Fisetin targets YB-1/RSK axis independent of its effect on ERK signaling: insights from *in vitro* and *in vivo* melanoma models

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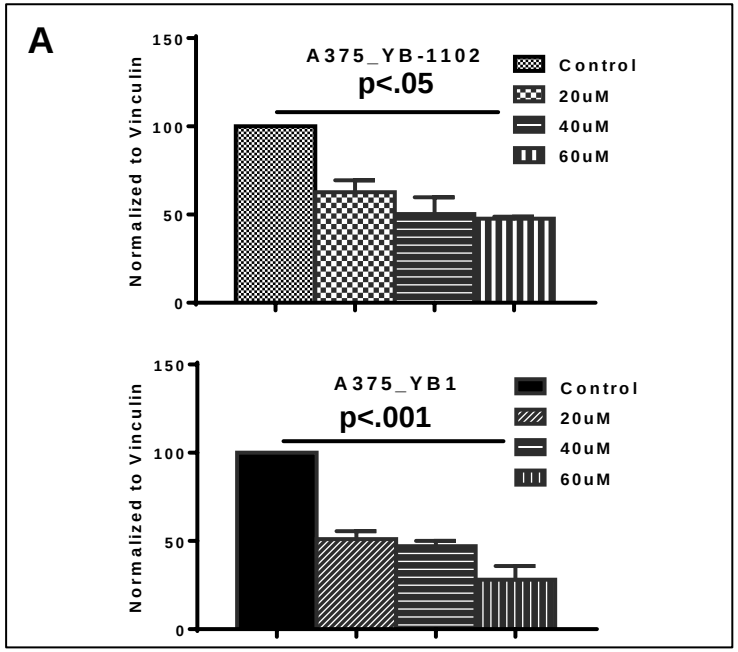

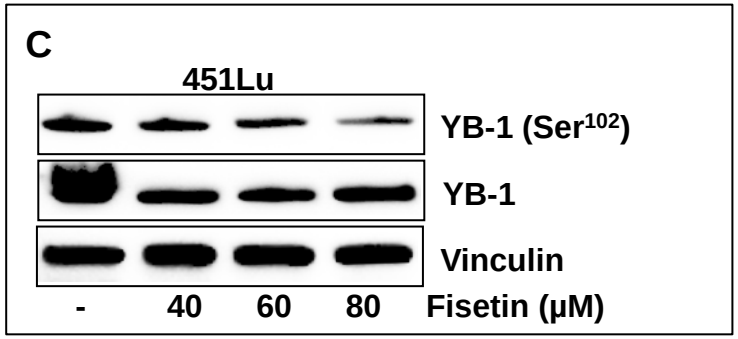

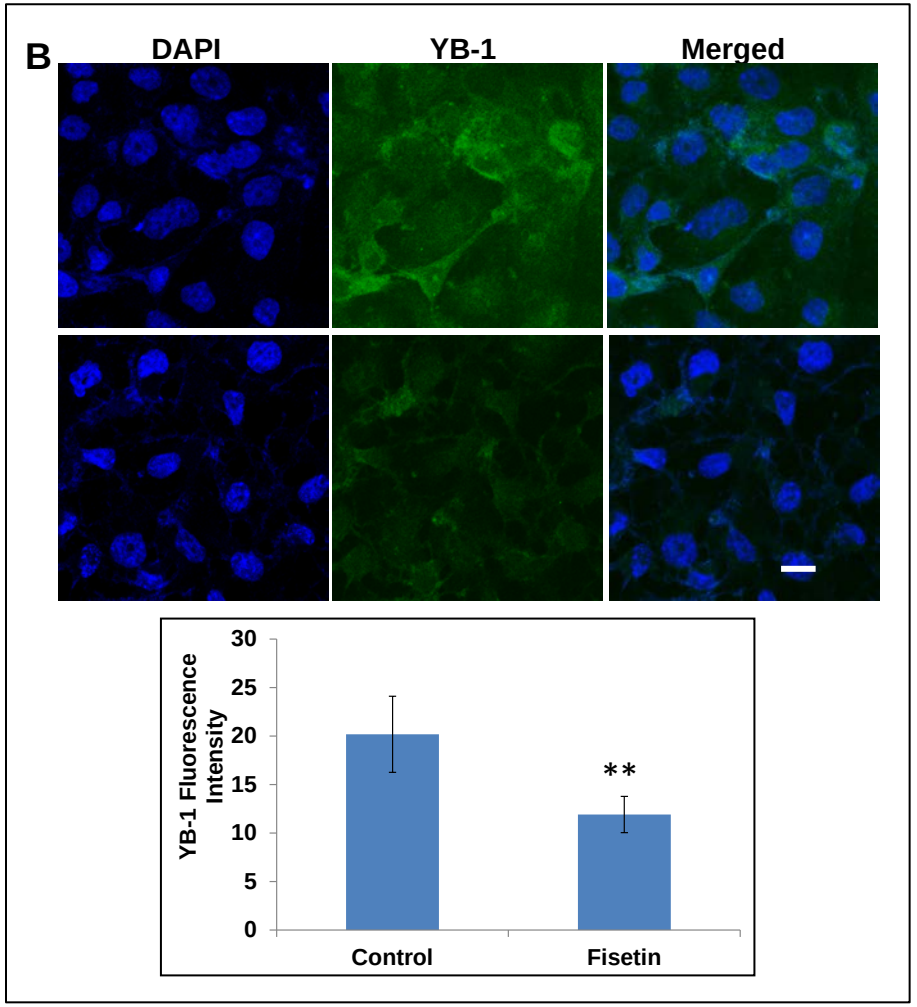

SupFig.1: **Fisetin inhibits YB-1 in BRAF mutant melanoma cells** (A) Densitomeric analysis of phosphorylated and total YB-1 expression in fisetin treated cells. Whole cell lysates of A375 melanoma cells, treated with fisetin (20-60μM:24h) were analyzed for p-YB-1and YB-1 protein expression. Relative density of the bands was normalized to vinculin. (B) (*Top*) Representative micrographs (20x) showing immunofluorescence for YB-1 (green) in A375 cells with or without fisetin (60μM:24h). Images were captured by a confocal microscope. Scale bar, 20μm. (*Bottom*) Histogram represents relative fluorescent intensity of YB-1, scored using ImageJ in A375 cells treated with/without fisetin. (C) Whole cell lysates of 451Lu melanoma cells treated with fisetin (40-80μM:24h) were analyzed for p-YB-1 and YB-1 expression. Equal loading was confirmed by reprobing for vinculin.

