mediated cell-cell adhesion. Our primary interest,

however, is on survival mechanisms used by aggressive carcinoma cells, which often exhibit a mesenchymal phenotype and express little, if any, E-cadherin (13). In fact, the carcinoma cell lines used in our study are mesenchymal when grown as adherent cells but retain the ability to engage in cell-cell adhesion upon matrix-detachment, a scenario that mimics the transition from aggressive primary carcinomas to circulating tumor cells, which tend to cluster (14).

Although studies on the nature of cell death triggered by matrix-detachment have focused largely on apoptosis, it is becoming apparent that other modes of cell death can occur that involve elevated ROS levels (15,16). Our findings establish ferroptosis as one such ROS-mediated form of cell death. They also provide insight into the cell biological parameters that influence ferroptosis by revealing how the clustering of matrix-detached carcinoma cells can promote ferroptosis as opposed to apoptosis. On a broader level, the role of ferroptosis in eliminating matrix-detached carcinoma cells is timely and significant based on the recent realization that aggressive carcinoma cells with a mesenchymal phenotype are highly sensitive to ferroptosis unless they enable mechanisms that sustain GPX4 expression (6). Based on our data, PVRL4/ α 6 β 4/Src signaling in the context of cell clustering could be one such mechanism.

EXPERIMENTAL PROCEDURES

Cell lines and reagents: MCF10-A cells were obtained from the Barbara Ann Karmanos Cancer Institute and SUM-159 cells were provided by Dr. Stephen Ethier (Medical College of South Carolina). Hs578T cells were provided by Dr. Dohoon Kim (UMass Medical School). MCF10-A cells were maintained in DMEM/F-12 (Gibco) with 5% horse serum, 1% pen/strep (Gibco), 20ng/mL EGF (PeproTech), 0.5mg/mL hydrocortisone (Gibco), 100ng/mL cholera toxin (Sigma), and 10ug/mL insulin (Sigma). SUM-159 cells were maintained in F-12 (Gibco) medium with 5% fetal bovine serum (Hyclone), 1% pen/strep, 0.5mg/mL hydrocortisone, and 10ug/mL insulin. Hs578t cells were maintained in DMEM high glucose (Gibco) medium with 10% fetal bovine serum, 1% pen/strep, and 10ug/mL insulin. All cells were checked quarterly for mycoplasma and authenticated using the

University of Arizona Genetic Core. The following antibodies were used: GPX4 (Abcam, ab125066), actin (Sigma-Aldrich), integrin β 4 (505 [Rabinovitz et al., 1999]), phospho-Src Y418 (R&D Systems MAB2685), total Src (Santa Cruz Biotechnology sc-8056), DECMA-1 (Abcam (ab11512), PVRL4 (R&D Systems 17402 and Cell Signaling Technology 17402). Other reagents used were: ZVAD-fmk (SelleckChem), ferrostatin-1 (Sigma-Aldrich), α -tocopherol (Sigma-Aldrich), PP2 (SelleckChem).

Matrix-detachment assays: Twenty-four well plates were coated with 800uL of polyHEMA (30mg/mL, Sigma-Aldrich) and dried overnight at room temperature. Cells were trypsinized and complete medium was used to quench the trypsin. Cells were counted and the remaining suspension was diluted to 2.5×10^4 cells per 100uL and plated (100uL per well) in cell-line specific serum-free medium with the reagents indicated in the figure legends for the times noted. Four wells were plated per condition as technical replicates. Cells were counted with a hemocytometer using trypan blue exclusion. Total cell number was calculated by multiplying average cells per square by dilution factor and chamber volume.

Biochemical experiments: For all experiments, three wells were plated per condition for each independent experiment as technical replicates, and at least three independent experiments were performed. For immunoblotting, cells collected in to pre-chilled tubes and spun down before washing twice in ice-cold PBS. Protein was extracted using RIPA buffer containing protease and phosphatase inhibitors (Boston Bioproducts, Worcester, MA, USA) by lysing cells 15 minutes on ice followed by centrifugation at maximum speed for 15 minutes at 4°C. Supernatant was collected and protein concentration quantified using Bradford reagent (Bio-Rad). Samples (25ug protein) were separated by SDS-PAGE and immunoblotted using the Abs specified in the figure legends. For qPCR, RNA was isolated from cells using the NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel). Briefly, cells were collected, lysed immediately following centrifugation, and RNA isolated following manufacturer's kit specifications. RNA concentrations were determined by Nanodrop and cDNA (1ug per

