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<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT</b> The hypothesis being tested in this proposal is that breast cancer cells, especially metastatic cells, are prone to rapid increases in ROS and lipid peroxidation caused by adverse micro-environmental conditions and that these oxidative bursts could have deleterious consequences including ferroptosis. Ferroptosis is defined as an iron-dependent, non-apoptotic form of programmed cell death characterized by the accumulation of intracellular soluble and lipid reactive oxygen species (ROS). For this reason, breast tumor cells must acquire mechanisms to protect against oxidative bursts and ferroptosis to metastasize. Work accomplished during the second year of this proposal has demonstrated that matrix-detached breast carcinoma cells cluster spontaneously and that this clustering triggers an increase in lipid peroxidation that is sufficient to induce ferroptosis. We also found that clustering occurs by 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. 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.						
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## Table of Contents

	<u>Page</u>
<b>1. Introduction.....</b>	<b>3</b>
<b>2. Keywords.....</b>	<b>3</b>
<b>3. Accomplishments.....</b>	<b>3</b>
<b>4. Impact.....</b>	<b>4</b>
<b>5. Changes/Problems.....</b>	<b>4</b>
<b>6. Products.....</b>	<b>4</b>
<b>7. Participants &amp; Other Collaborating Organizations.....</b>	<b>4</b>
<b>8. Special Reporting Requirements.....</b>	<b>5</b>
<b>9. Appendices.....</b>	<b>5</b>

## 1. INTRODUCTION

The goal of this Breakthrough Award application is to open a new direction in our understanding of the mechanisms that sustain the survival of breast tumor cells, a problem of paramount importance in the biology and therapy of breast cancer. Adverse conditions in the tumor microenvironment such as detachment from matrix (anoikis) and hypoxia result in cell death, and tumor cells must acquire mechanisms to resist such death to survive and progress to metastatic disease. Our interest in this area has been awakened by the discovery of a novel mode of programmed cell death termed ferroptosis, which is defined as an iron-dependent, non-apoptotic form of programmed cell death characterized by the accumulation of intracellular soluble and lipid reactive oxygen species (ROS). The overarching hypothesis to be tested is that breast cancer cells, especially metastatic cells, are prone to rapid increases in ROS and lipid peroxidation caused by adverse micro-environmental conditions and that these oxidative bursts could have deleterious consequences including ferroptosis. For this reason, breast tumor cells must acquire mechanisms to protect against oxidative bursts and ferroptosis to metastasize. One novel mechanism involves expression of the  $\alpha 6\beta 4$  integrin, which we postulated in the original application has the ability to regulate enzymes that buffer intracellular ROS and diminish lipid peroxidation.

Two specific aims were proposed to address our hypothesis. The first aim was to establish that the  $\alpha 6\beta 4$  integrin protects breast tumor cells against ferroptosis caused by adverse tumor microenvironments and the second aim was to determine the mechanism by which the  $\alpha 6\beta 4$  integrin protects tumor cells against ferroptosis. As reported below, we have made significant progress in achieving the goals that we outlined and the impact of the data that we have obtained has been strengthened by other developments in the field.

## 2. KEYWORDS

Breast cancer, Integrins, Ferroptosis, Cell Death, Metastasis, Lipid Peroxidation

## 3. ACCOMPLISHMENTS

### *What were the major goals of the project?*

The major goals of the project were divided into two major tasks:

Task 1. Establish that the  $\alpha 6\beta 4$  integrin protects breast tumor cells against ferroptosis caused by adverse tumor microenvironments. Months 1-24.

Task 2. Determine the mechanism by which the  $\alpha 6\beta 4$  integrin protects tumor cells against ferroptosis. Months 6-24.

### *What was accomplished under these goals?*

**Task 1. Establish that the  $\alpha 6\beta 4$  integrin protects breast tumor cells against ferroptosis caused by adverse tumor microenvironments.** We finalized this task and published our second manuscript on this project (Brown et al., *Journal of Biological Chemistry*, 2018, 293:12741-12748). The progress we made impacted all of the three sub-tasks of Task 1. Specifically, we discovered that matrix-detached breast carcinoma cells cluster spontaneously and that this clustering triggers an increase in lipid peroxidation that is sufficient to induce ferroptosis (see Figures 1 and 2 in the Brown et al. publication). We also found that clustering occurs by 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 (see Figures 3-5 in the Brown et al. publication). 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.

The results we report 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 (Pavlova et al., 2013, *eLife* 2, ee00358). 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.