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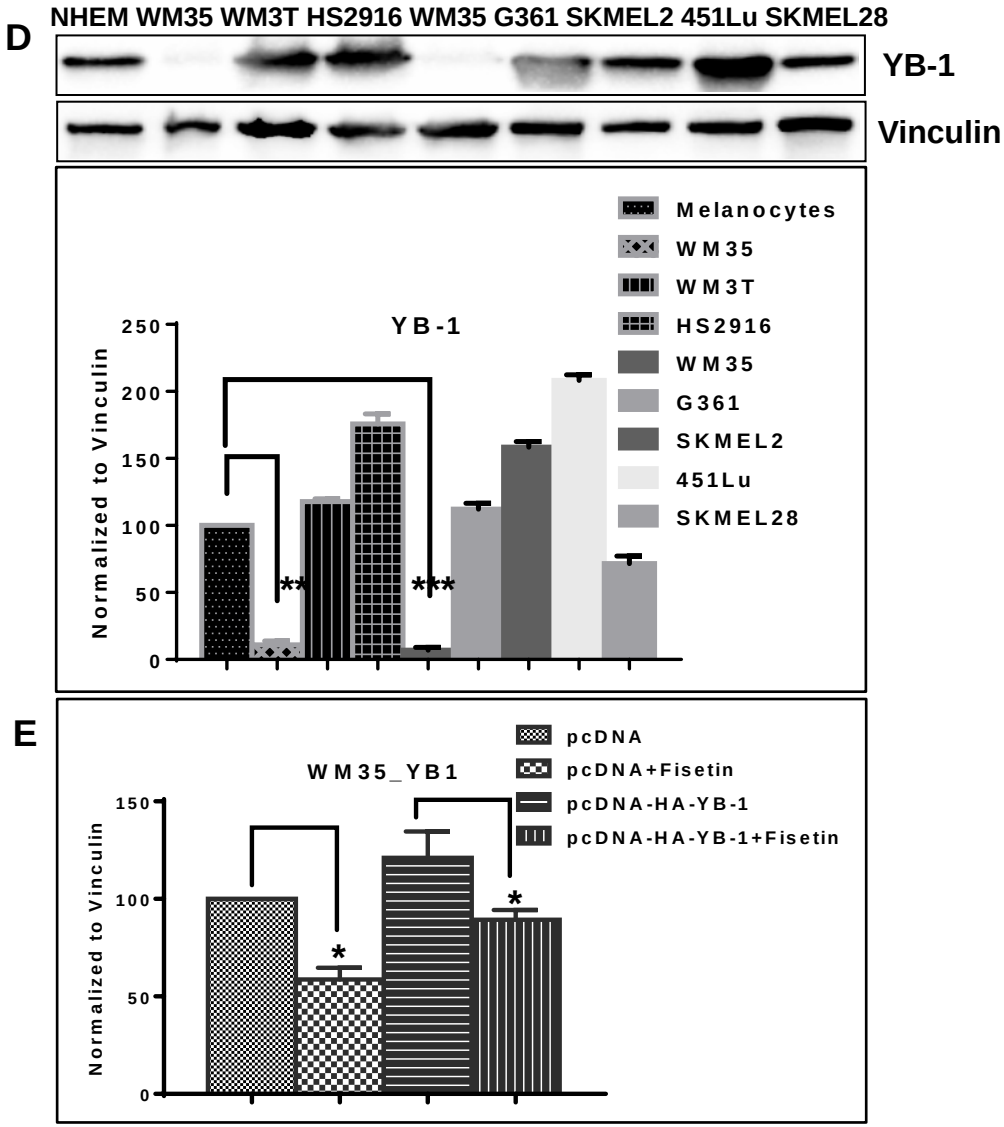

SupFig.1: **Fisetin inhibits YB-1 in BRAF mutant melanoma cells:** (D) Whole cell lysates of melanoma cell lines were analyzed for basal YB-1 expression. WM35 melanoma cells from two different passages were loaded twice. Equal loading was confirmed by reprobing for vinculin. (*top*). Relative density of the bands normalized to vinculin (*bottom*) (E) Densitometric analysis of YB-1 expression in WM35 melanoma cells. WM35 melanoma cells transfected with pcDNA-HA-YB-1 and treated with fisetin (60μM:24h) were analyzed for YB-1 expression. Relative density of the bands were normalized to vinculin. Data shown are representative of three independent experiments with similar results.

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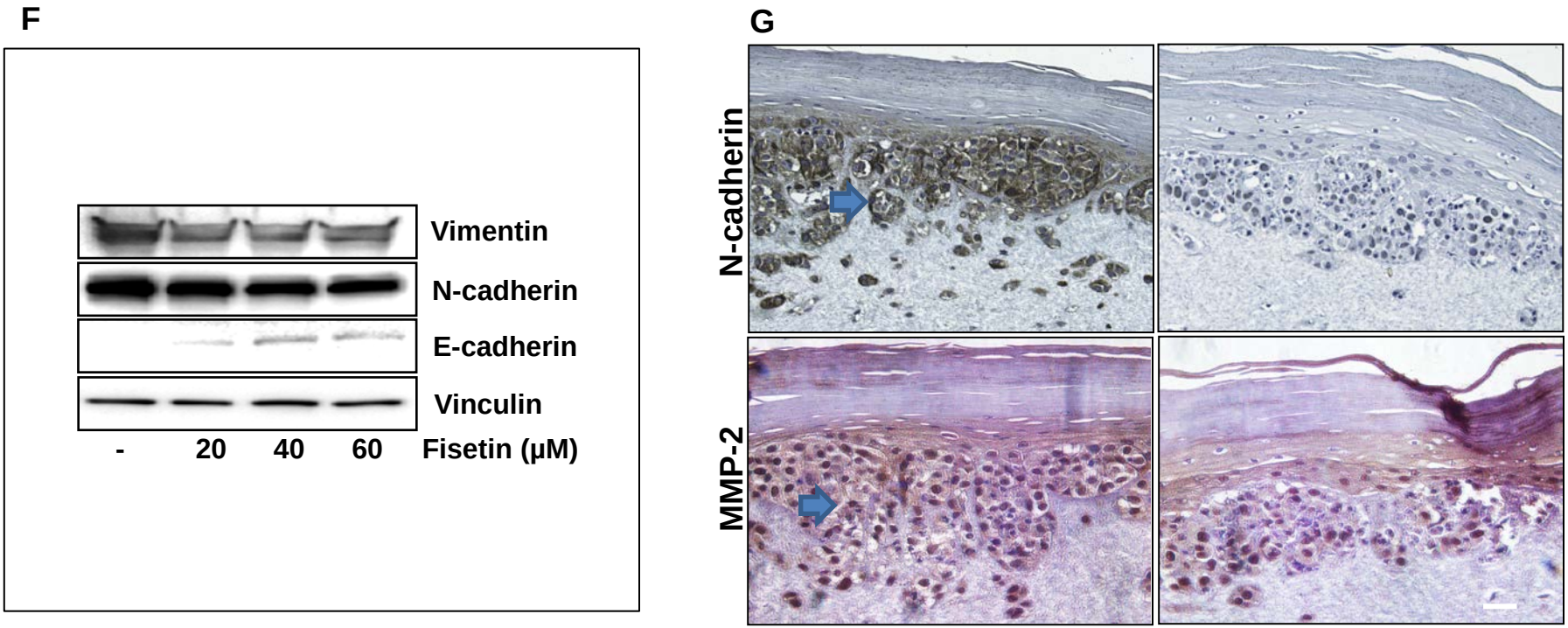

SupFig.1: **Fisetin inhibits YB-1 in BRAF mutant melanoma cells:** (F) Whole cell lysates of A375 melanoma cells treated with fisetin (20-60μM:24h) were analyzed for EMT regulatory proteins. Equal loading was confirmed by reprobing for vinculin. (G) Representative micrographs (10x) showing MMP2 and N-cadherin expression in A375 melanoma constructs treated with fisetin (80μM), harvested at day 12 post treatment. Scale bar, 20μm. Data shown are representative of three independent experiments with similar results.

