

SUPPLEMENTARY INFORMATION

Acsl4 Dictates Ferroptosis Sensitivity by Shaping Cellular Lipid Composition

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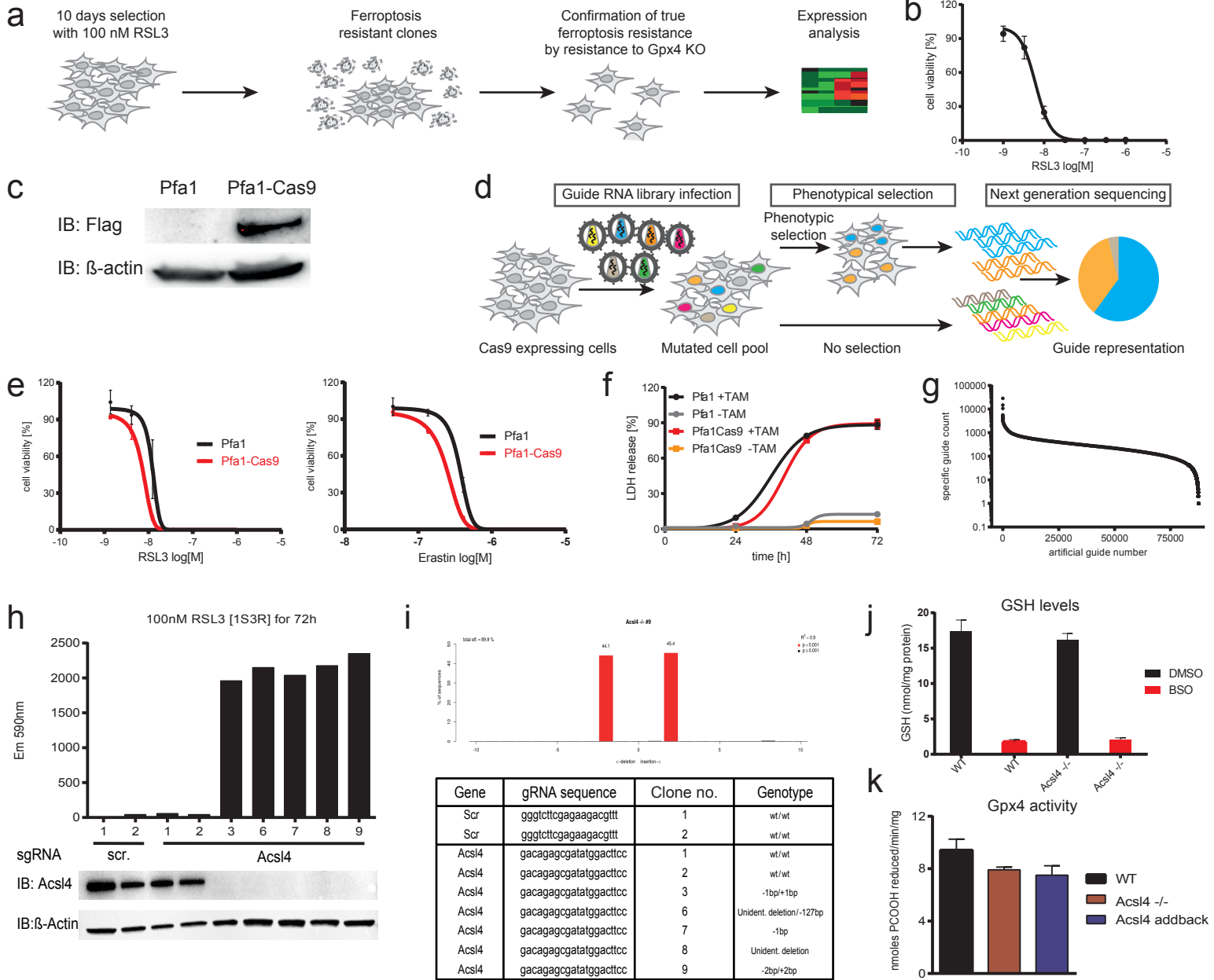
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Supplementary Results