**Task 2. Determine the mechanism by which the  $\alpha6\beta4$  integrin protects tumor cells against ferroptosis.** The results described above also impacted the goals of Task 2. Specifically, these data, in concert with our 2017 Journal of Cell Biology paper (see last year's report), provide a mechanism for how the  $\alpha6\beta4$  integrin. We now know that this mechanism involves PVRL4-dependent cell clustering and consequent  $\alpha6\beta4$ -mediated Src activation. We were also able to resolve Task 2b and conclude that this mechanism is independent of  $\alpha6\beta4$  binding to its ligand, which are the laminins. This result is consistent with conclusion of the Elledge paper mentioned above.

***What opportunities for training and professional development has the project provided?***

Nothing to report

***How were the results disseminated to communities of interest?***

Nothing to report

***What do you plan to do during the next reporting period to accomplish the goals?***

The funding period for this grant ended on January 14, 2019. However, we requested and were granted a no-cost extension to finalize the experiments outlined in Task 2c.

#### **4. IMPACT**

The findings that we have made during the second year of this award continue to have significant implications for the therapeutic treatment of aggressive cancers. 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. 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 (Viswanathan et al., *Nature*, 2017, 547:453-457). Based on our data, PVRL4/ $\alpha6\beta4$ /Src signaling in the context of cell clustering could be one such mechanism. A major effort is underway to develop effective GPX4 inhibitors (Hangauer et al. *Nature*, 2017, 551:247-250) and a similar effort to develop inhibitors of  $\alpha6\beta4$  signaling seems warranted based on our work.

#### **5. CHANGES/PROBLEMS**

Nothing to report.

#### **6. PRODUCTS**

The major product to report is our journal publication Brown CW, Amante JJ, Mercurio AM. PVRL4-mediated clustering is necessary for ferroptosis resistance promoted by the  $\alpha6\beta4$  integrin in matrix-detached carcinoma cells. *Journal of Biological Chemistry*, 2018, 293:12741-12748. This publication is included in the Appendix.

#### **7. PARTICIPANTS and OTHER COLLABORATING ORGANIZATIONS**

***What individuals worked on the project?***

Arthur M. Mercurio (No Change)

Caitlin W. Brown (No Change)

John J. Amante (No Change)

***Has there been a change in the active support of PI or key personnel?***

We are thankful that this Breakthrough enabled us to obtain a new NIH R01 grant. This grant was awarded recently and will allow us to continue our studies on the regulation and function of ferroptosis in breast cancer with an emphasis on therapy.

**1R01 CA218085-01A1 (Mercurio, PI) 07/11/18 – 06/30/23**

NIH/NCI

*Integrin Regulation of Non-apoptotic Death in Breast Cancer*

The goal of this application is to understand mechanisms that enable breast tumor cells to survive under conditions that have the potential to kill them with an emphasis on ferroptosis.

***What other organizations were involved as partners?***

None

**8. APPENDICES**

We include 1 publication in the Appendix.



# Cell clustering mediated by the adhesion protein PVRL4 is necessary for $\alpha6\beta4$ integrin–promoted ferroptosis resistance in matrix-detached cells

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Edited by Alex Tokar

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 GSH 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  $\alpha6\beta4$  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/ $\alpha6\beta4$ /Src signaling axis that sustains GPX4 expression and buffers against lipid peroxidation. In the absence of  $\alpha6\beta4$ , 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  $\alpha6\beta4$ , 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  $\alpha6\beta4$  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)<sup>3</sup> 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 GSH

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<sub>C</sub><sup>-</sup>, which imports cystine into the cell in exchange for glutamate, also has a critical role in protecting against ferroptosis because cystine, the monomeric form of cystine, is a building block for the antioxidant GSH, which is a co-factor for GPX4 (3, 4). Molecules that inhibit system X<sub>C</sub><sup>-</sup>, 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 the 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, then 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 depends 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