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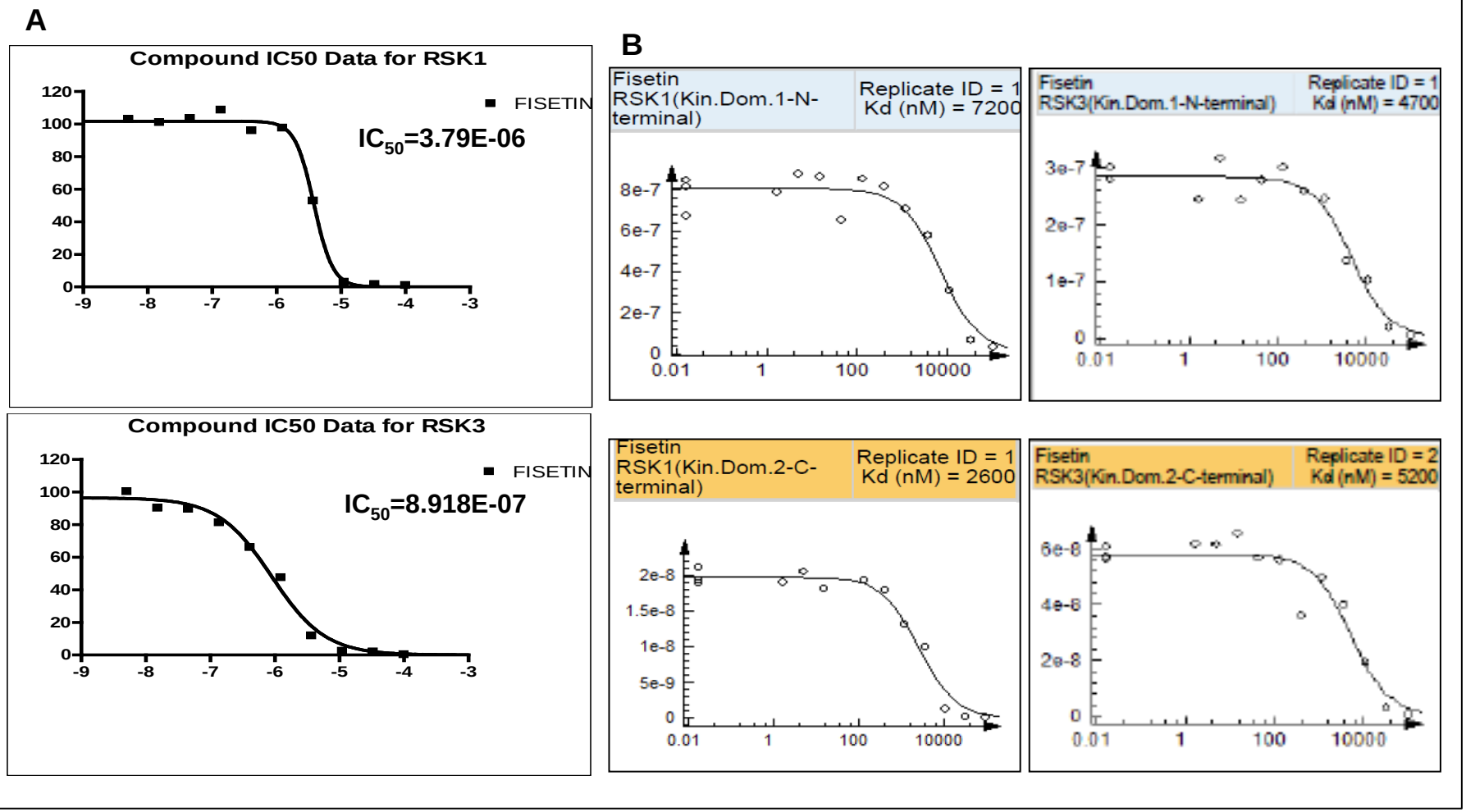

SupFig.2: **Fisetin binds to RSK and suppresses its kinase activity (A)** Representative curves of % kinase activity for RSK isoforms. Fisetin was tested for kinase activity inhibition against RSK1 and RSK3 in 10-dose IC_{50} mode with 3-fold serial dilution starting at 100μM. Reactions were carried out at 30μM ATP. **(B)** Representative curves of competition binding assay for RSK1 and RSK3. An 11-point 3-fold serial dilution of fisetin was prepared in 100% DMSO at 100X final test concentration and diluted to 1X in the assay. Kds were determined using a top concentration of 100μM. The amount of kinase measured by qPCR (signal; *y*-axis) is plotted against the corresponding compound concentration in log10 scale (*x*-axis).