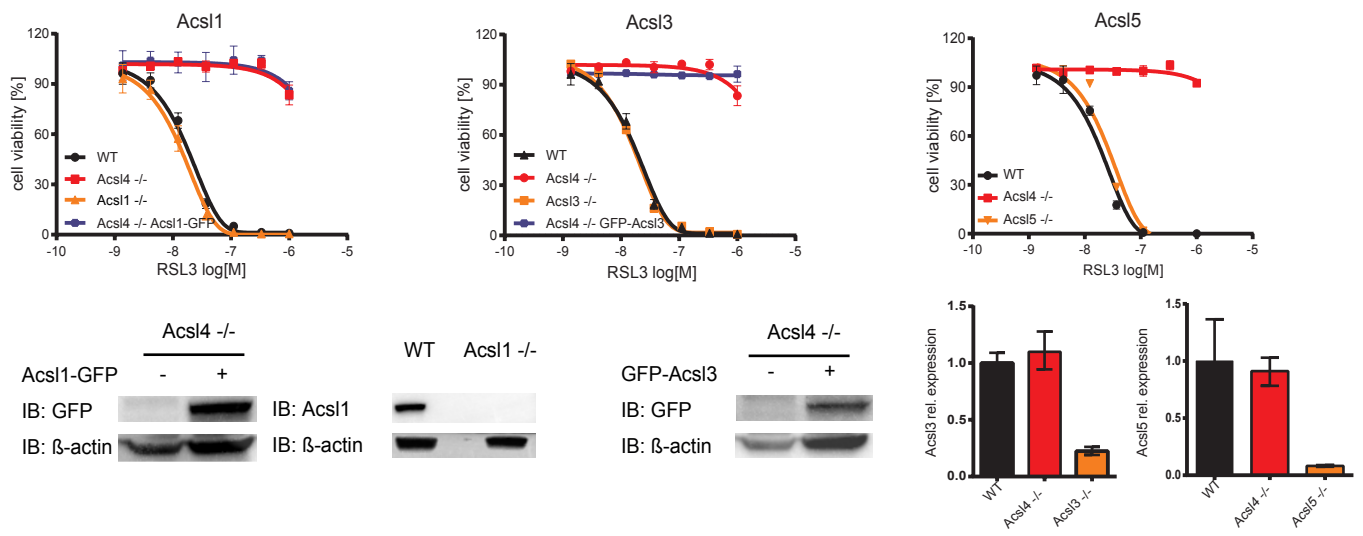
Supplementary figure 1



Supplementary Figure 1 | Generation of Pfa1-Cas9 cells and set-up of genome-wide screening for novel ferroptosis players. (a) Scheme of the generation and selection procedure for the analysis of ferroptosis-resistant cell clones. (b) Dose-dependent killing of Pfa1 cells. Cell viability was assessed 24 h after treatment using AquaBluer. (c) Immunoblot analysis of NLS-FLAG-Cas9 expression in Pfa1 cells and Pfa1-Cas9 cells using an antibody against the FLAG epitope. (d) Scheme depicting the pooled CRISPR-based screen in Pfa1_CAS9 cells treated with RSL3 and erastin (ERA). (e) Dose-dependent cytotoxicity of RSL3 and ERA. Cell viability was assessed 24 h after treatment using AquaBluer. (f) Time-dependent increase of LDH release in Pfa1 and Pfa1-Cas9 upon Gpx4 deletion. (g) Deep-sequencing analyses of the gRNAs in the lentiviral plasmid DNA library. 99.71 % of all gRNAs of the lentiviral library were present. (h) Acsl4 KO is associated with high ferroptosis resistance in Pfa1 cells. Cell viability of single cell clones was measured 24 h after RSL3 treatment using AquaBluer. (i) Genotype of different single cell clones shown in **Supplementary Figure 1h**. Deletions and insertions in clones were detected using the tool tide.nki.nl (TIDE results of the clone Acsl4^{-/-} #9 are shown exemplarily). (j) Acsl4 KO cells do not show increased levels of GSH. (k) Gpx4 activity is not elevated in Acsl4 KO cells compared to wt cells. Data shown represents the mean \pm s.d. of $n = 3$ from a representative experiment performed independently three times (b, e and f); j and k represents the mean \pm s.d of $n = 1$ performed independently three times.

Supplementary figure 2

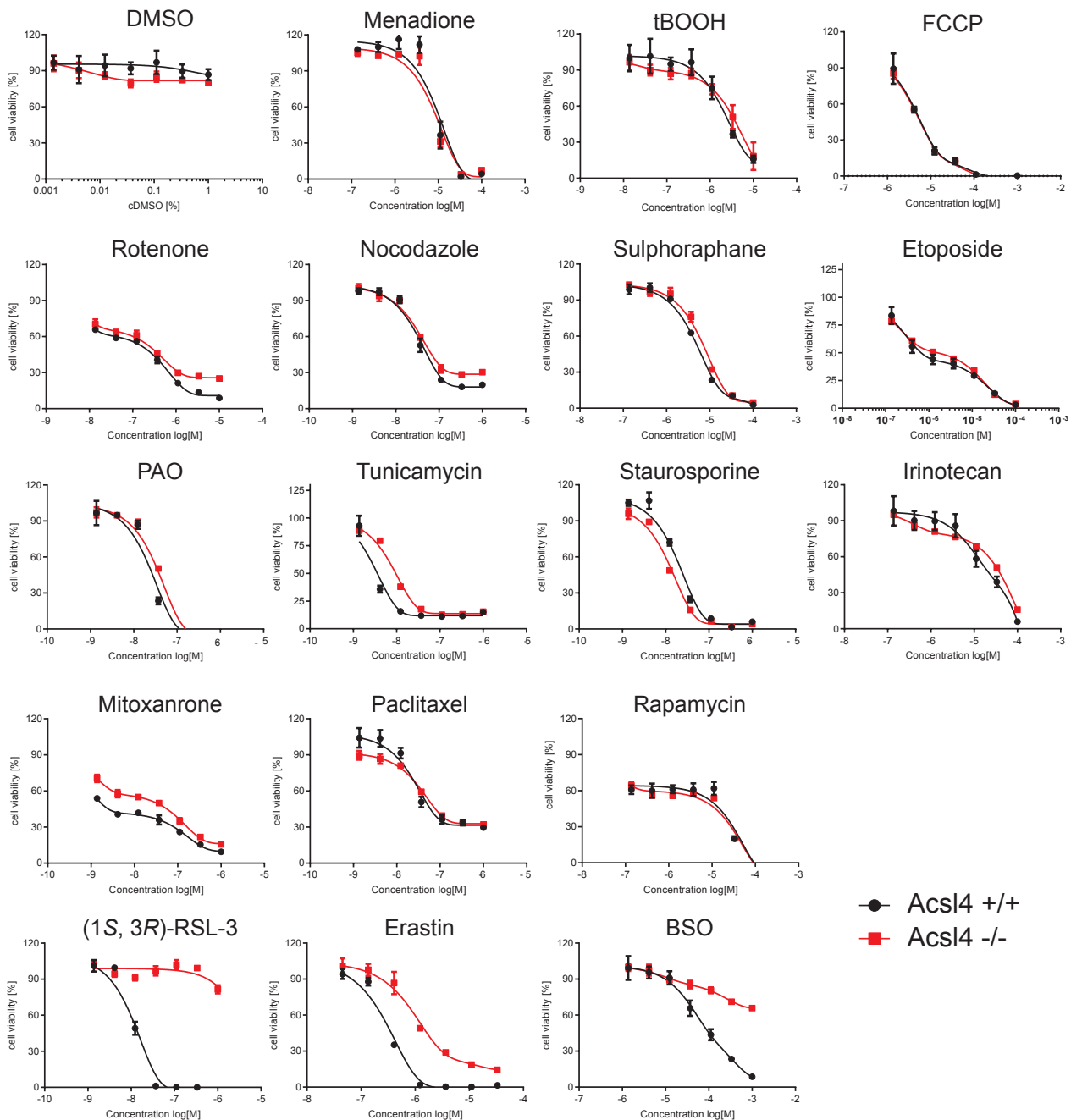
a



b

Gene	Clone	sgRNA sequence	Genotype (frameshifts)
Acs1	#4	GGACTCCTACGGCAGTGATC	-25/-19/-11/-8 bp
Acs3	#4	GAGTCCGGTTTGGAACTGAC	-1/+1 bp
Acs5	#10	GTGGCCGTCCAAACGTCAGG	-20/-11 /-2bp
Lpcat3	#7	GAAACCACCCCTCGCTTCAA	-8/-4/-1 bp
Pparγ	#12	GATAAATAAGCTTCAATCGGA	-16/-8 bp

c



Supplementary Figure 2 | Specificity of Acsl4 in sensitizing to ferroptosis. (a) Pfa1_Acsl1, Pfa1_Acsl3, Pfa1_Acsl5 KO cells are not resistant to RSL3-induced ferroptosis and overexpression of Acsl1 and Acsl3 does not re-sensitize Acsl4 KO cells to ferroptosis. Cell viability was assessed by AquaBluer assay 24 h after RSL3 treatment. (b) List of all single cell clone genotypes used for cell viability studies. Well-characterized out-of-frame mutations for each cell clone (analysed using tide.nki.nl) are listed as well as the gRNAs used for CRISPR/Cas9-mediated KO of the indicated genes. (c) Acsl4 KO cells do not show multi-drug resistance properties while being highly resistant to ferroptosis inducing agents, such as RSL3, erastin and BSO. Cell viability was assessed 48 h after treatment using AquaBluer. Data shown represents the mean \pm s.d. of n = 3 wells of a 96-well plate performed once.