sample) was produced using the All-In-One cDNA Synthesis SuperMix (BioScript). qPCR was performed using a Cyber Green master mix (BioTool). Sequences for primers used are as follows: 18s Forward 5'-AACCCGTTGAACCCATT-3', Reverse 5'-CCATCCAATCG GTAGTAGCG-3', derived from NT_167214.1, GPX4 Forward 5'-GAGGCAAGACCGAAGTAACTAC-3', Reverse 5'-CCGAACTGGTTACACGGGAA-3', derived from NC_000019.10.

To assay lipid peroxidation, the Malondialdehyde (MDA) Lipid Peroxidation microplate assay (Sigma-Aldrich) was used according to the manufacturer's specifications. Briefly, cells (1×10^6) were cultured in cell-line specific, serum-free medium on polyHEMA-coated plates for 4 hours. Three wells were plated per condition for each independent experiment as technical replicates. Cells were collected, lysed and reacted with thiobarbituric acid (TBA) for one hour at 95°C. An aliquot of lysate (5 uL) was saved for protein concentration determination using the Bradford assay prior to reaction with TBA. Fluorescence was read at 532(ex)/590(em). Lipid peroxidation levels were normalized to protein concentration.

Molecular biology experiments: For CRISPR-mediated deletion of the $\beta 4$ integrin subunit, guide RNAs (sgRNA) targeting exon 1 of the $\beta 4$ sequence were selected using two websites, CRISPR Design (<http://crispr.mit.edu>) and

CRISPRdirect (<https://crispr.dbcls.jp>). Four guide RNAs were tested and the two most efficient knockouts were. Guide RNA sequences used were:

B4-3 5'
CACCGTTGTCCAGATCATCGGACA 3',
5'AAACTGTCCGATGATCTGG ACAAC 3'.
B4-4 5' CACCGAAATCCAATAGTGTAGTCGC
3', AAACGCGACTACACTAT TGGATTTC 3'.

Cells were sub-cloned by FACS and screened for loss of protein expression by immunoblot. New CRISPR-depleted cells were generated every three months to control for potential secondary effects caused by long-term gene depletion. To express GPX4, a plasmid construct for GPX4 was purchased from Origene (RC208065). Cells were transfected with 10 μ g of DNA using Lipofectamine 2000 (ThermoFisher) and incubated for 48 hours prior to use.

Statistical Analysis: The bars in graphs represent mean \pm standard deviation. All experiments were repeated at least three times. P-values were calculated using ANOVA and a p-value less than 0.05 was considered significant.

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Conflict of Interest:

The authors declare that they have no conflicts of interest with the contents of this article.

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FIGURE LEGENDS

Figure 1. Breast epithelial and carcinoma cells preferentially cluster upon matrix detachment (A) MCF10-A, SUM-159 and Hs578t cells (vector and $\beta 4$ -depleted) were detached and allowed to cluster spontaneously or treated with either 2mM EDTA or 0.5% methylcellulose to generate single cell suspensions. Photomicrographs were taken 2 hrs post-detachment. Bar = 10 microns. **(B)** Quantification of MCF10-A, SUM-159 and Hs578t clusters was performed by counting the total number of single and clustered cells, total number of clusters (defined as ≥ 3 cells). Six independent photos of each cell line and each condition were quantified. **(C)** The $\beta 4$ integrin subunit was depleted in Hs578t cells by CRISPR/CAS9 using two independent guide RNAs ($\beta 4$ -3 and $\beta 4$ -4). Depletion of $\beta 4$ expression was verified by immunoblotting.