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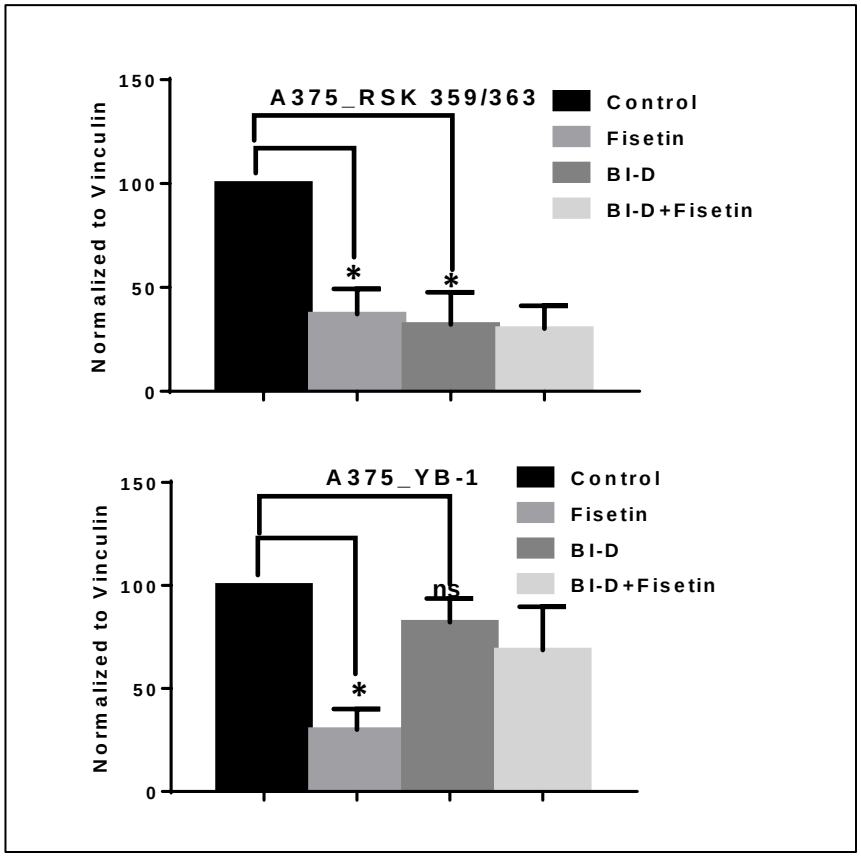
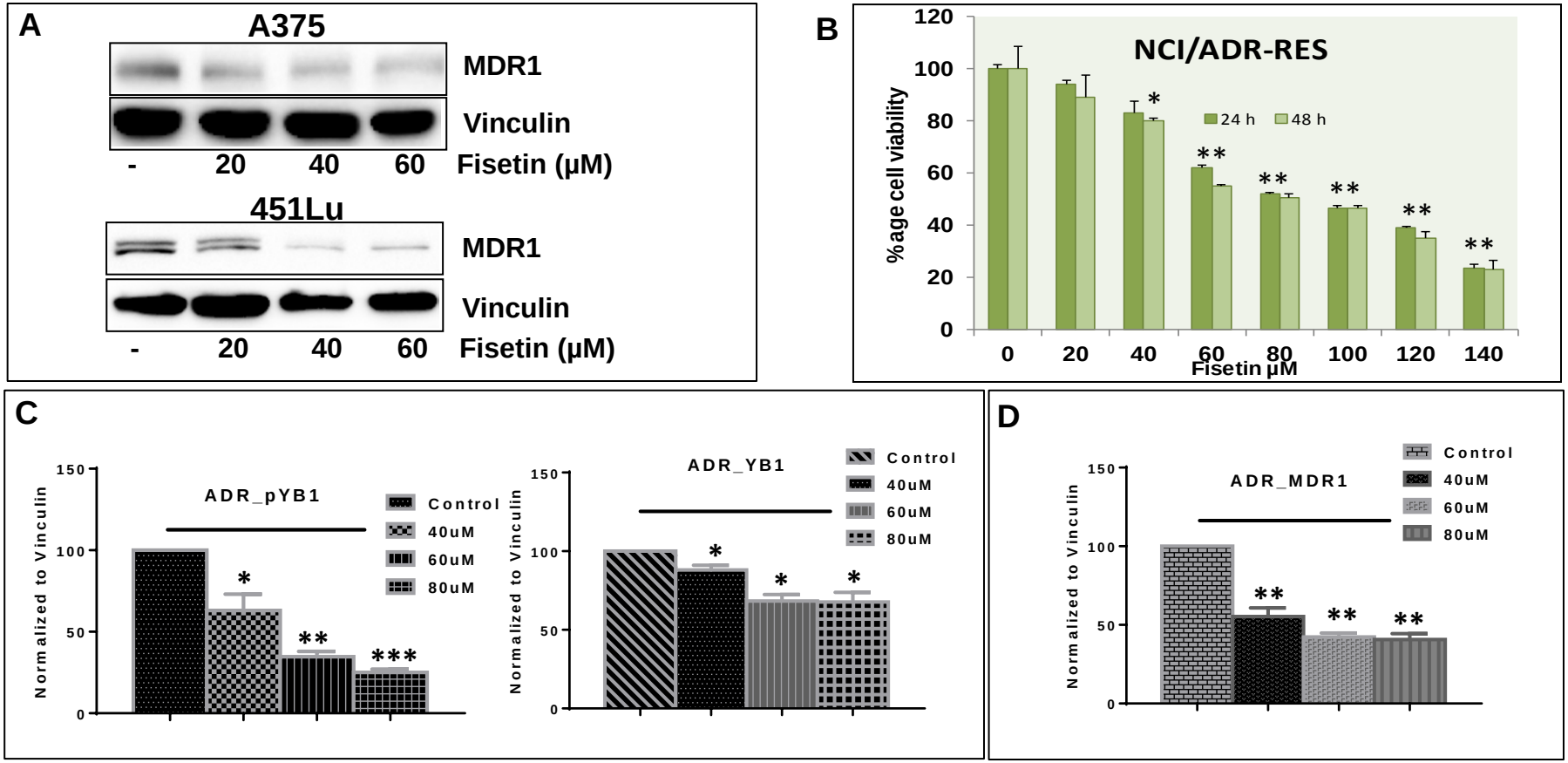
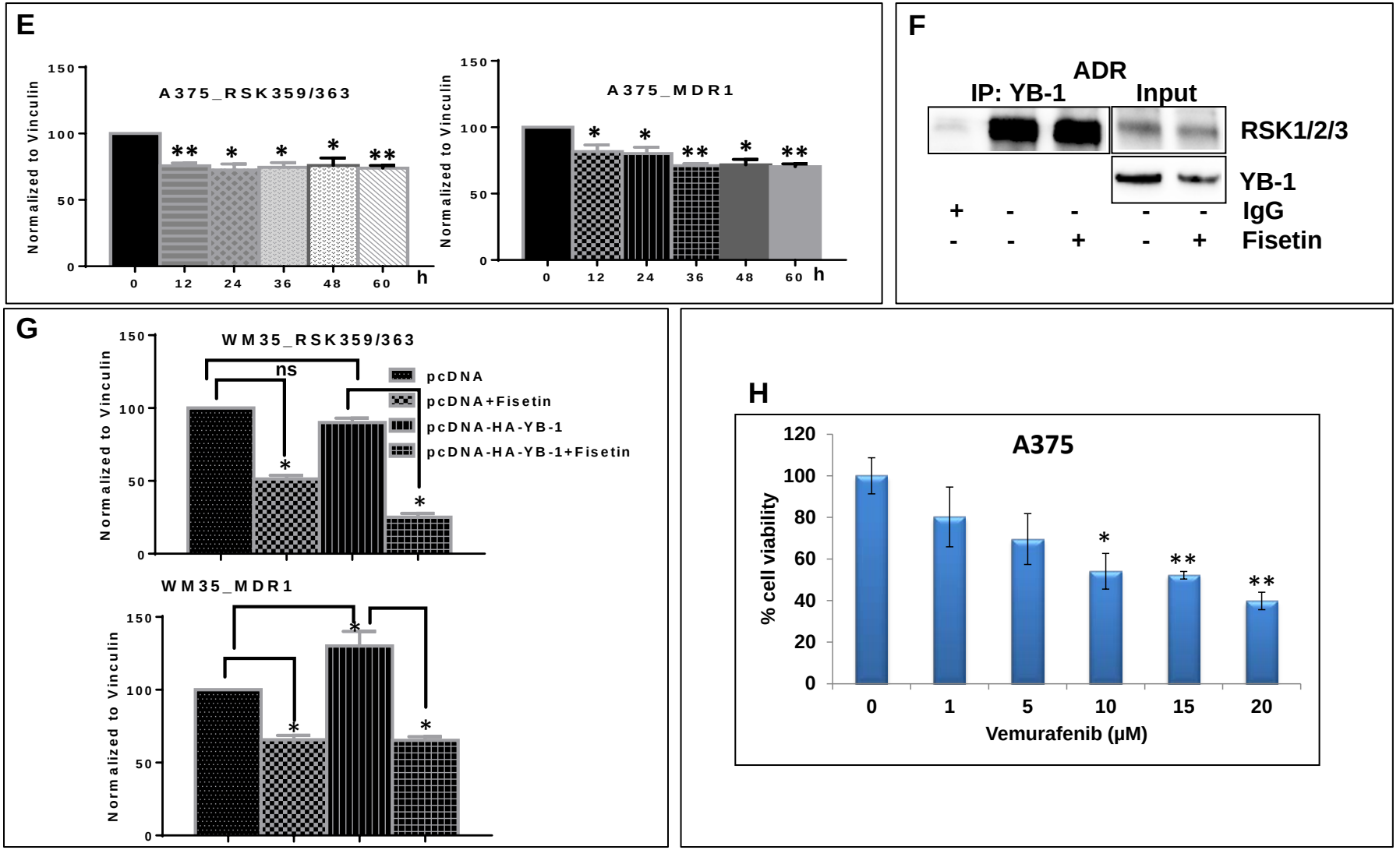