Supplementary Figure 3 | Arachidonic acid (AA) and adrenic acid (AdA) preferentially sensitize *Acs14* ^{-/-} *Pfa1* cells to undergo ferroptosis. (a) ω 6 fatty acids preferentially sensitize cells to ferroptosis. *Pfa1_Acs14_KO* cells were incubated overnight with a mixture of fatty acids/BSA at the indicated concentrations. Upon wash out, ferroptosis was triggered with 100 nM RSL3 and cell viability was assessed after 4h. Data shown represents the mean \pm s.d. from $n = 3$ wells of a 96-well plate from a representative experiment performed three times. An increase in red intensity indicates a sensitization to ferroptosis through the applied fatty acid. (b) Quantitative assessment of free AA (20:4) (left panel) and AdA (22:4) (right panel) in control wt and *Acs14* ^{-/-} cells. Data are mean \pm SD. $n = 4$. (c-e) Quantitative assessment of phosphatidylinositol (PI) (c), phosphatidylcholine (PC) (d), and phosphatidylserine (PS) (e) molecular species containing AA (20:4) (left panels) and AdA (22:4) (right panels) in control wt and *Acs14* ^{-/-} cells. Data are mean \pm SD. $n = 4$.

Supplementary figure 4

Basal

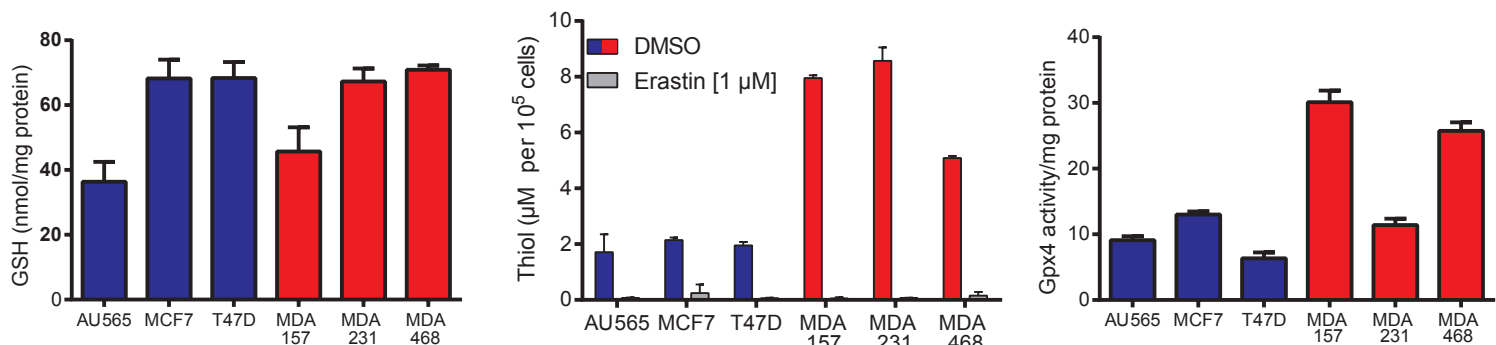
Luminal

a

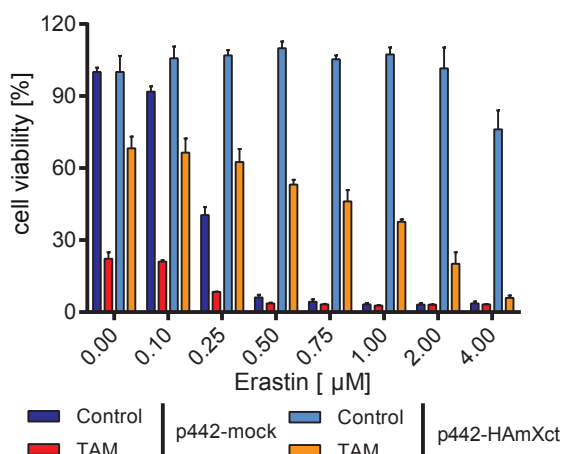
RSL3 [nM]	MDA157	MDA231	MDA436	MDA468	BT549	H38	H1143	H1937	SK-BR-3	T47D	MCF7	AU565	MDA453	BT474
0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0
1	101,0	97,6	97,3	96,5	105,3	93,3	92,6	96,2	96,0	98,6	97,4	97,8	101,2	100,8
3	102,2	96,7	87,8	94,2	102,4	92,4	102,0	98,9	97,9	99,1	94,8	99,5	96,6	94,2
10	41,4	92,1	90,5	94,1	102,6	88,9	97,2	81,5	98,4	98,4	98,6	91,0	93,7	98,9
30	0,8	50,5	86,9	86,8	96,0	88,3	95,3	37,6	82,0	95,1	95,6	90,7	91,6	85,5
100	0,8	24,0	93,3	55,8	78,2	75,5	79,2	26,0	56,6	89,4	95,6	88,0	94,3	78,0
300	0,7	28,0	84,2	28,0	41,9	48,9	30,6	16,7	33,7	75,3	90,8	70,2	87,7	36,9
1000	0,8	9,6	60,3	13,7	38,5	30,1	21,8	9,6	20,9	68,7	74,7	26,0	62,9	18,6