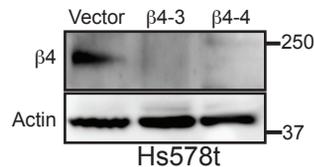
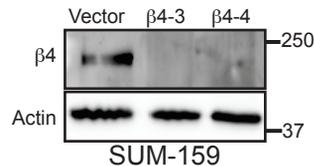
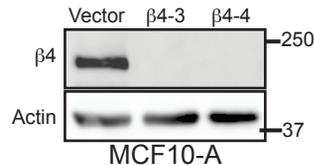
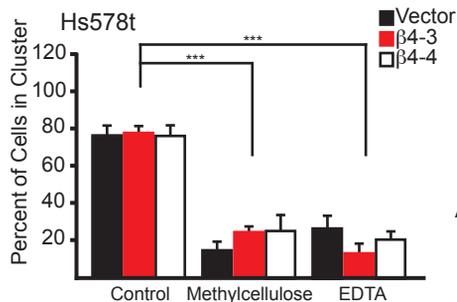
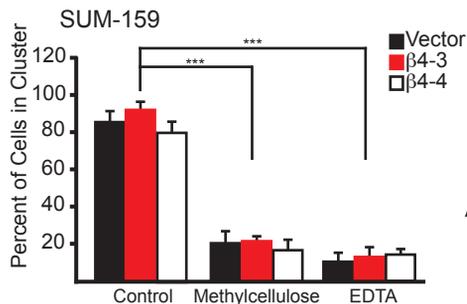
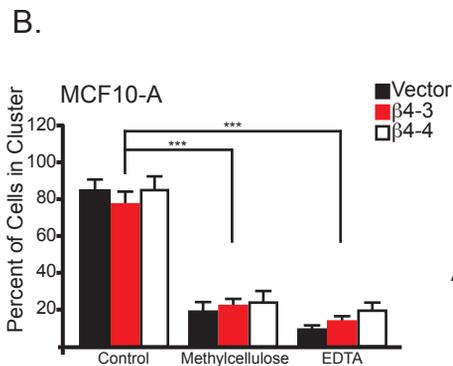
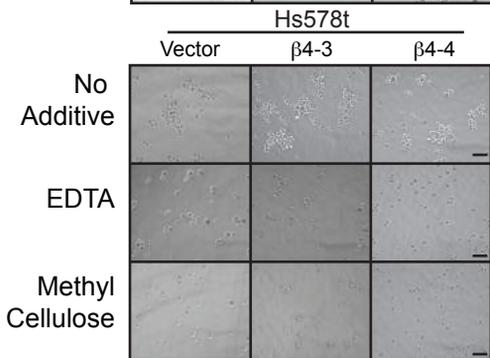
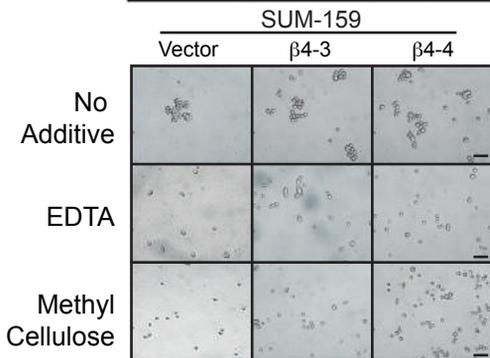
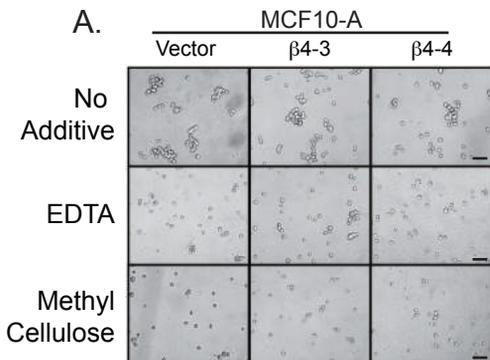
Figure 2. Clustering of matrix-detached cells influences the relative levels of ferroptosis and apoptosis in the absence of $\alpha 6\beta 4$. **(A)** Control and $\beta 4$ -depleted cells were detached for 24 hrs in the presence of either DMSO, ferrostatin-1 (2 μ M), ZVAD-fmk (25 μ M) or ferrostatin-1 and ZVAD-fmk and the number of viable cells was quantified. **(B)** Control and $\beta 4$ -depleted cells were detached for 24 hrs with 2mM EDTA in the presence of either DMSO, ferrostatin-1 (2 μ M), ZVAD-fmk (25 μ M) or ferrostatin-1 and ZVAD-fmk and the number of viable cells was quantified. **(C)** Control and $\beta 4$ -depleted cells were detached for 24 hrs with 0.5% methylcellulose in the presence of either DMSO, ferrostatin-1 (2 μ M), ZVAD-fmk (25 μ M) or ferrostatin-1 and ZVAD-fmk and the number of viable cells was quantified.