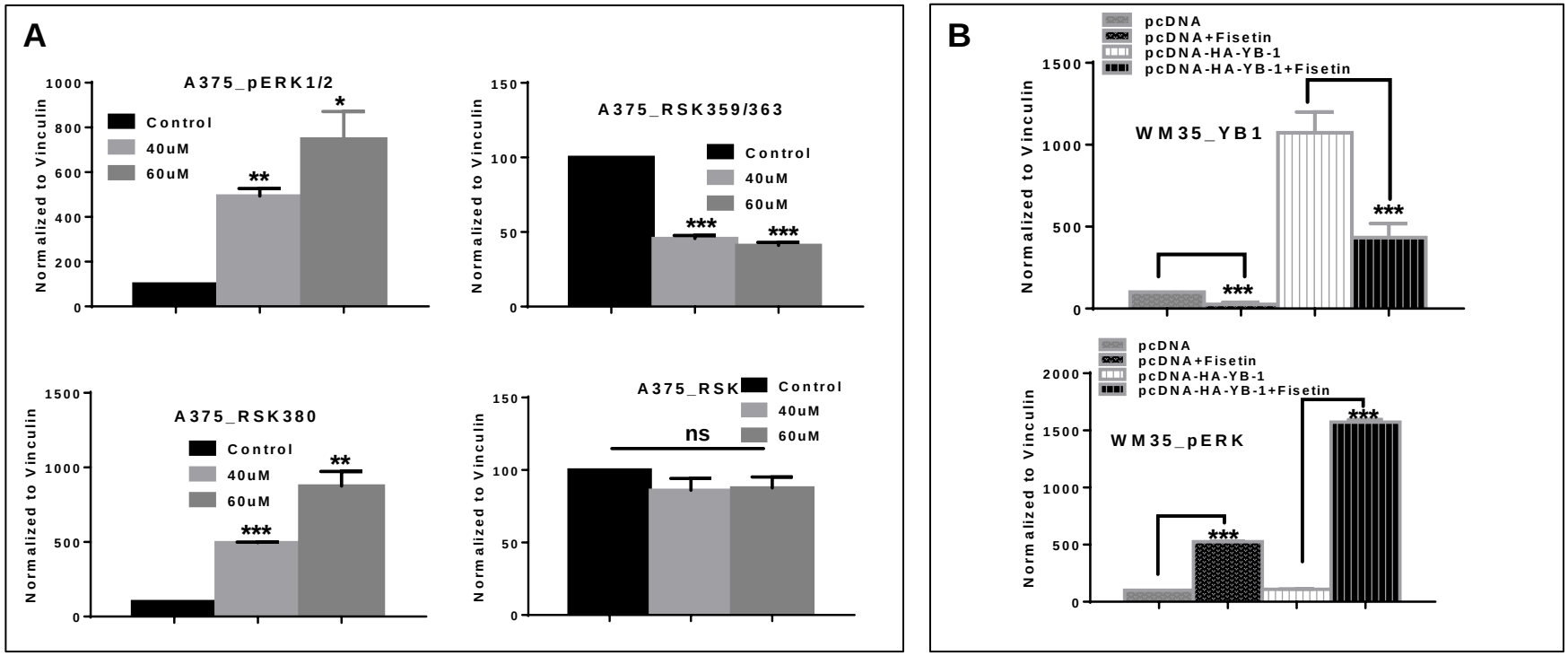
SupFig.3: **Fisetin/RSK2 complex augments its binding to YB-1:** Whole cell lysates of fisetin-treated A375 melanoma cells (60μM:24h) with/without RSK inhibitor BI-D1870 were analyzed for RSK (top) and YB-1 expression (*bottom*). Relative density of the bands were normalized to vinculin. Data shown are representative of three independent experiments with similar results.



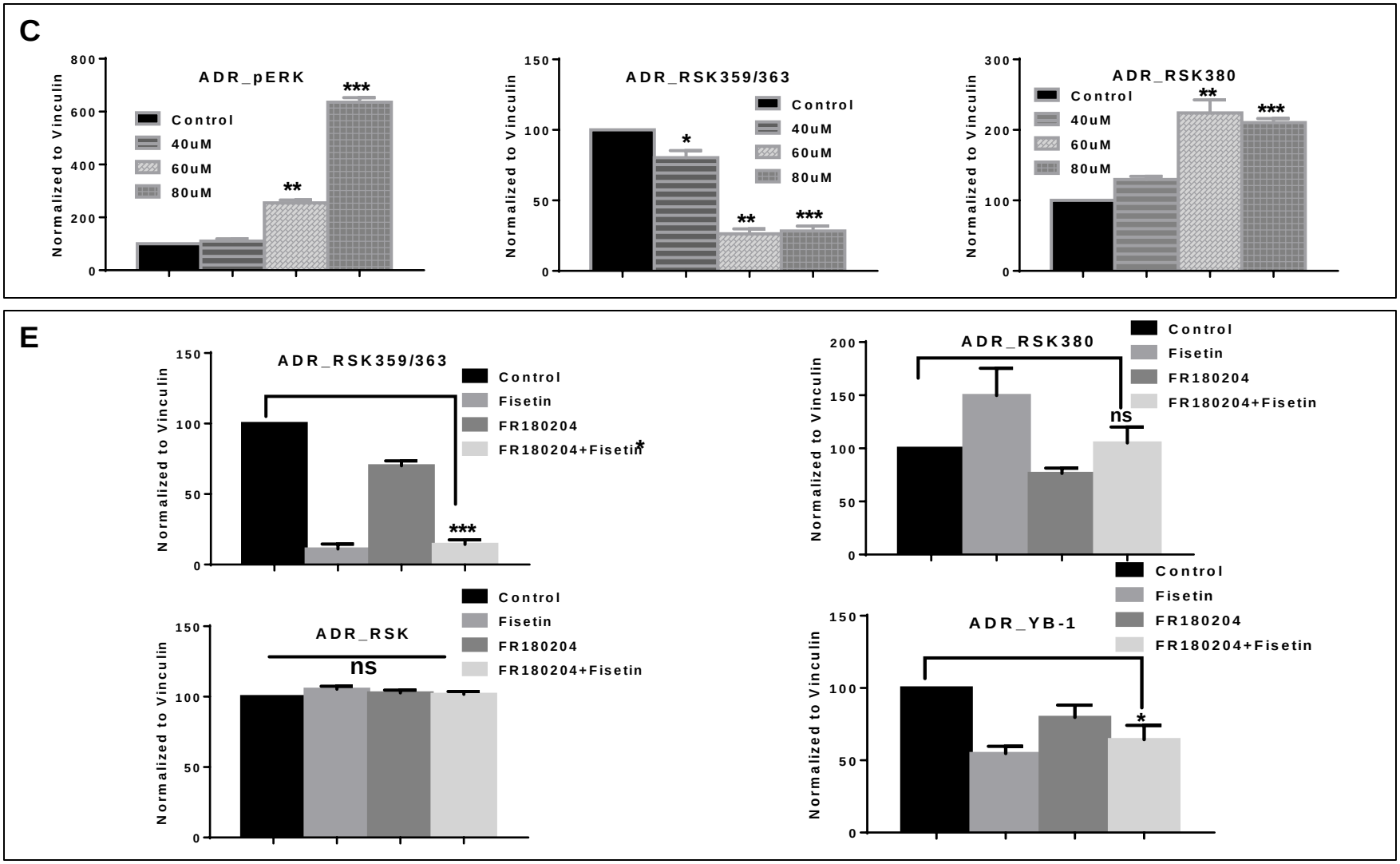
SupFig.4: **Fisetin induced downregulation of YB-1/RSK signaling is associated with decrease in MDR1 (A)** Whole cell lysates of fisetin treated A375 and 451Lu melanoma cells (24 h) were analyzed for MDR1 expression. Equal loading was confirmed by reprobing for vinculin. **(B)** Cell viability studies in fisetin-treated NCI-ADR-Res ovarian cancer cells, 24 and 48h post treatment was assessed by MTT assay. The data expressed as the percentage cell viability represent the mean±SE of three experiments. **(C&D)** Whole cell lysates of NCI/ADR-Res ovarian cancer cells treated with fisetin (40-80μM:24h) were analyzed for p-YB-1, YB-1 and MDR1 expression. Histograms represent relative density of bands normalized to vinculin. Data shown are representative of three independent experiments with similar results.



