Electronic supplementary material

A Framework for the *in vitro* Evaluation of Cancer-relevant Molecular Characterisations and Mitogenic Potency of Insulin Analogues

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SUPPLEMENTAL METHODS

Cell lines and ligands

The mutation data for colon cancer cell lines was obtained from the COSMIC (Catalogue of somatic mutations in cancer) database (http://cancer.sanger.ac.uk/cpsmic); additionally, KRAS and PI3KCA mutations were confirmed in-house by mutational analysis (Table S1). All cells were Mycoplasma free.

Table S1 Mutational characteristics of colon cancer cell lines

	KRAS	**BRAF**	**PI3KCA**	**TP53**
HCT 116	G13D	WT	H1047R	WT
HT-29	WT	V600E	P449T	R273H
COLO 205	WT	V600E	WT	Y103

WT: wildtype

Chemicals and antibodies

The primary antibodies used for western blotting (to assess IR/IGF-IR signalling pathways) were from Cell Signalling Technologies (Danvers, MA, USA) apart from: anti-IR antibody (Calbiochem, San Diego, CA, USA), anti-tubulin antibody (Abcam, Boston, MA, USA), anti-actin antibody (Sigma Aldrich, Poole, UK) and anti-phosphorylated Tyr_{612} IRS-1 antibody (Millipore, Billerica, MA, USA). The antibody pIGF-IRβ (Tyr 1131) / IR β (Tyr 1146) was used

to determine phosphorylation status on both IGF-IR and IR. The antibody p-Tyr (PY99) used for immunoprecipitation was purchased from Santa Cruz Biotechnology, Inc. The secondary antibodies were horseradish peroxidise HRP-conjugated anti-rabbit and HRP-conjugated anti-mouse, both obtained from Dako (Ely, UK).

Western blotting and immunoprecipitation

Whole-cell lysates were prepared directly in Laemmli sample buffer (Biorad Laboratories, Hemel Hempstead, UK). Samples were boiled for 5 min prior to storage at -20°C until analysis. Proteins were resolved using an Invitrogen SureLock mini gel system and transferred to a nitrocellulose membrane and probed using the antibodies. Immunocomplexes were detected using Western Lightning chemiluminescence reagent (Applied Biosystems, Warrington, UK) and images were captured using a Fujifilm Imagestation LAS1000 (Raytek Scientific, Sheffield, UK). Western signals were quantified with Aida Advanced Image Data Analyzer software (v.3.28.001, Raytek Scientific, Sheffield, UK), divided by the densitometric signal for control protein load (actin or tubulin) or non-phosphorylated protein.

Cells were seeded in 6-well plates in complete medium and left to adhere for 24 hr followed by starvation in serum-free medium for 24 hr. The cells were stimulated with insulin, X10 and IGF-I at the indicated concentrations for 15 min. Whole cell lysates were prepared and phospho-tyrosine containing proteins were immunoprecipitated overnight at 4^{o}C using PY99 antibody. Proteins in the immunocomplexes were detected by western blotting using the indicated antibodies. Sample input into the immunoprecipitations was determined using actin and tubulin detection in a small volume of total cell lysate.

SUPPLEMENTAL RESULTS

Table S2 Q-PCR relative quantification of IR isoforms in our four cell types

	hIRA	hIRB
MCF7	86%	14%
HCT 116	91%	9%
HT-29	95%	5%
COLO 205	95%	5%

Results expressed as average of 3 experiments.

The effects of insulin, X10 and IGF-I on apoptosis in HT-29 and HCT 116 cells were different

Both proliferation assays showed that in HT-29 cells insulin, X10 and IGF-I did not induce any proliferation. Contrary, the number of cells decreased as the concentration of ligands increased. A decrease in cell number is likely to be related to increased cell death. Caspase 3/7 activity may be a potential marker of cell death. A very moderate increase in caspase 3/7 activity was observed when HT-29 cells were treated with insulin whereas IGF-I and the highest concentration of X10 (100 nM) caused significant increase in caspase 3/7 activity measured after 24 hr (ESM Fig. 6). A longer incubation (48 hr) with insulin, X10 and IGF-I in HT-29 cells induced around two- to five-fold increase in the activity of caspase 3/7. IGF-I and 100 nM X10 induced the highest activity of caspase 3/7. In contrast, in HCT 116 cells no increase in caspase activity was observed after 24 or 48 hr incubation with insulin, X10 or IGF-I.