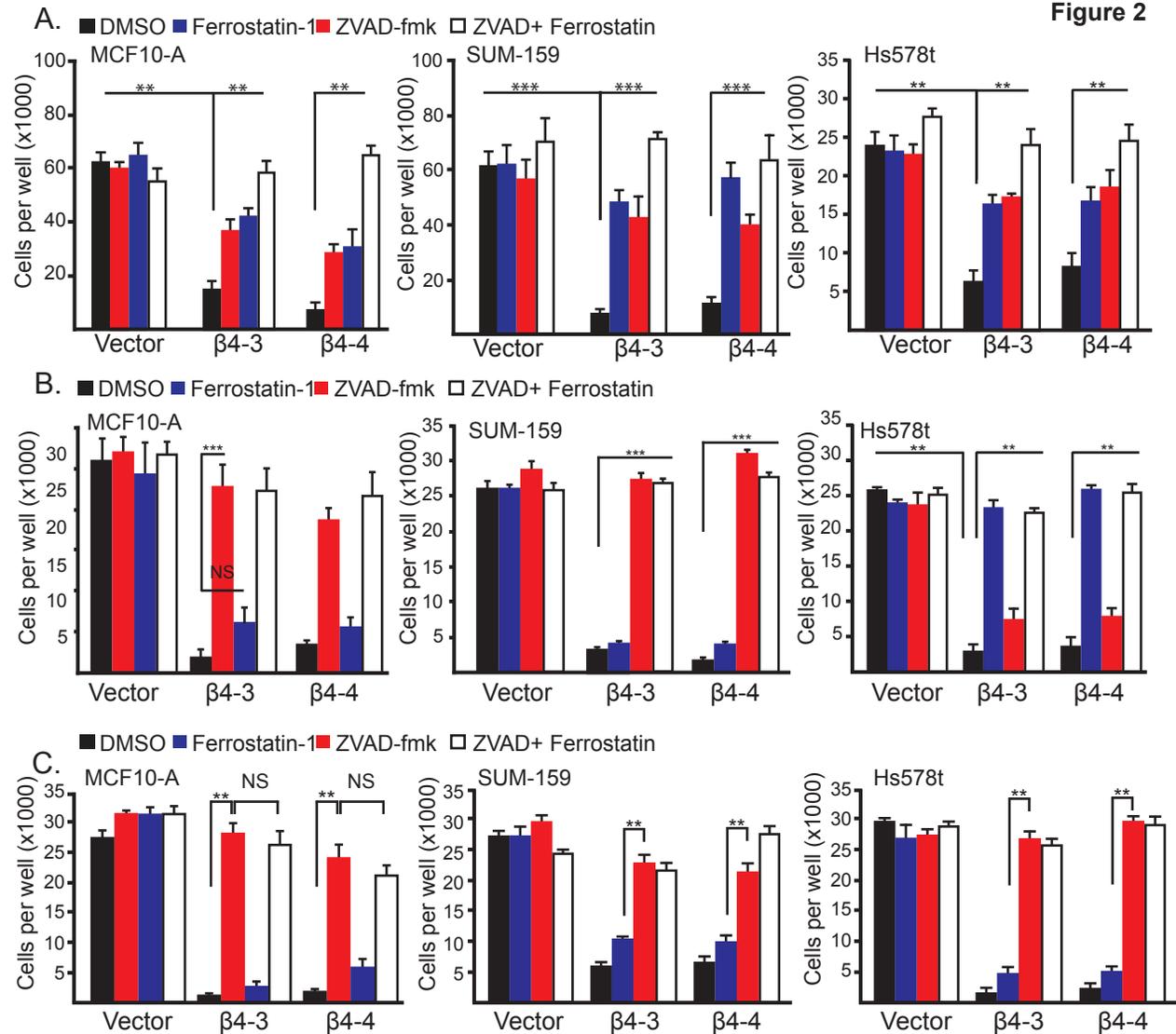
Figure 3. Clustering of matrix-detached cells influences lipid peroxidation, GPX4 expression and Src activity. **(A)** Lipid peroxidation was quantified using the MDA assay in control and $\beta 4$ -depleted MCF10-A and SUM-159 cells under either adherent, detached clustered (no additive) or detached single (0.5% methylcellulose) conditions for 4 hrs. Similar results were obtained using 2mM EDTA. **(B)** GPX4 mRNA expression was quantified by qPCR in control and $\beta 4$ -depleted cells MCF10-A and SUM-159 cells under either adherent, detached clustered (no additive) or detached single (0.5% methylcellulose) conditions for 2 hrs. **(C)** GPX4 protein expression was assessed by immunoblotting in control MCF10-A, SUM-159 and Hs578t cells in detached clustered (no additive) or detached single (0.5% methylcellulose) conditions for 4 hrs. Immunoblots were quantified by densitometry and the ratio of the intensity of the GPX4/actin bands relative to adherent cells is shown under the blots. **(D)** SUM-159 $\beta 4$ -depleted cells were detached for 24 hrs with either no additive (Clustered) or 2mM EDTA (Single) in the presence of either DMSO or 500mM α -tocopherol, and the number of viable cells was quantified. **(E)** Control and