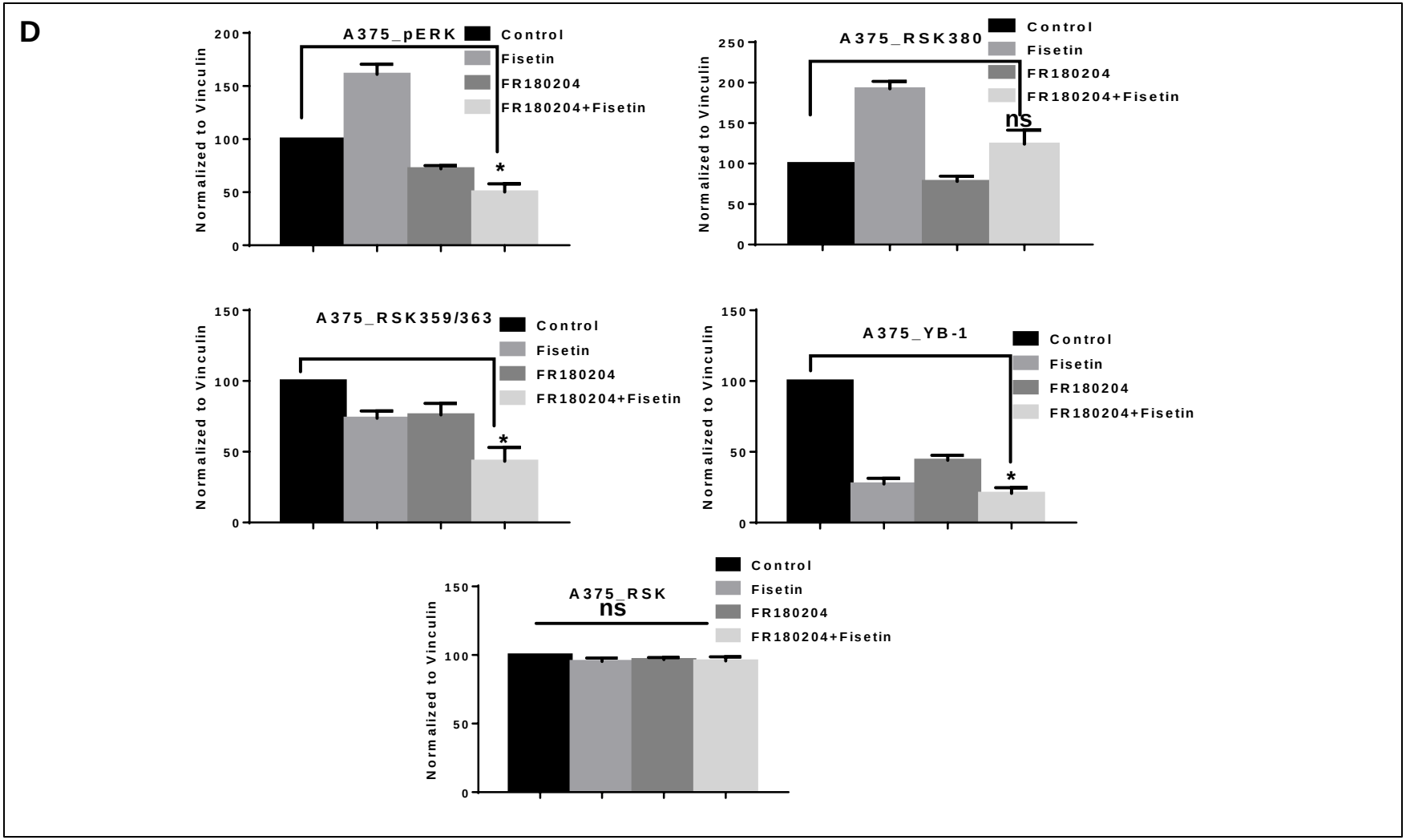
SupFig.4: **Fisetin induced downregulation of YB-1/RSK signaling is associated with decrease in MDR1 (E)** Whole cell lysates of A375 melanoma cells treated with fisetin (20-60μM:24h) were analyzed for p-RSK and MDR1 expression at specified time points. Histograms represent relative density of bands normalized to vinculin. **(F)** Equal amounts of NCI-ADR cell lysates treated with/without fisetin (60μM:24h) were immunoprecipitated with YB-1 antibody followed by western blot analysis for RSK1/2/3 and YB-1. **(G)** WM35 melanoma cells transfected with pcDNA-HA-YB-1, treated with fisetin (60μM:24h) were analyzed for p-RSK and MDR1 expression. Histograms represent relative density of bands normalized to vinculin. Data shown are representative of three independent experiments with similar results. (**H**) A375 melanoma cells were treated with varying doses of vemurafenib and MTT assay was performed after 24h. The data expressed as the percentage of cell viability represent the mean+SE of three experiments



SupFig.5: **Fisetin mediated decrease in RSK/YB-1 does not require suppression of ERK signaling (A)** Whole cell lysates of A375 cells treated with fisetin (40-60μM:24h) were analyzed for ERK1/2 and RSK protein expressions. Histograms represent relative density of the bands normalized to vinculin. **(B)** WM35 melanoma cells, transfected with pcDNA-HA-YB-1, treated with fisetin (60μM:24h) were analyzed for p-ERK1/2 and YB-1 expression. Histograms represent relative density of the bands normalized to vinculin. Data shown are representative of three independent experiments with similar results.