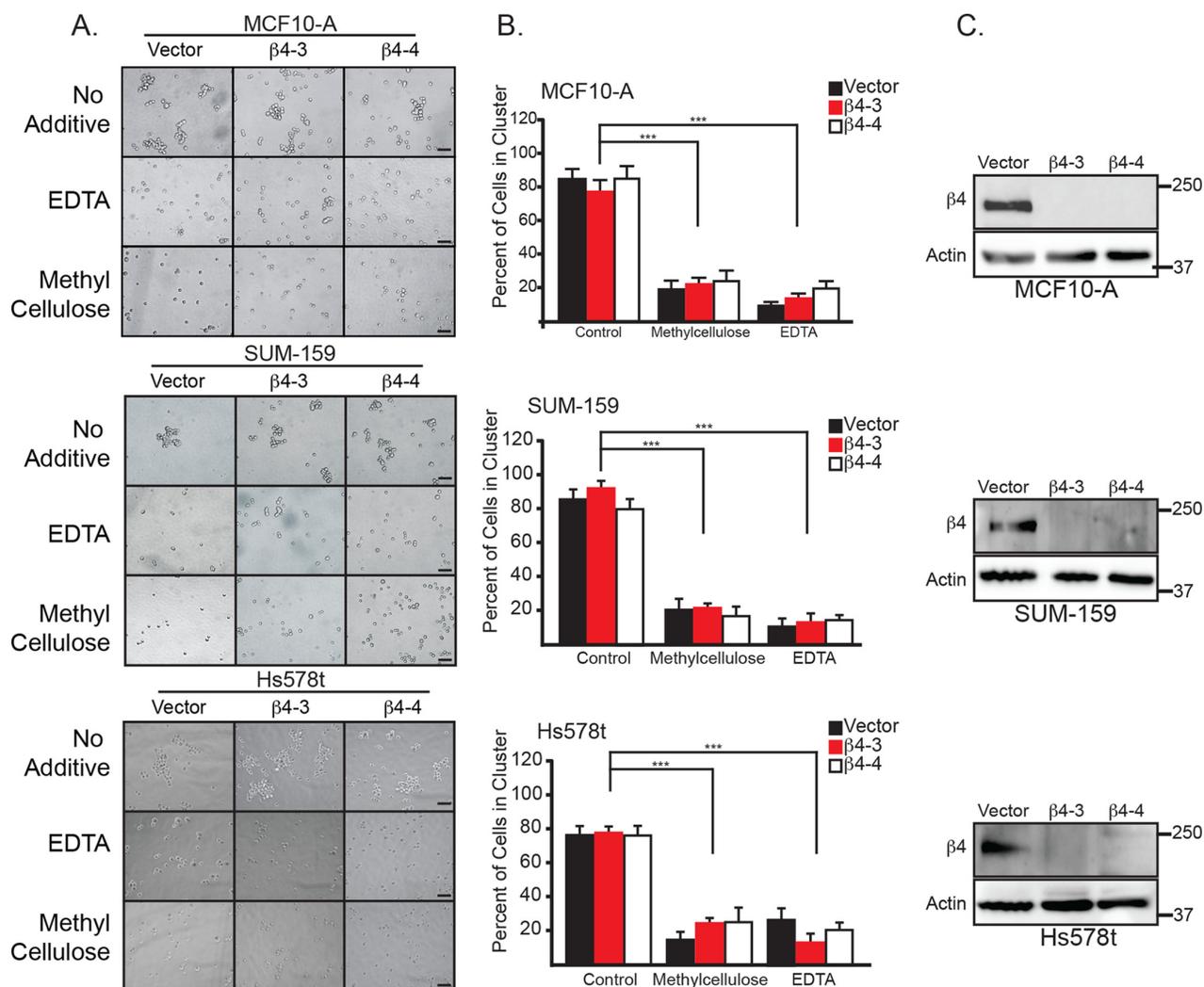
This work was supported by Department of Defense Grant W81XWH-17-1-0009. The authors declare that they have no conflicts of interest with the contents of this article.

<sup>1</sup> Supported by American Cancer Society Grant 130451-PF-17-105-01-CSM.

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<sup>3</sup> The abbreviations used are: ROS, reactive oxygen species; Z-VAD-fmk, benzoyloxycarbonyl-VAD-fluoromethyl ketone; MDA, malondialdehyde; Ab, antibody; pen/strep, penicillin/streptomycin; qPCR, quantitative PCR.