RSL3 [nM] + Erastin	MDA157	MDA231	MDA436	MDA468	BT549	H38	H1143	H1937	SK-BR-3	T47D	MCF7	AU565	MDA453	BT474
0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0
1	94,4	97,8	97,5	95,4	96,0	92,2	98,7	92,2	100,4	93,0	98,2	90,8	86,6	102,8
3	66,5	97,9	98,3	89,8	93,2	77,7	102,3	77,7	104,3	89,9	98,3	87,5	85,9	97,0
10	14,7	34,5	93,7	72,8	73,7	32,1	85,7	32,1	86,6	92,3	97,5	84,3	83,6	96,5
30	2,5	9,4	92,8	48,1	46,3	10,6	41,0	10,6	1,3	86,4	101,5	78,8	80,7	83,7
100	1,9	6,1	91,2	10,1	26,8	5,7	26,3	5,7	0,3	90,3	97,7	74,1	78,4	72,0
300	1,9	6,4	68,6	6,4	13,3	5,5	6,1	5,5	0,6	74,1	92,6	69,6	84,3	39,7
1000	2,0	6,3	48,8	5,6	6,0	3,6	5,2	3,6	0,5	70,4	79,5	26,3	47,7	15,8

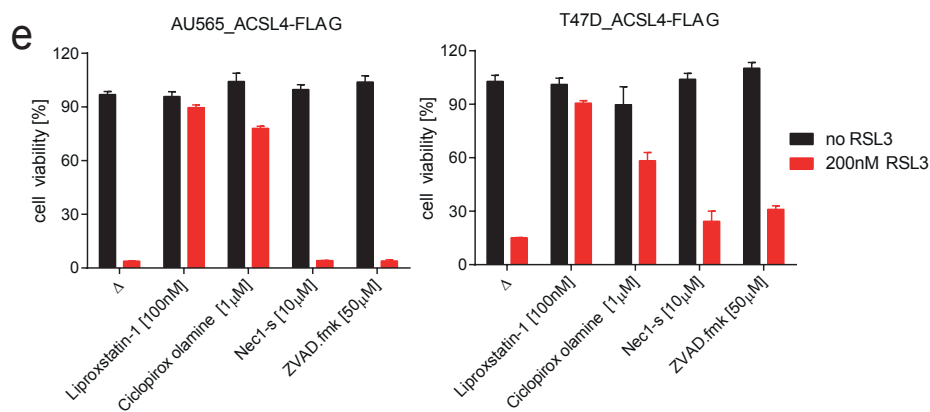
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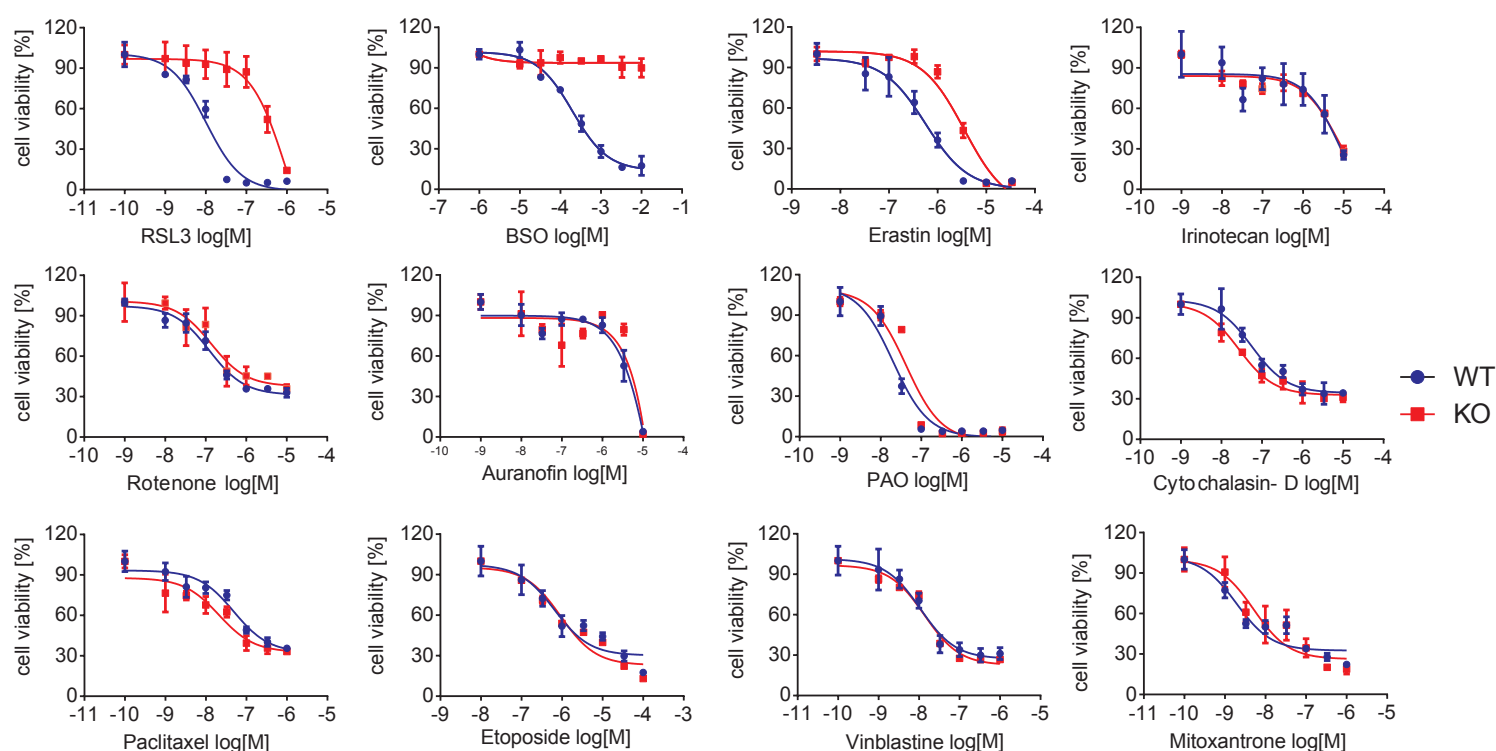
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e