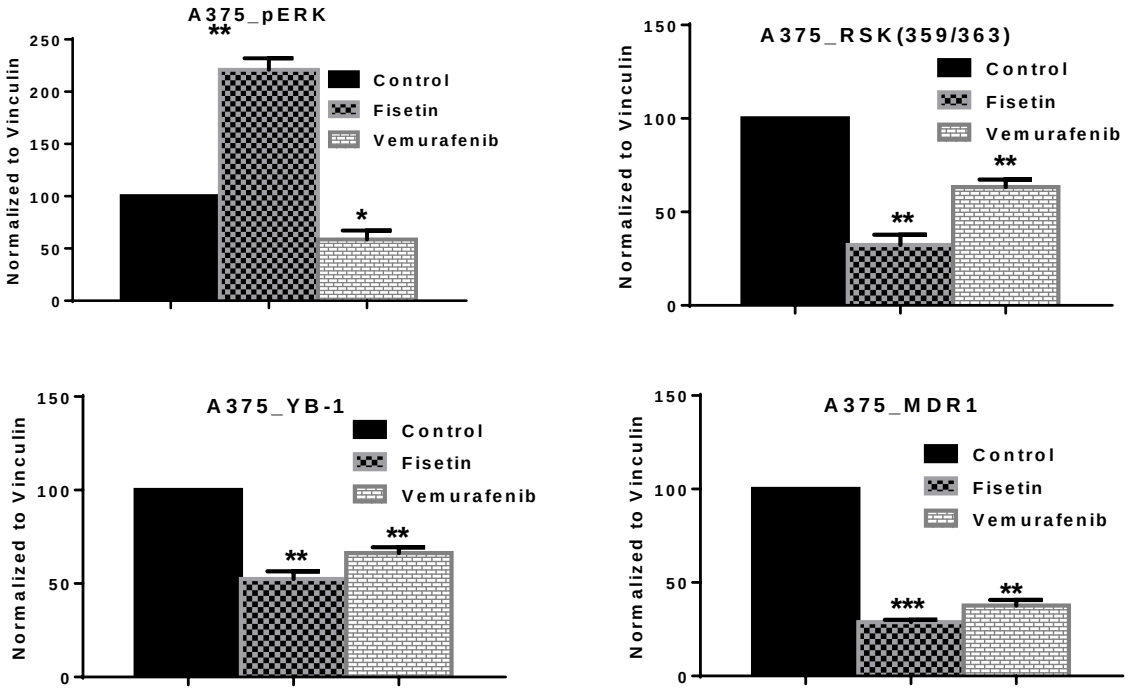


SupFig.5 **Fisetin mediated decrease in RSK/YB-1 does not require suppression of ERK signaling (C)** Whole cell lysates of NCI/ADR-RES ovarian cancer cells treated with fisetin (40-80µM:24h) were analyzed for phosphorylated RSK and ERK1/2 protein expressions. Histograms represent relative density of the bands normalized to vinculin. **(E)** Whole cell lysates of NCI/ADR-RES ovarian cancer cells, treated with fisetin (60µM:24h) and/or ERK1/2 inhibitor FR180204 (10µM) were analyzed for phosphorylated RSK and YB-1 protein expressions. Histograms represent relative density of the bands normalized to vinculin. Data shown are representative of three independent experiments with similar results.



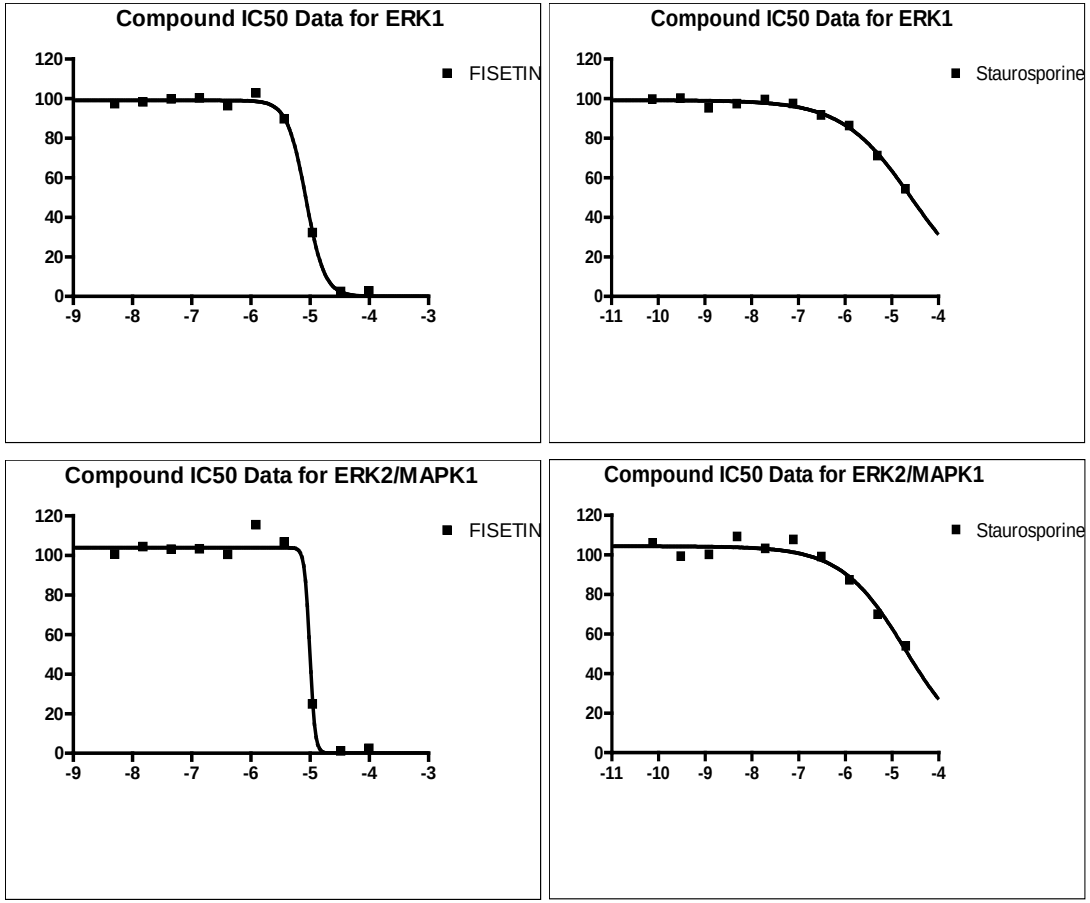
SupFig.5 **Fisetin mediated decrease in RSK/YB-1 does not require suppression of ERK signaling (D)** Whole cell lysates of A375 melanoma treated with fisetin (60µM:24h) and/or ERK1/2 inhibitor FR180204 (10µM) were analyzed for phosphorylated RSK and YB-1 protein expressions. Histograms represent relative density of the bands normalized to vinculin. Data shown are representative of three independent experiments with similar results.