SUPPLEMENTAL DISCUSSION

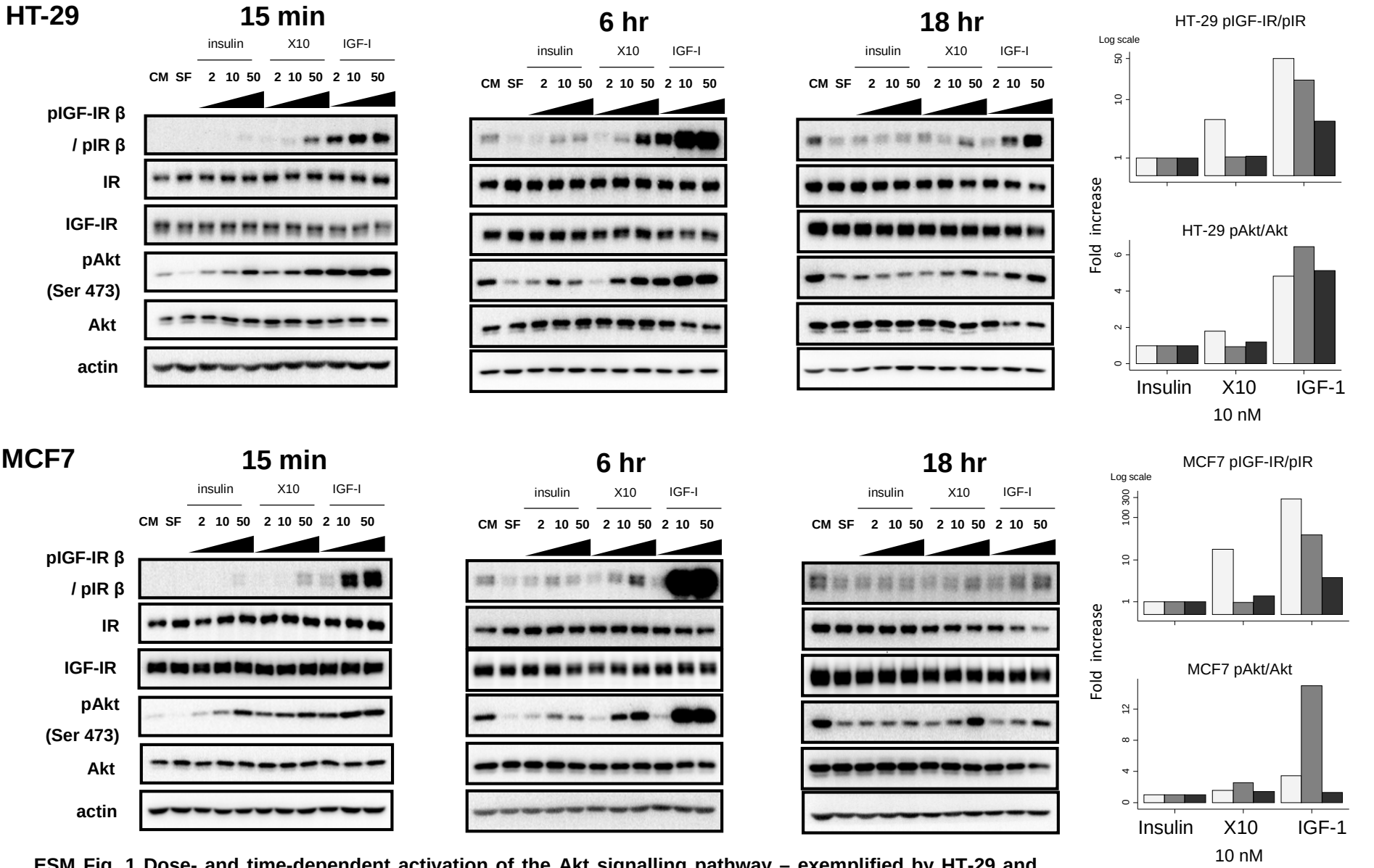
Lack of cell growth in HT29 cells

We failed to elicit growth curves in HT-29 cells by either cell proliferation assay. We checked this several times – rather, cell number decreased with increasing concentration of the ligands. This is an apparent paradox and we sought to explain.