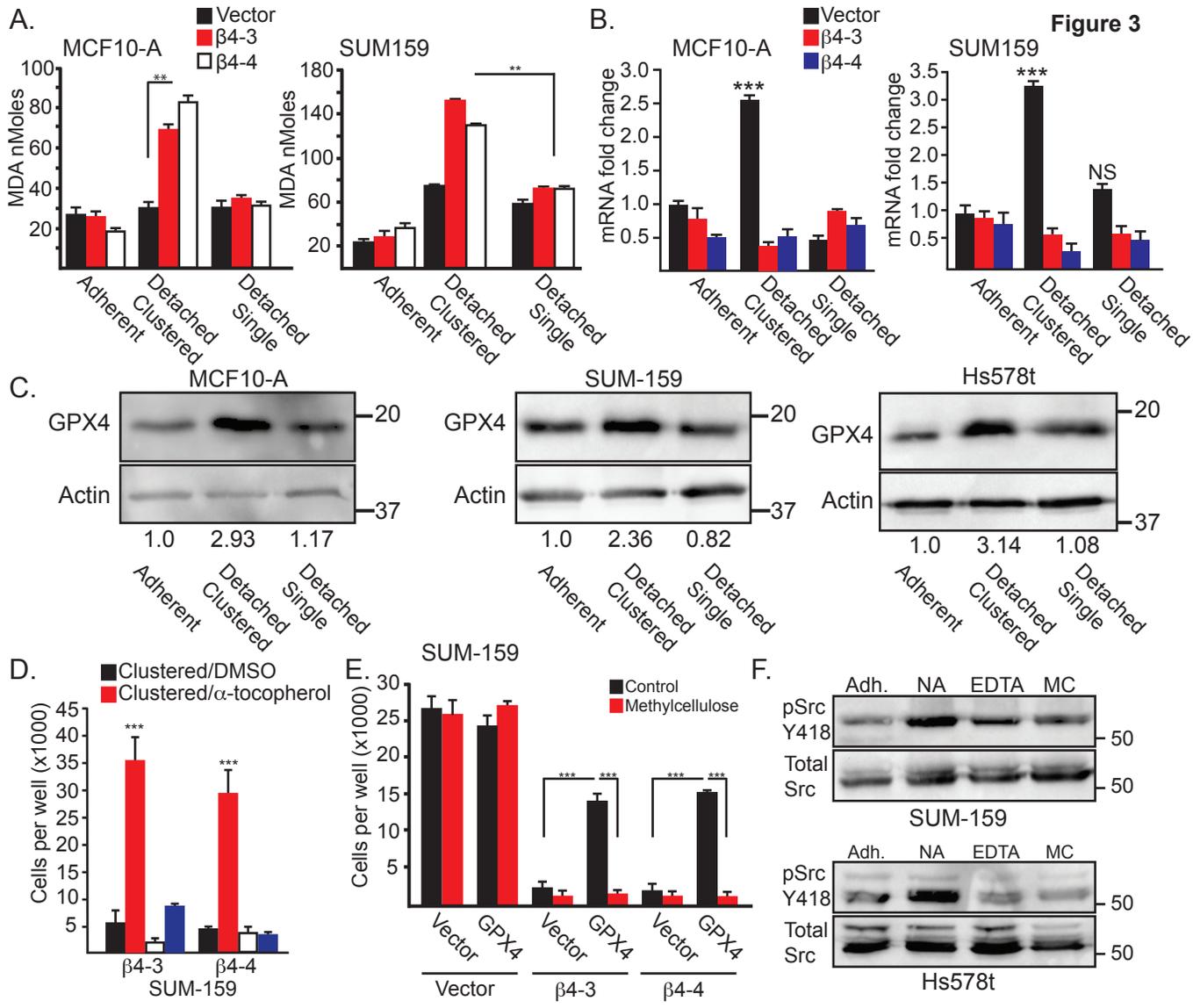
β 4-depleted cells SUM-159 cells that had been transfected with either a vector control or a GPX4 expression vector were detached for 24 hours with no additive (Control) or 0.5% methylcellulose, and the number of viable cells was quantified. (F) SUM-159 and Hs578t control cells were assessed for phosphorylated (Y418) Src by immunoblotting in adherent conditions as well as following two hours of matrix-detachment with no additive (NA), 2mM EDTA (EDTA), or 0.5% methylcellulose (MC).