## PVRL4-mediated cell clustering influences ferroptosis



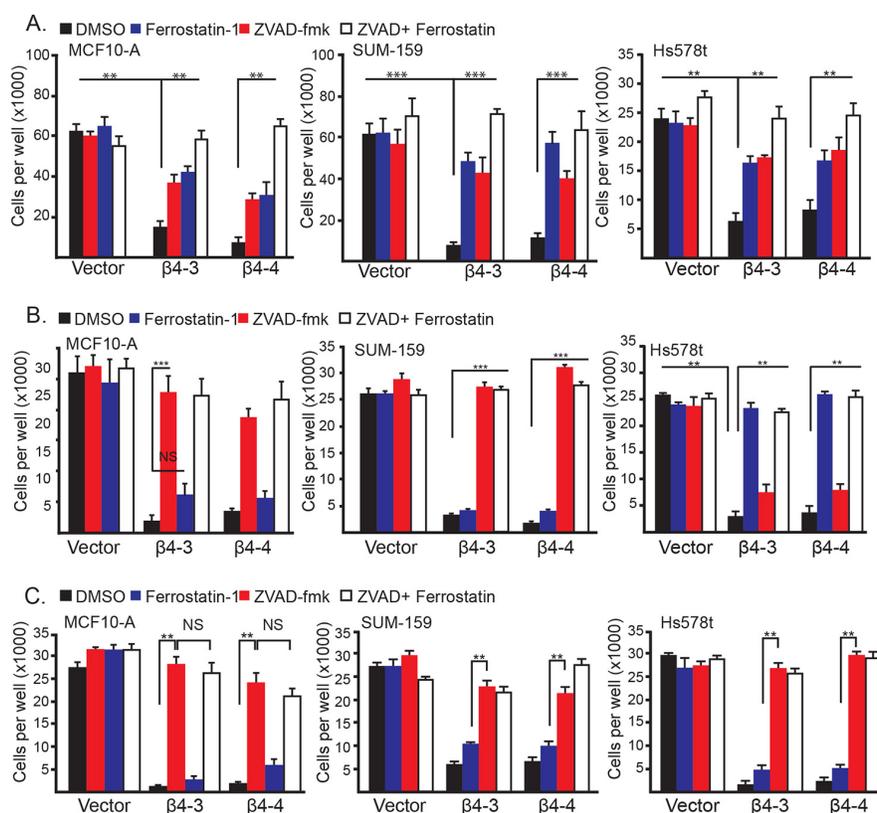
**Figure 1. Breast epithelial and carcinoma cells preferentially cluster upon matrix detachment.** A, MCF10-A, SUM-159, and Hs578t cells (vector and  $\beta 4$ -depleted) were detached and allowed to cluster spontaneously or treated with either 2 mM EDTA or 0.5% methylcellulose to generate single-cell suspensions. Photomicrographs were taken 2 h post-detachment. Scale bars = 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  $\geq 3$  cells). Six independent photos of each cell line and each condition were quantified. \*\*\*,  $p < 0.005$ . 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.

priated by the observation that immortalized mammary epithelial cells (MCF10-A) and breast carcinoma cells (SUM-159 and Hs578t) cluster spontaneously upon detachment (Fig. 1, A and B). These clusters could be dissociated into single cells with either 0.5% methylcellulose or 2 mM EDTA (Fig. 1, A and B). Interestingly, we did not observe significant differences in cluster number upon depletion of  $\alpha 6\beta 4$  using CRISPR/Cas9 to knock down the  $\beta 4$  subunit using two guide RNAs that target different regions of the first exon of ITGB4 (referred to as  $\beta 4$ -3 and  $\beta 4$ -4) (Fig. 1, B and 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 $\alpha 6\beta 4$

The observations described above prompted us to assess the role of cell clustering in ferroptosis that is triggered by the loss

of  $\alpha 6\beta 4$ . For this purpose, we assayed cell viability 24 h after matrix detachment as a function of  $\alpha 6\beta 4$  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  $\alpha 6\beta 4$ , remained viable 24 h after matrix detachment regardless of their clustering status (Fig. 2, A–C). In response to loss of  $\alpha 6\beta 4$  by CRISPR/Cas9-mediated knockdown of the  $\beta 4$  subunit, however, a dramatic decrease in viability was seen under all conditions (Fig. 2, A–C), confirming the importance of the  $\alpha 6\beta 4$  integrin in maintaining survival in response to stress. It is important to note that CRISPR-mediated depletion of the  $\beta 4$  subunit in these cells does affect expression of the  $\alpha 6$  integrin subunit, and they express the  $\alpha 6\beta 1$  integrin (7). Specificity is also evidenced by our previous finding that expression of the  $\beta 4$  construct that cannot be targeted by these CRISPRs in  $\beta 4$ -depleted cells rescued their



**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 h in the presence of either DMSO, ferrostatin-1 (2  $\mu\text{M}$ ), Z-VAD-fmk (25  $\mu\text{M}$ ), or ferrostatin-1 and Z-VAD-fmk, and the number of viable cells was quantified. B, control and  $\beta 4$ -depleted cells were detached for 24 h with 2 mM EDTA in the presence of either DMSO, ferrostatin-1 (2  $\mu\text{M}$ ), Z-VAD-fmk (25  $\mu\text{M}$ ), or ferrostatin-1 and Z-VAD-fmk, and the number of viable cells was quantified. C, control and  $\beta 4$ -depleted cells were detached for 24 h with 0.5% methylcellulose in the presence of either DMSO, ferrostatin-1 (2  $\mu\text{M}$ ), Z-VAD-fmk (25  $\mu\text{M}$ ), or ferrostatin-1 and Z-VAD-fmk, and the number of viable cells was quantified. NS, not significant. \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.005$ .

viability under matrix-detached conditions (7). Moreover, the  $\alpha 3\beta 1$  integrin does not promote ferroptosis resistance in these cells (7).