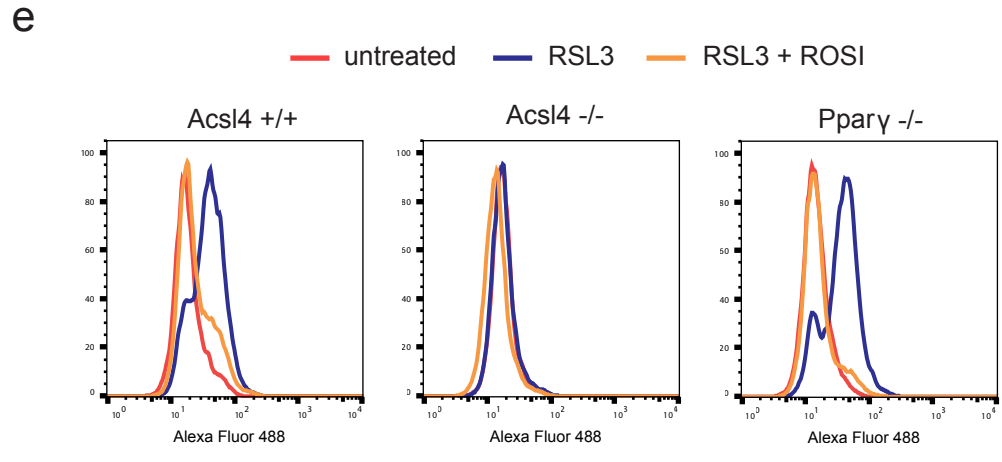
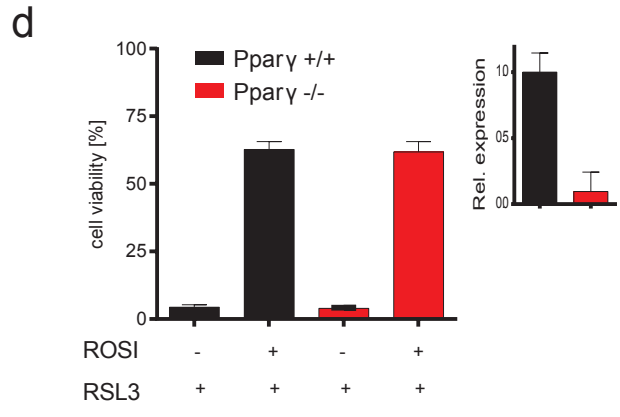
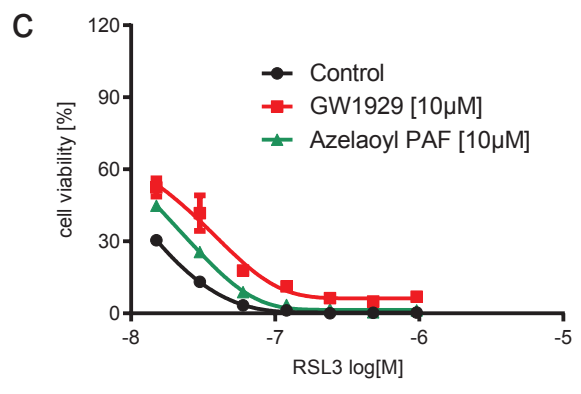
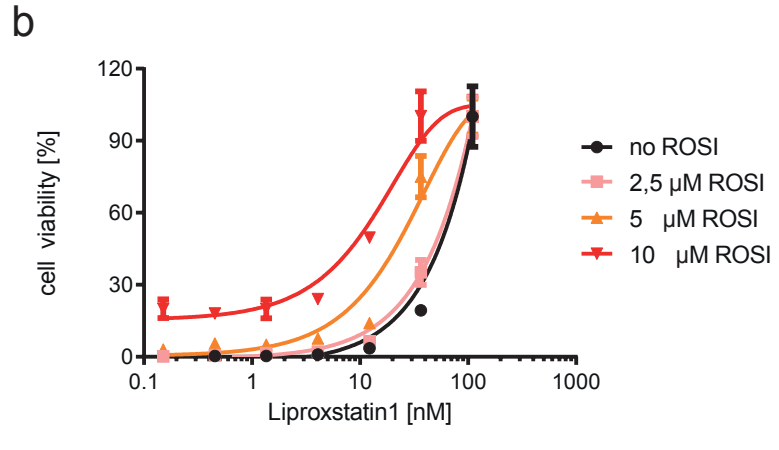
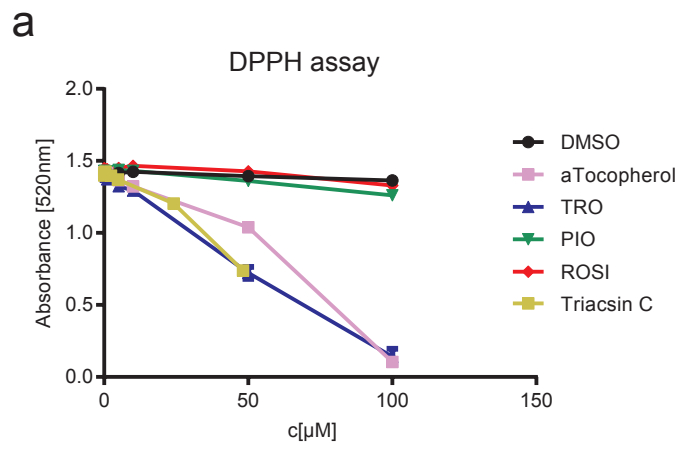


d



Supplementary Figure 4 | Sensitivity of different basal and luminal breast cancer cells to ferroptosis induction. (a) Sensitivity of breast cancer cells to ferroptosis induction. Treatment using increasing concentrations of RSL3 in the presence or absence of Erastin (1 μ M) was performed for 48 h and cell viability was assessed using AquaBluer. (b) Evaluation of critical ferroptosis parameters were evaluated measuring of total GSH levels, system X_c^- activity (Thiol release) and Gpx4 specific-activity. (c) Ferroptosis was induced by Gpx4 deletion in Pfa1 cells stably expressing xCT or empty plasmid (mock). Increasing concentrations of ERA were added 24h after KO induction and viability was assessed using AquaBluer 24 h after ERA treatment. (d) MDA157 WT (blue) and KO for ACSL4 (2000 cells, red) were treated each with lethal compounds at increasing concentrations for 48 h, and cell viability was assessed using AquaBluer. (e) Forced expression of ACSL4-FLAG in AU565 and T47D cells re-sensitized these cells to RSL3-induced ferroptosis. Cell death was inhibited by the lipophilic antioxidant Liproxstatin-1 (Lip1) as well as the iron chelator ciclopirox olamine but not by the RIP kinase inhibitor Necrostatin-1s (Nec-1s) or the caspase inhibitor ZVAD.fmk. Data shown represents the mean \pm s.d. of $n = 3$ (b,c,e) or 4 (d) wells of a 96-well plate from an experiment performed two (b,d) or three times (c,e).