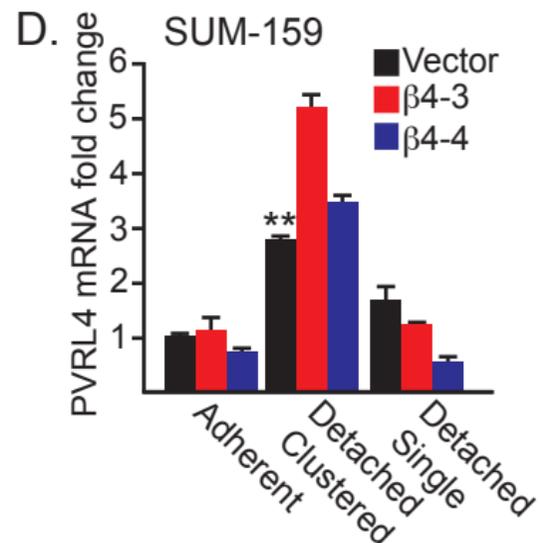
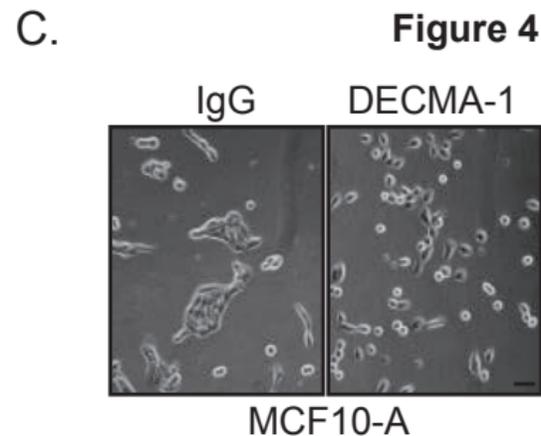
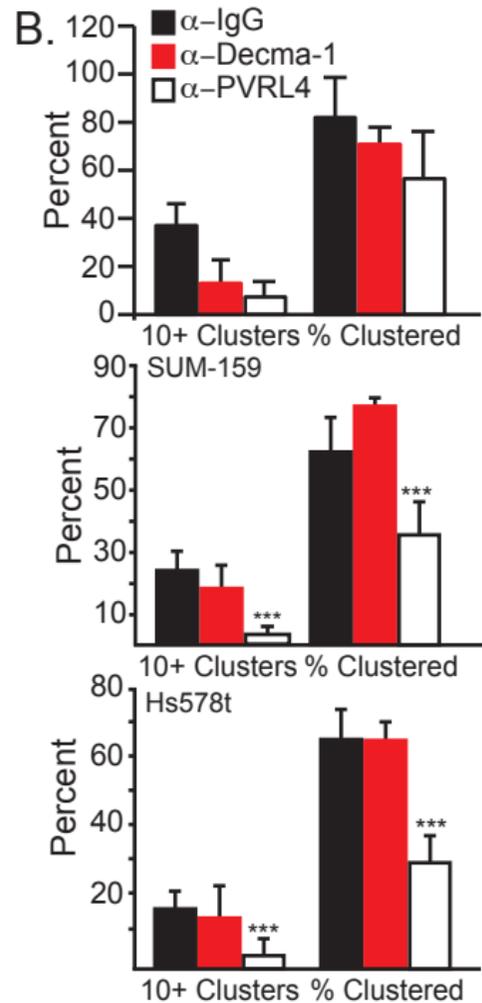
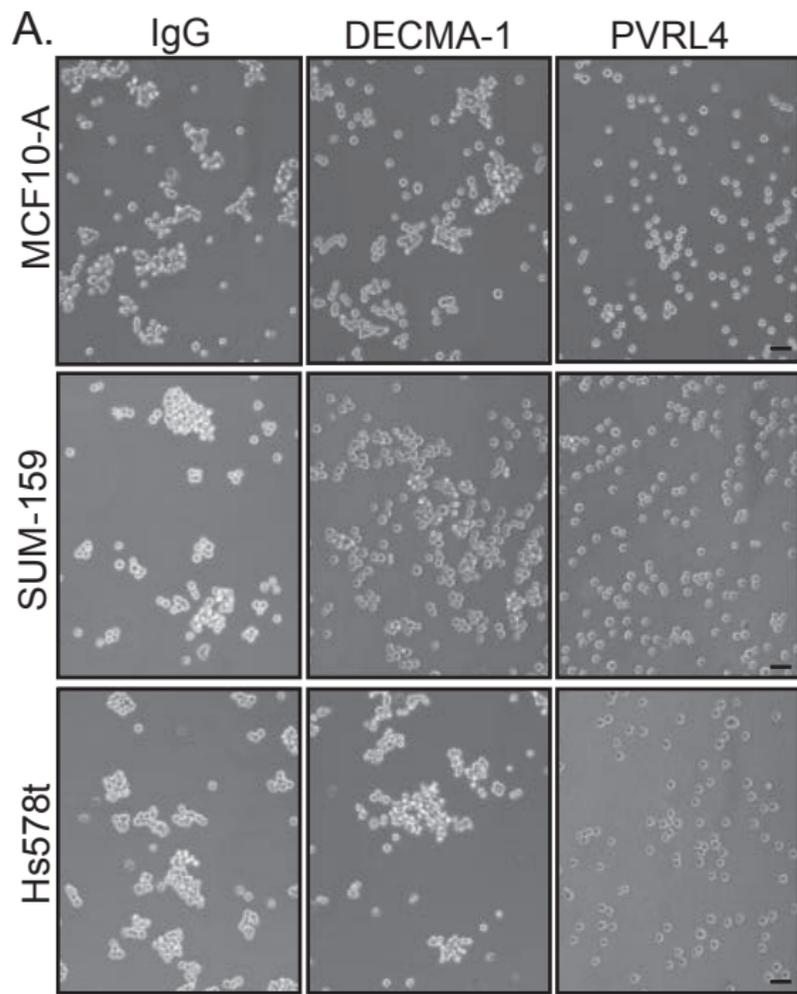
Figure 4. PVRL4 mediates clustering upon matrix-detachment. (A) MCF10-A, SUM-159, and Hs578t vector control cells were detached and allowed to aggregate in the presence of 4 μ g/mL of either IgG, DECMA-1 or a PVRL4-blocking Ab. Photomicrographs were taken 2 hrs post-detachment. Bar = 10 microns. (B) Quantification of MCF10-A, SUM-159 and Hs578t clusters was performed by counting the total number of single and clustered cells, total number of clusters (defined as ≥ 3 cells) and the number of large clusters (defined as ≥ 10 cells). (C) MCF10-A cells were detached and allowed to re-adhere in the presence of either IgG or DECMA-1. Photomicrographs were taken at 24h. Bar = 10 microns. (D) SUM-159 control and β 4-depleted cells under adherent, detached clustered or detached single cell conditions were assessed for expression of PVRL4 mRNA by qPCR.