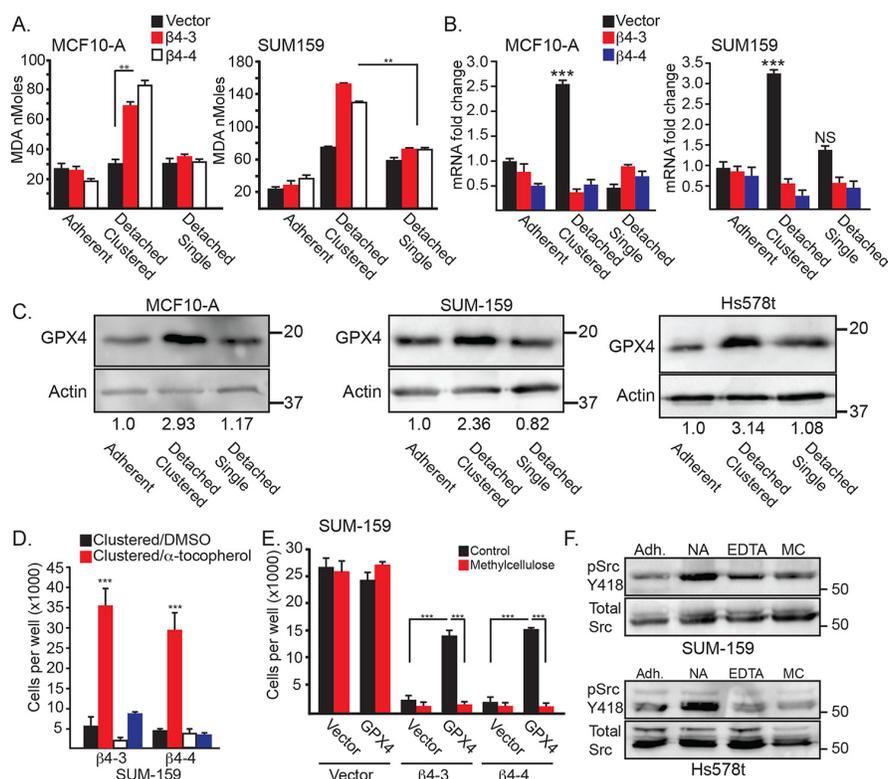
To determine whether the nature of cell death differed between clustered and single detached cells in the absence of  $\alpha 6\beta 4$ , we compared the ability of ferrostatin-1, Z-VAD-fmk, or both inhibitors to rescue the viability of detached,  $\alpha 6\beta 4$ -depleted cells in the presence or 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 (Fig. 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 Z-VAD-fmk did rescue viability almost completely (Fig. 2, B and C). These results indicate that cell clustering predisposes cells to ferroptosis in the absence of  $\alpha 6\beta 4$  and that disruption of these clusters results in apoptosis. Moreover, the finding that  $\alpha 6\beta 4$ -depleted cells are prone to both ferroptosis and apoptosis in the absence of EDTA and methylcellulose probably reflects the fact that this population is comprised of a mixture of clustered and single cells (Fig. 1A).

#### Lipid peroxidation is significantly higher in clustered, detached cells compared with single cells in the absence of $\alpha 6\beta 4$

To understand why clustered but not single cells are prone to ferroptosis in the absence of  $\alpha 6\beta 4$ , we quantified lipid peroxidation, which is the root cause of ferroptosis, using the malondialdehyde (MDA) assay. Indeed, depletion of  $\alpha 6\beta 4$  resulted in a marked increase in lipid peroxidation in clustered but not single cells (Fig. 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  $\alpha 6\beta 4$  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  $\alpha 6\beta 4$  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  $\alpha 6\beta 4$  to induce GPX4 expression and activity in these distinct populations. We observed that GPX4 mRNA (Fig. 3B) and protein (Fig. 3C) expression increased significantly upon matrix detachment of clustered cells (2 h), but this increase was less