Supplementary figure 5



Supplementary Figure 5 | Assessment of antioxidant activity of thiazolidinediones and potential involvement of Ppar γ in ferroptosis. (a) Analysis of radical trapping/antioxidant potential of thiazolidinediones (troglitazone (TRO), rosiglitazone (ROSI), pioglitazone (PIO)) and Triacsin C compared to α -tocopherol. Antioxidant activity was assessed using the DPPH assay. Absorption was measured at 517nm. (b) ROSI and Liproxstatin-1 present an additive effect in preventing ferroptosis. Pfa1 were treated with sub-rescuing concentrations of ROSI in the presence of increasing concentrations of Liproxstatin-1 upon Gpx4 deletion and cell death was assessed using AquaBluer 72 h thereafter. (c) Cells treated with the PPAR γ agonists GW1929 and Azelaoyl PAF for 3 days did not increase resistance to ferroptosis induced by RSL3. Cell viability was assessed 24 h after treatment using AquaBluer. (d) ROSI prevents RSL3 (100 nM)-induced ferroptosis in a Ppar γ -independent manner. (e) ROSI prevents lipid oxidation independently of Ppar γ by specifically inhibiting Acsl4 as assessed by BODIPY 581/591 C11 oxidation using flow cytometry analysis. Data shown represents the mean \pm s.d. of n=3 wells of a 96-well plate of an experiment performed independently once (a,c) or three times (b,d).

Supplementary figure 6

Fig. 1a

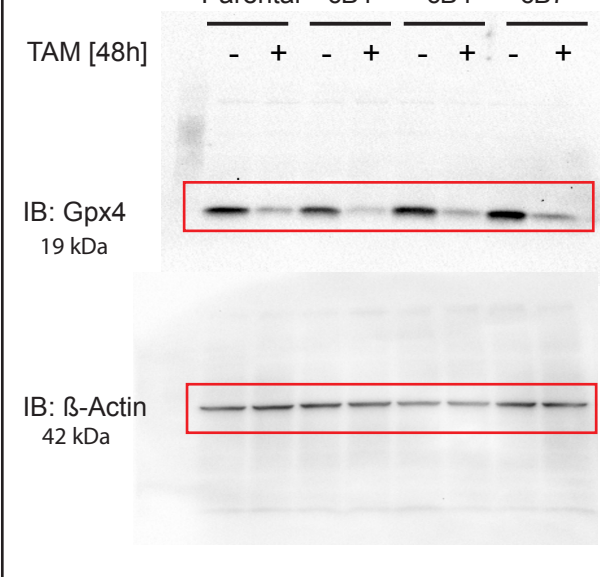


Fig. 1d

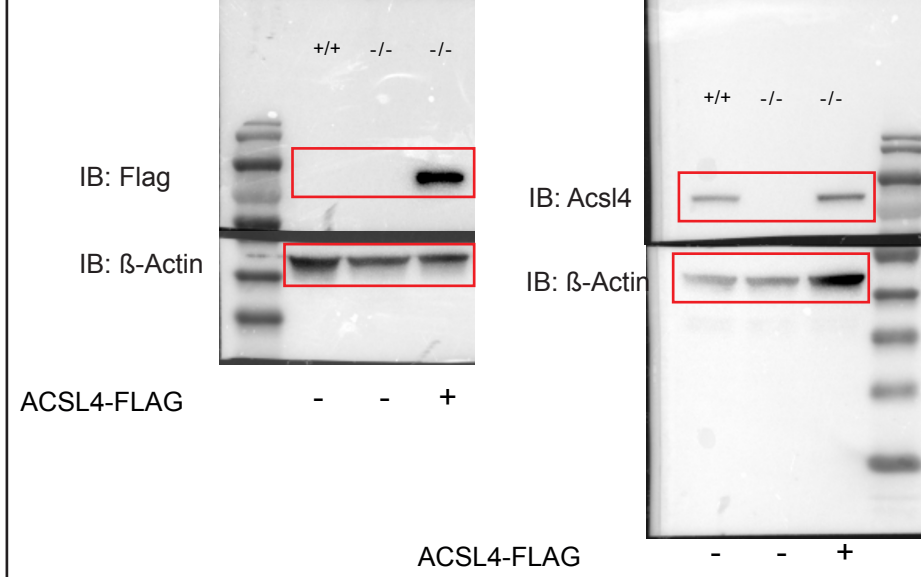
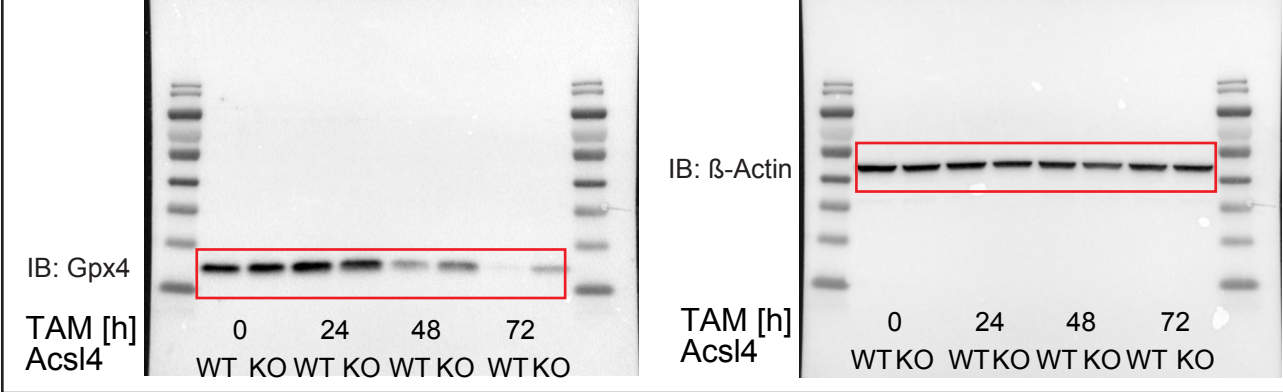
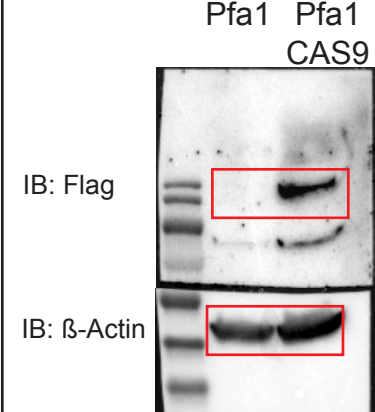