A

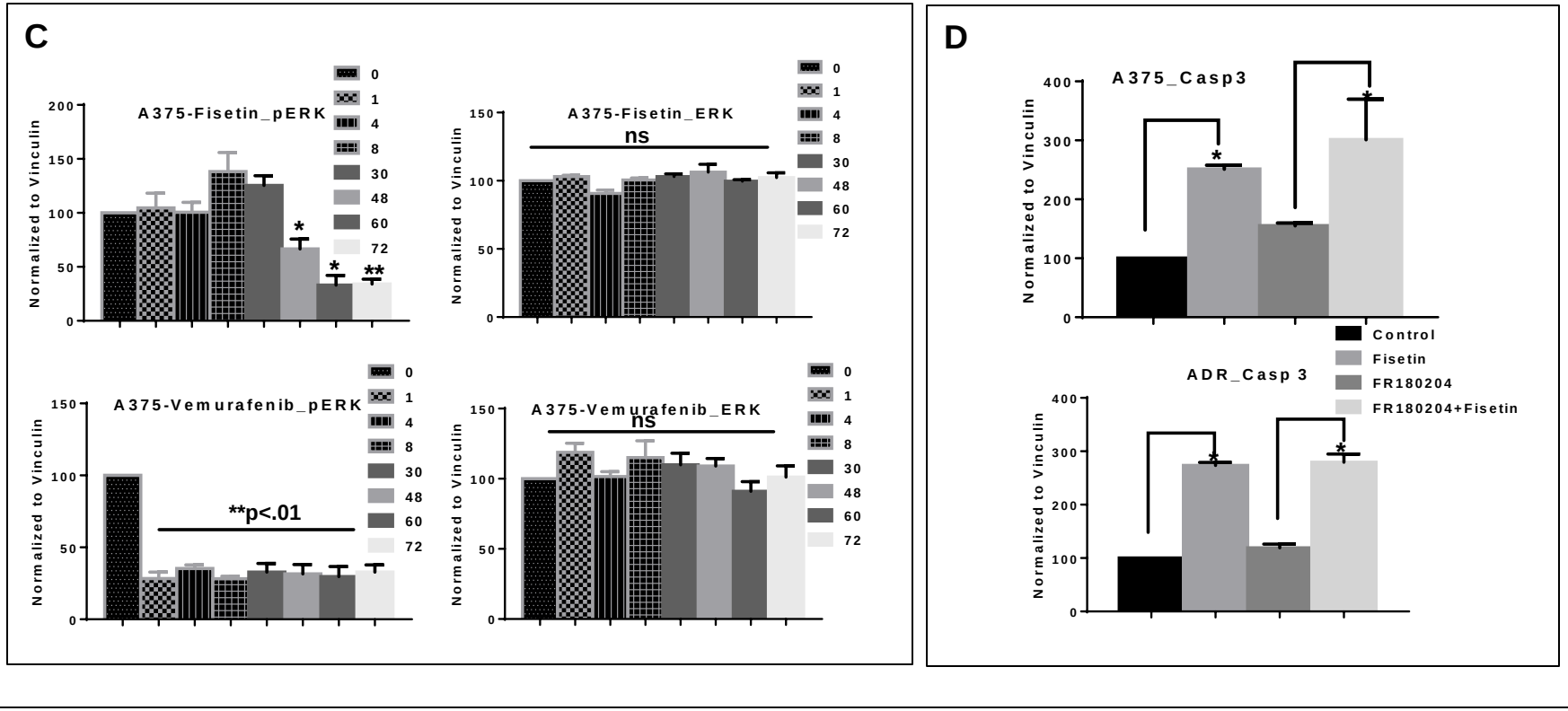


SupFig.6 **Comparable inhibition of melanoma tumor growth by fisetin and vemurafenib albeit through divergent regulation of ERK signaling (A)** Whole cell lysates of A375 melanoma cells treated with fisetin (60µM) or vemurafenib (10µM) for 24h were analyzed for phosphorylated RSK, ERK1/2, total YB-1 and MDR1. Histograms represent relative density of the bands normalized to vinculin. Data shown are representative of three independent experiments with similar results.

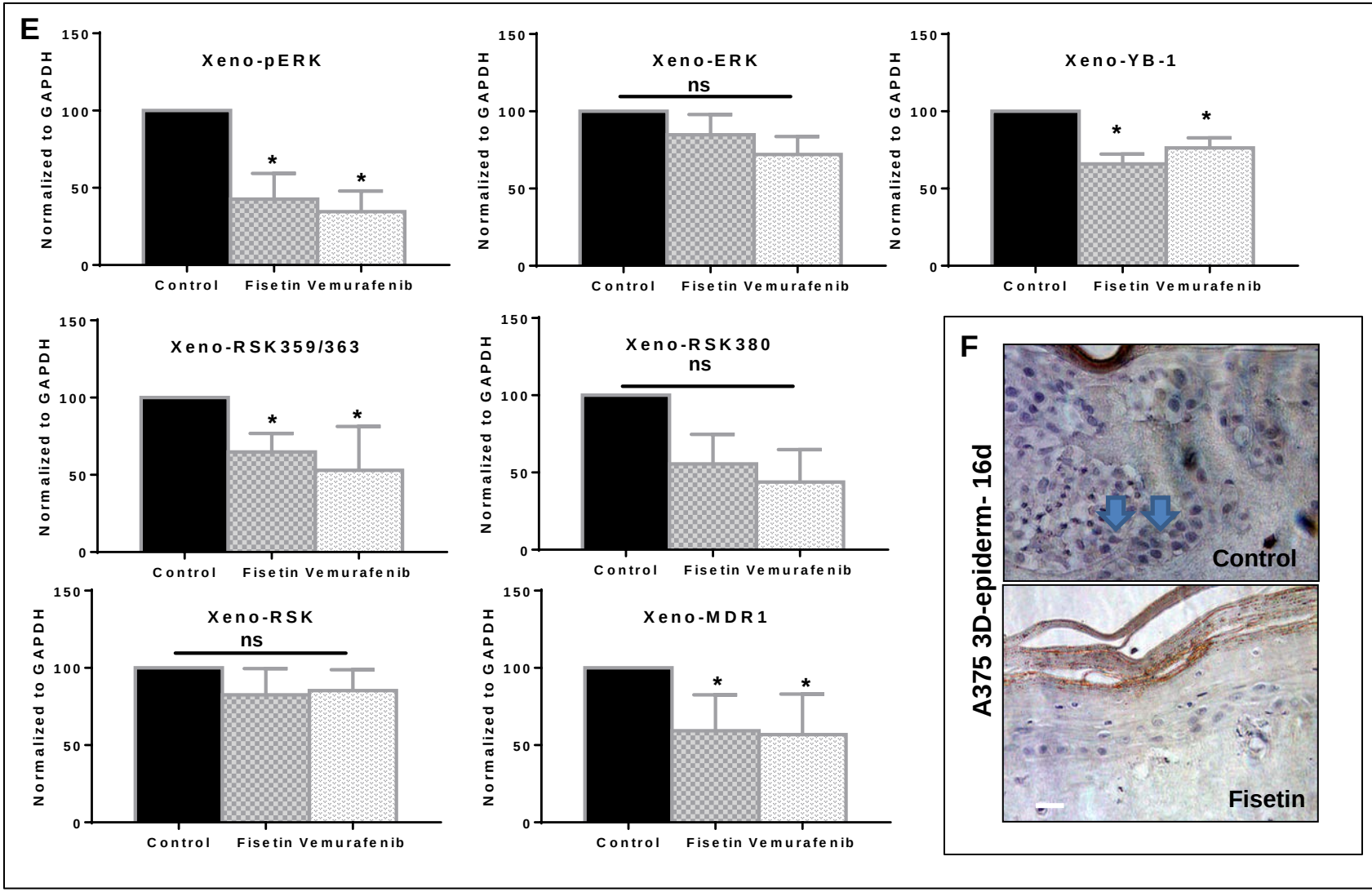
B



SupFig.6 **Comparable inhibition of melanoma tumor growth by fisetin and vemurafenib albeit through divergent regulation of ERK signaling (B)** Representative curve of % kinase inhibitory activity for ERK1/2 with fisetin and staurosporine. Fisetin was tested for kinase activity along with reference compound staurosporine in 10-dose IC_{50} mode with 3-fold serial dilution starting at 100μM. Staurosporine was tested in 10-dose IC_{50} mode with 4-fold serial dilution starting at 20μM. Reactions were carried out at 30μM ATP.



SupFig.6 **Comparable inhibition of melanoma tumor growth by fisetin and vemurafenib albeit through divergent regulation of ERK signaling (C)** Whole cell lysates of A375 melanoma cells treated with fisetin (60μM) or vemurafenib (10μM) for specified time points were analyzed for phosphorylated and total ERK1/2. Histograms represent relative density of the bands normalized to vinculin. **(D)** Whole cell lysates of A375 melanoma and NCI/ADR-RES ovarian cancer cells, treated with fisetin (60μM) and/or ERK1/2 inhibitor FR180204 (10μM) for 24h were analyzed for cleaved caspase-3 expression. Histograms represent relative density of the bands normalized to vinculin. Data shown are representative of three independent experiments with similar results.



SupFig.6 **Comparable inhibition of melanoma tumor growth by fisetin and vemurafenib albeit through divergent regulation of ERK signaling (E)** Whole cell lysates of tumor tissues from DMSO/fisetin/vemurafenib-treated mice analyzed by western blot. Histograms represent relative density of the bands normalized to GAPDH. **(F)** Representative micrographs (10x) showing pERK1/2 expression (arrow) in A375 melanoma constructs treated with/without fisetin (80µM), harvested at day 16 post treatment. Scale bar, 10 µm. Data represent samples from each group repeated twice with similar results.