## PVRL4-mediated cell clustering influences ferroptosis



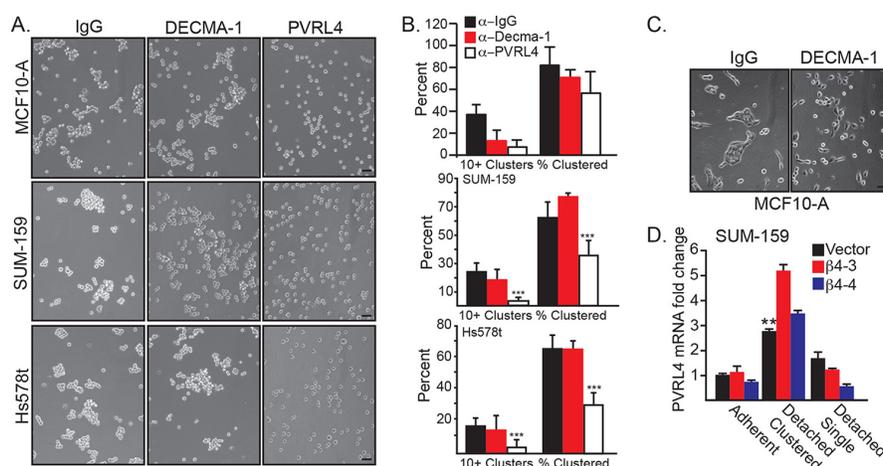
**Figure 3. Clustering of matrix-detached cells influences lipid peroxidation, GPX4 expression, and Src activity.** A, lipid peroxidation was quantified using the MDA assay in control and  $\beta 4$ -depleted MCF10-A and SUM-159 cells under either adherent, detached clustered (no additive), or detached single (0.5% methylcellulose) conditions for 4 h. Similar results were obtained using 2 mM EDTA. B, GPX4 mRNA expression was quantified by qPCR 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 2 h. C, GPX4 protein expression was assessed by immunoblotting in control MCF10-A, SUM-159, and Hs578t cells under detached clustered (no additive) or detached single (0.5% methylcellulose) conditions for 4 h. 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 h with either no additive (clustered) or 2 mM EDTA (single) in the presence of either DMSO or 500  $\mu$ M  $\alpha$ -tocopherol, and the number of viable cells was quantified. E, control and  $\beta 4$ -depleted cells SUM-159 cells that had been transfected with either a vector control or a GPX4 expression vector were detached for 24 h 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 (Tyr-418) Src by immunoblotting under adherent conditions (Adh) as well as following 2 h of matrix detachment with no additive (NA), 2 mM EDTA (EDTA), or 0.5% methylcellulose (MC). NS, not significant. \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.005$ .

evident in single cells. The increase in GPX4 expression depends on  $\alpha 6\beta 4$ , consistent with our previous results (7). Moreover, the viability of the clustered,  $\alpha 6\beta 4$ -depleted cells was rescued by either the lipophilic antioxidant  $\alpha$ -tocopherol (Fig. 3D) or exogenous expression of GPX4 (Fig. 3E). In contrast, neither  $\alpha$ -tocopherol nor GPX4 re-expression in  $\alpha 6\beta 4$ -depleted, single cells rescued viability. These data infer that  $\alpha 6\beta 4$  signaling differs between clustered and single, matrix-detached cells. Given that the induction of GPX4 expression upon matrix detachment depends on  $\alpha 6\beta 4$ /Src signaling (7), we assessed Src activation in detached clustered and single cells as a function of  $\alpha 6\beta 4$  expression. Matrix detachment caused an increase in activated Src in clustered cells, as assayed by Tyr-418 immunoblotting, that was dependent on  $\alpha 6\beta 4$  expression and was not detected in either EDTA- or methylcellulose-treated cells (Fig. 3F).

### PVRL4 mediates clustering of matrix-detached cells and ferroptosis resistance

An important issue that arose from our data concerns 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 observed no effect on clustering (Fig. 4A). Interestingly, however, DECMA-1 had no effect on the percentage of MCF10-A cells in clusters, although it did cause a significant decrease in the number of large (10+ cells) clusters (Fig. 4B). In contrast, treatment of adherent MCF10-A cells with DECMA-1 disrupted cell–cell adhesion (Fig. 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  $\alpha 6\beta 4$  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



**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  $\mu\text{g/ml}$  of either IgG, DECMA-1, or a PVRL4-blocking Ab. Photomicrographs were taken 2 h post-detachment. Scale bars = 10  $\mu\text{m}$ . B, quantification of MCF10-A, SUM-159, and Hs578t clusters was performed by counting the total number of single and clustered cells, the total number of clusters (defined as  $\geq 3$  cells), and the number of large clusters (defined as  $\geq 10$  cells). \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.005$ . C, MCF10-A cells were detached and allowed to reaggregate in the presence of either IgG or DECMA-1. Photomicrographs were taken at 24 h. Scale bar = 10  $\mu\text{m}$ . D, SUM-159 control and  $\beta 4$ -depleted cells under adherent, detached clustered, or detached single cell conditions were assessed for expression of PVRL4 mRNA by qPCR.