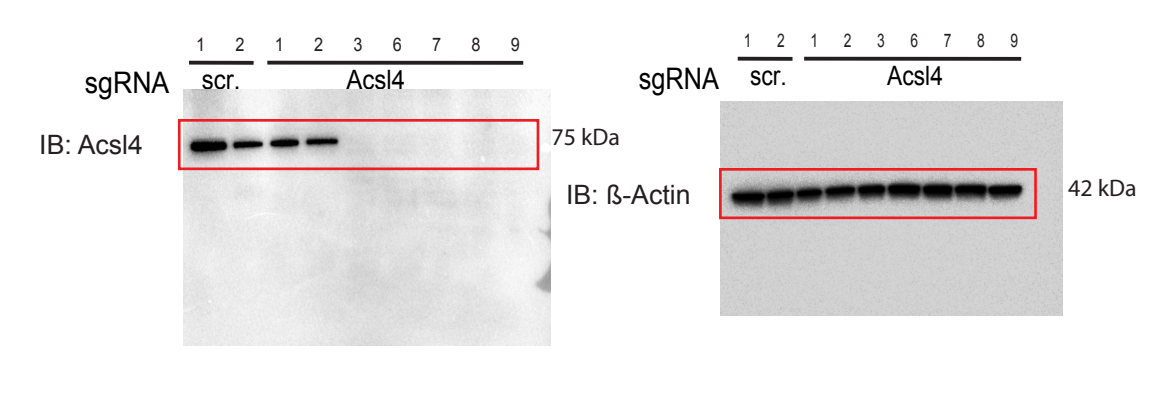
Fig. 1e



Suppl. fig. 1c



Suppl. fig. 1h



Suppl. fig. 2a

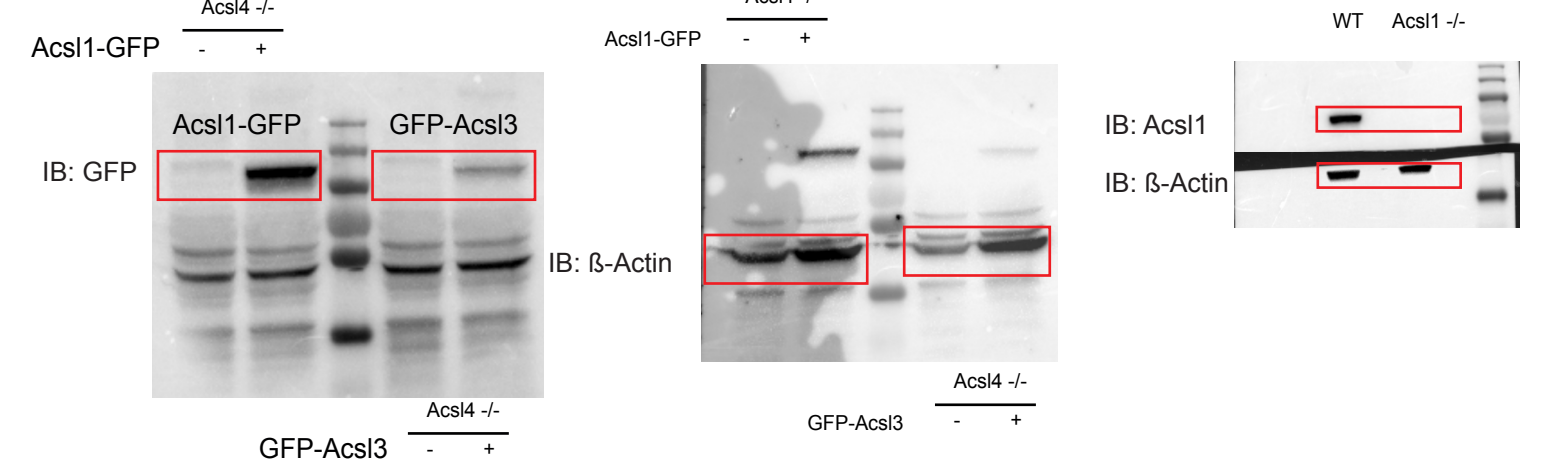


Fig. 4b

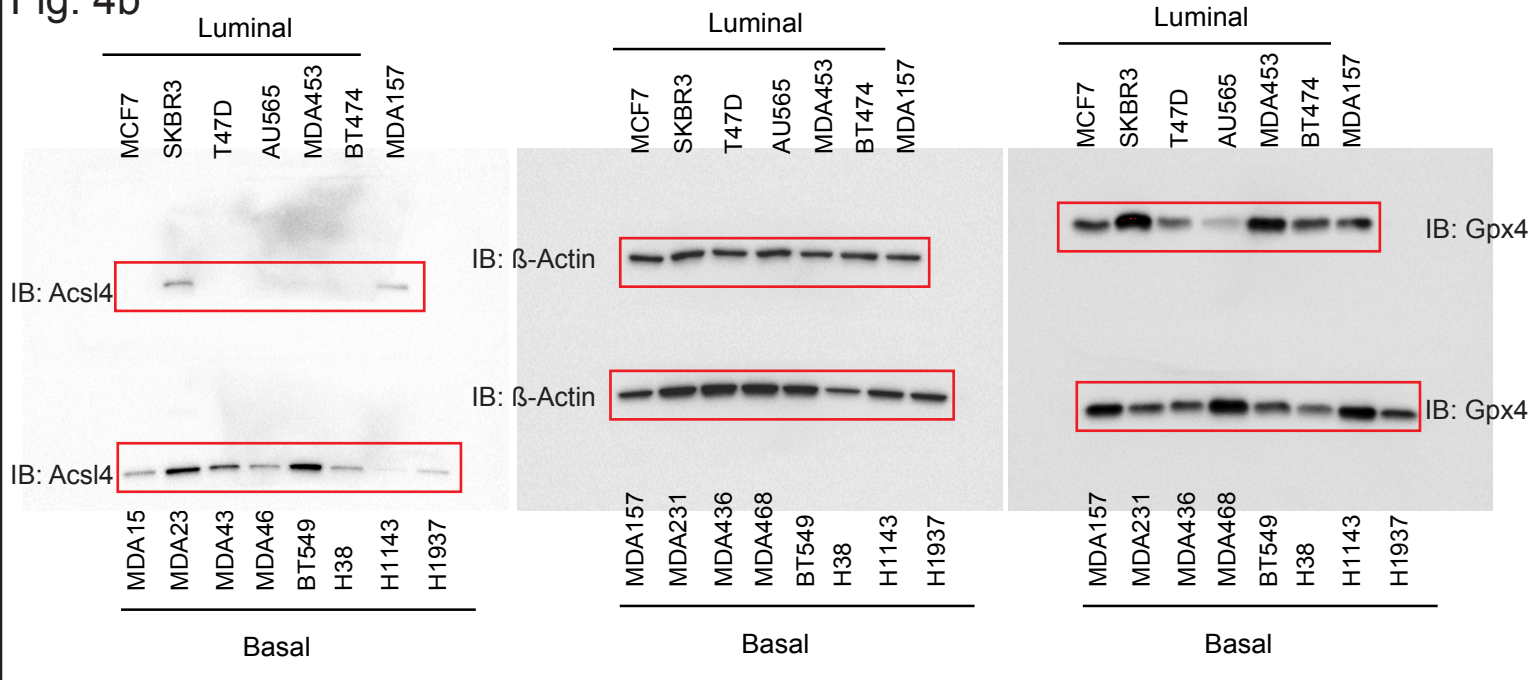


Fig. 4c