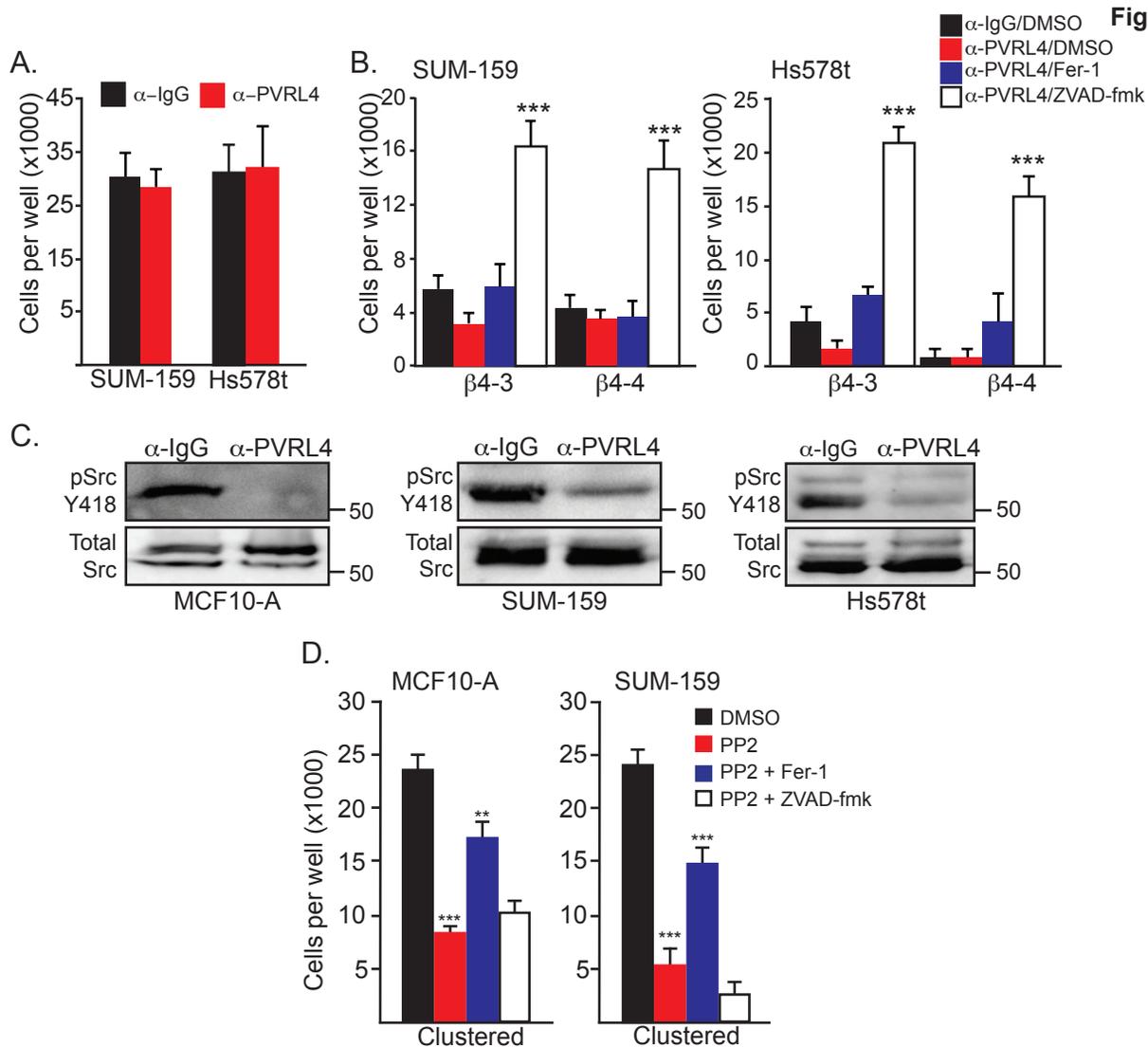
Figure 5. PVRL4-mediated cell clustering is necessary for α 6 β 4-dependent Src activation. (A) SUM-159 and Hs578t control cells were detached for 24 hrs in the presence of either IgG or a PVRL4 Ab and the number of viable cells was quantified. (B) SUM-159 and Hs578t β 4-depleted cells were detached for 24 hrs in the presence of either IgG and DMSO, PVRL4 Ab, PVRL4 Ab and ferrostatin-1 (2 μ M) or PVRL4 and ZVAD-fmk (25 μ M), and the number of viable cells was quantified. (C) MCF10-A, SUM-159 and Hs578t control cells were detached for 2 hours in the presence of either IgG or a PVRL4-blocking Ab, and phosphorylation of Src (Y418) was assessed by immunoblotting. (D) Cells (MCF10-A and SUM-159) were assessed for viability after 24 h of detachment (clustered conditions) in the presence of either DMSO, PP2 (10 μ M) or PP2 in combination with either ferrostatin-1 (2 μ M) or ZVAD-fmk (25 μ M).











**Cell clustering mediated by the adhesion protein PVRL4 is necessary for $\alpha 6\beta 4$ integrin
–promoted ferroptosis resistance in matrix-detached cells**

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