matrix-detached cells and found that this Ab was as effective as either EDTA or methylcellulose in disrupting clusters into single cells (Fig. 4A). Depletion of  $\beta 4$  did not significantly affect PVRL4 expression in  $\beta 4$ -depleted cells (Fig. 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 the IgG control (Fig. 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 Z-VAD-fmk and not ferrostatin-1 (Fig. 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.

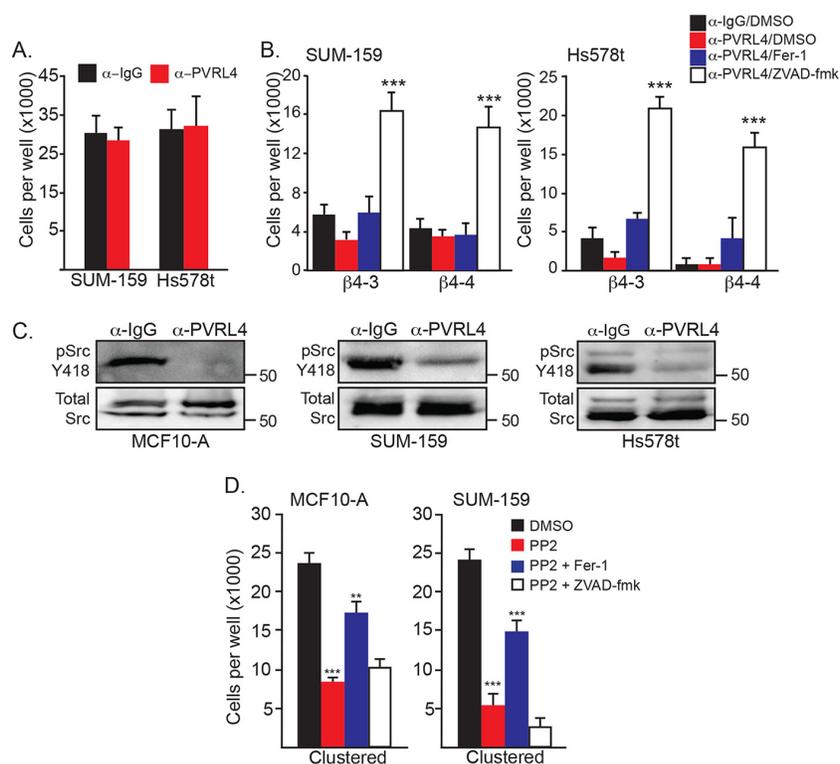
Given that  $\alpha 6\beta 4$ -mediated Src signaling maintains the survival of matrix-detached cells (7), we examined the impact of the PVRL4 function-blocking antibody on Src activation in matrix-detached cells. Indeed, disruption of cell clustering by this antibody resulted in a significant decrease in Src activation compared with control cells, as assessed by phospho-Src (Tyr-418) immunoblotting (Fig. 5C). A causal role for Src activation in ferroptosis resistance is indicated by the observation that 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 Z-VAD-fmk (Fig. 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 when 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 co-workers (10) that identified PVRL4 in a gain-of-function screen for genes that promote the survival of matrix-detached carcinoma cells. 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.

## PVRL4-mediated cell clustering influences ferroptosis



**Figure 5. PVRL4-mediated cell clustering is necessary for  $\alpha$ 6 $\beta$ 4-dependent Src activation.** A, SUM-159 and Hs578t control cells were detached for 24 h in the presence of either IgG or a PVRL4 Ab, and the number of viable cells was quantified. B, SUM-159 and Hs578t  $\beta$ 4-depleted cells were detached for 24 h in the presence of either IgG and DMSO, PVRL4 Ab, PVRL4 Ab and ferostatin-1 (2  $\mu$ M), or PVRL4 and Z-VAD-fmk (25  $\mu$ M), and the number of viable cells was quantified. C, MCF10-A, SUM-159 and Hs578t control cells were detached for 2 h in the presence of either IgG or a PVRL4-blocking Ab, and phosphorylation of Src (Tyr-418) 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  $\mu$ M), or PP2 in combination with either ferrostatin-1 (2  $\mu$ M) or Z-VAD-fmk (25  $\mu$ M). \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.005$ .