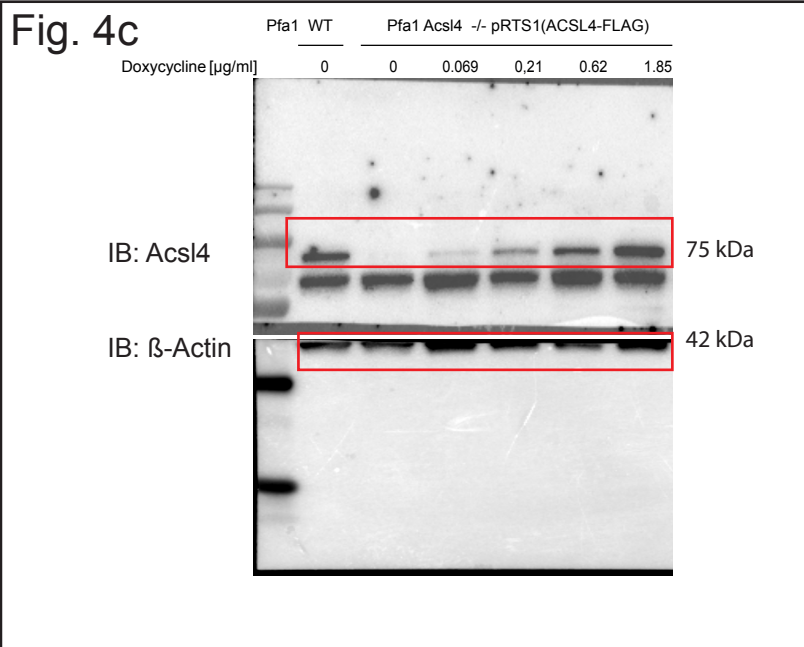


Fig. 4e

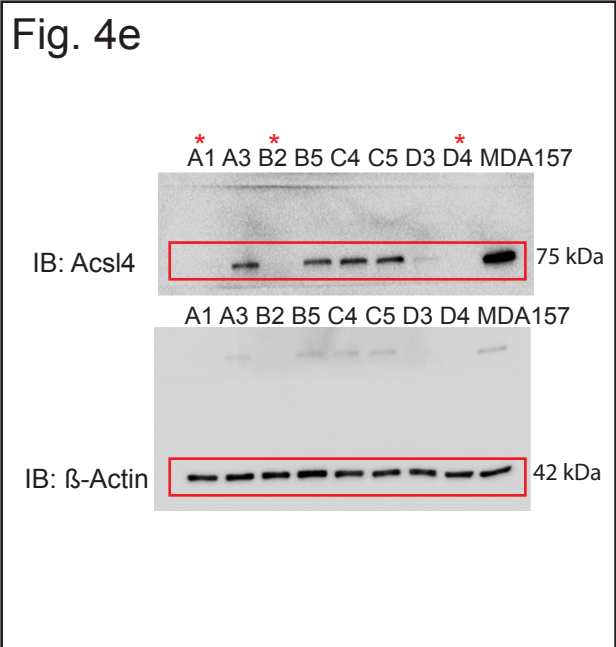
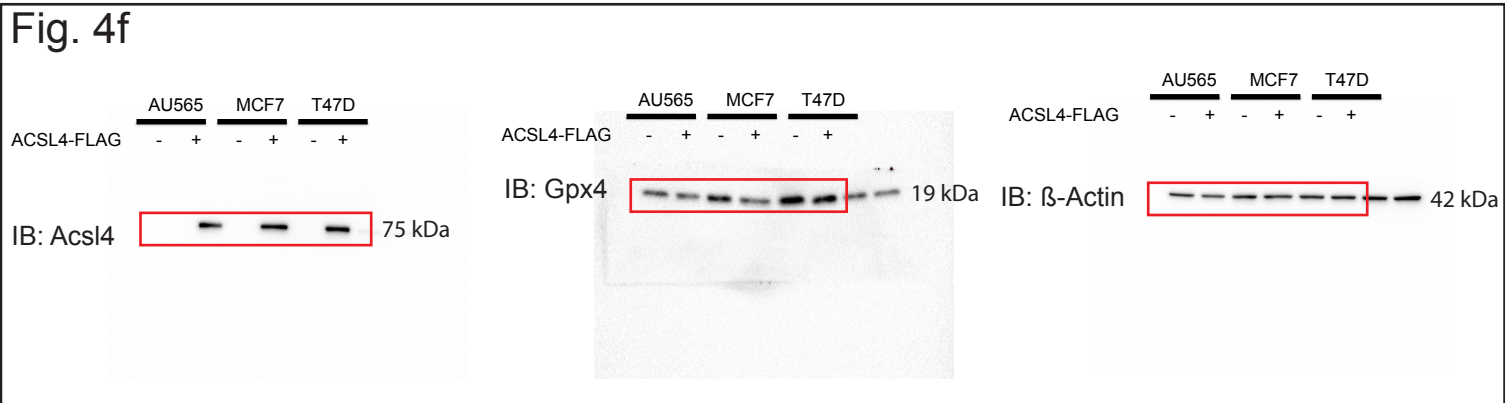


Fig. 4f



Supplementary Figure 6 | Complete blots used for construction of the respective figures as indicated.