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 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 of 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/ $\alpha$ 6 $\beta$ 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 Dulbecco’s modified Eagle’s medium/F-12 (Gibco) with 5% horse serum, 1% pen/strep (Gibco), 20 ng/ml epidermal growth factor (Peprotech), 0.5 mg/ml hydrocortisone (Gibco), 100 ng/ml cholera toxin (Sigma), and 10  $\mu$ g/ml insulin (Sigma). SUM-159 cells were maintained in F-12 (Gibco) medium with 5% fetal bovine serum (Hyclone), 1% pen/strep, 0.5 mg/ml hydrocortisone, and 10  $\mu$ g/ml insulin. Hs578t cells were maintained in Dulbecco’s modified Eagle’s medium high-glucose (Gibco) with 10% fetal bovine serum, 1% pen/strep, and 10  $\mu$ g/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  $\beta 4$  (505; Ref. 18), phospho-Src Y418 (R&D Systems, MAB2685), total Src (Santa Cruz Biotechnology, sc-8056), DECMA-1 (Abcam, ab11512), PVRL4 (R&D Systems, 17402; Cell Signaling Technology, 17402). Other reagents used were as follows: Z-VAD-fmk (SelleckChem), ferrostatin-1 (Sigma-Aldrich),  $\alpha$ -tocopherol (Sigma-Aldrich), and PP2 (SelleckChem).

#### Matrix detachment assays

Twenty-four-well plates were coated with 800  $\mu$ l of poly-HEMA (30 mg/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  $2.5 \times 10^4$  cells/100  $\mu$ l and plated (100  $\mu$ l/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 prechilled tubes and spun down before washing twice in ice-cold PBS. Protein was extracted using radioimmune precipitation assay buffer containing protease and phosphatase inhibitors (Boston Bioproducts, Worcester, MA) by lysing cells for 15 min on ice, followed by centrifugation at maximum speed for 15 min at 4 °C. Supernatant was collected, and protein concentration was quantified using Bradford reagent (Bio-Rad). Samples (25  $\mu$ g of 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 and lysed immediately following centrifugation, and RNA was isolated following the manufacturer's specifications. RNA concentrations were determined by Nanodrop, and complementary DNA (1  $\mu$ g/sample) was produced using All-In-One cDNA Synthesis SuperMix (BioScript). qPCR was performed using Cyber Green Master Mix (BioTool). The sequences for primers used are as follows: 18S, 5'-AACCCGTTGAACCCATT-3' (forward) and 5'-CCATCCAATCG GTAGTAGCG-3' (reverse), derived from NT\_167214.1; GPX4, 5'-GAGGCAAGACCGAAGTAA-ACTAC-3' (forward) and 5'-CCGAACTGGTTACACGG-GAA-3' (reverse), derived from NC\_000019.10.

To assay lipid peroxidation, the MDA lipid peroxidation microplate assay (Sigma-Aldrich) was used according to the manufacturer's specifications. Briefly, cells ( $1 \times 10^6$ ) were cultured in cell-line specific, serum-free medium on polyhydroxyethylmethacrylate (polyHEMA)-coated plates for 4 h. Three wells were plated per condition for each independent experiment as technical replicates. Cells were collected, lysed, and reacted with thiobarbituric acid for 1 h at 95 °C. An aliquot of lysate (5  $\mu$ l) was saved for protein concentration determina-

tion using the Bradford assay prior to reaction with thiobarbituric acid. Fluorescence was read at 532 (excitation)/590 (emission). 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 CRISPR Design (<http://crispr.mit.edu>)<sup>4</sup> and CRISPRdirect (<https://crispr.dbcls.jp>; 17).<sup>4</sup> Four guide RNAs were tested, and the two most efficient knockouts were selected. The guide RNA sequences used were as follows: B4-3, 5'-CACCGTTGTCCAGATCATCGGACA-3' and 5'-AAAC-TGTCCGATGATCTGG ACAAC-3'; B4-4, 5'-CACCGAAA-TCCAATAGTGTAGTCGC-3' and 5'-AAACGCGACTACACTAT TGGATTTC-3'. Cells were subcloned by FACS and screened for loss of protein expression by immunoblotting. New CRISPR-depleted cells were generated every 3 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  $\mu$ g of DNA using Lipofectamine 2000 (Thermo Fisher) and incubated for 48 h prior to use.

#### Statistical analysis

The error bars in graphs represent mean  $\pm$  S.D. All experiments were repeated at least three times. The *p* values were calculated using ANOVA, and a *p* value of less than 0.05 was considered significant.

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## PVRL4-mediated cell clustering influences ferroptosis

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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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