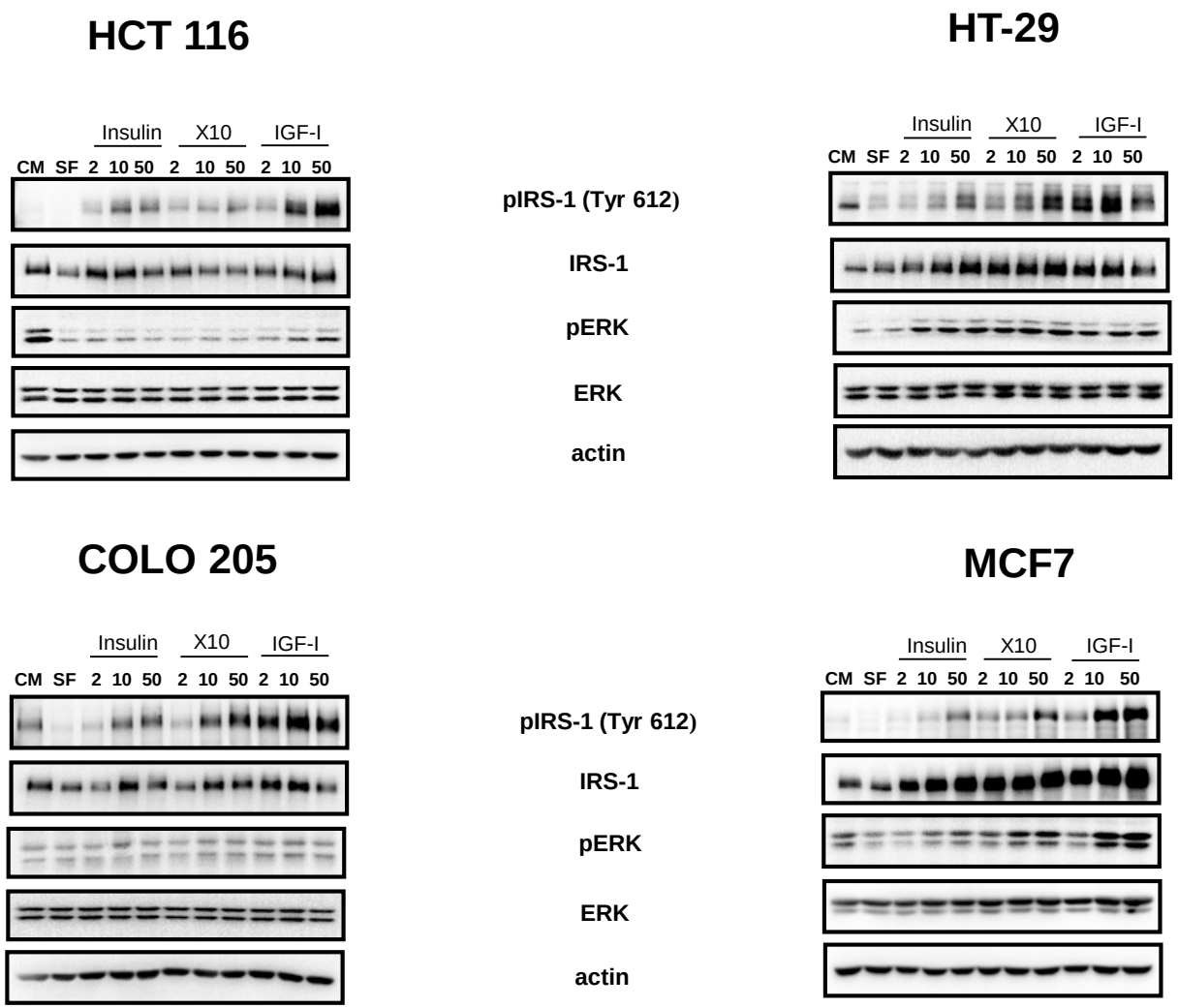
In ESM Figure 6, increased caspase-3/7 activity was found in HT29 cells in response to all three ligands after both 24 and 48h. Insulin's ability to activate caspase-3/7 was lower than that seen by both X10 and IGF-I. This coincided with X10 and IGF-I's greater ability to activate both IGF-I/IR and Akt signalling compared with insulin, providing a possible link between receptor activation, Akt activation, caspase-3/7 activity and apoptosis. Of note, sustained activation of Akt signalling can be detrimental to cell survival as increased glycolysis, the formation of reactive oxygen species, inhibition of FOXO transcription factors, inhibition of reactive oxygen species scavengers and caspase activation can result in apoptosis (1,2).

References to ESM

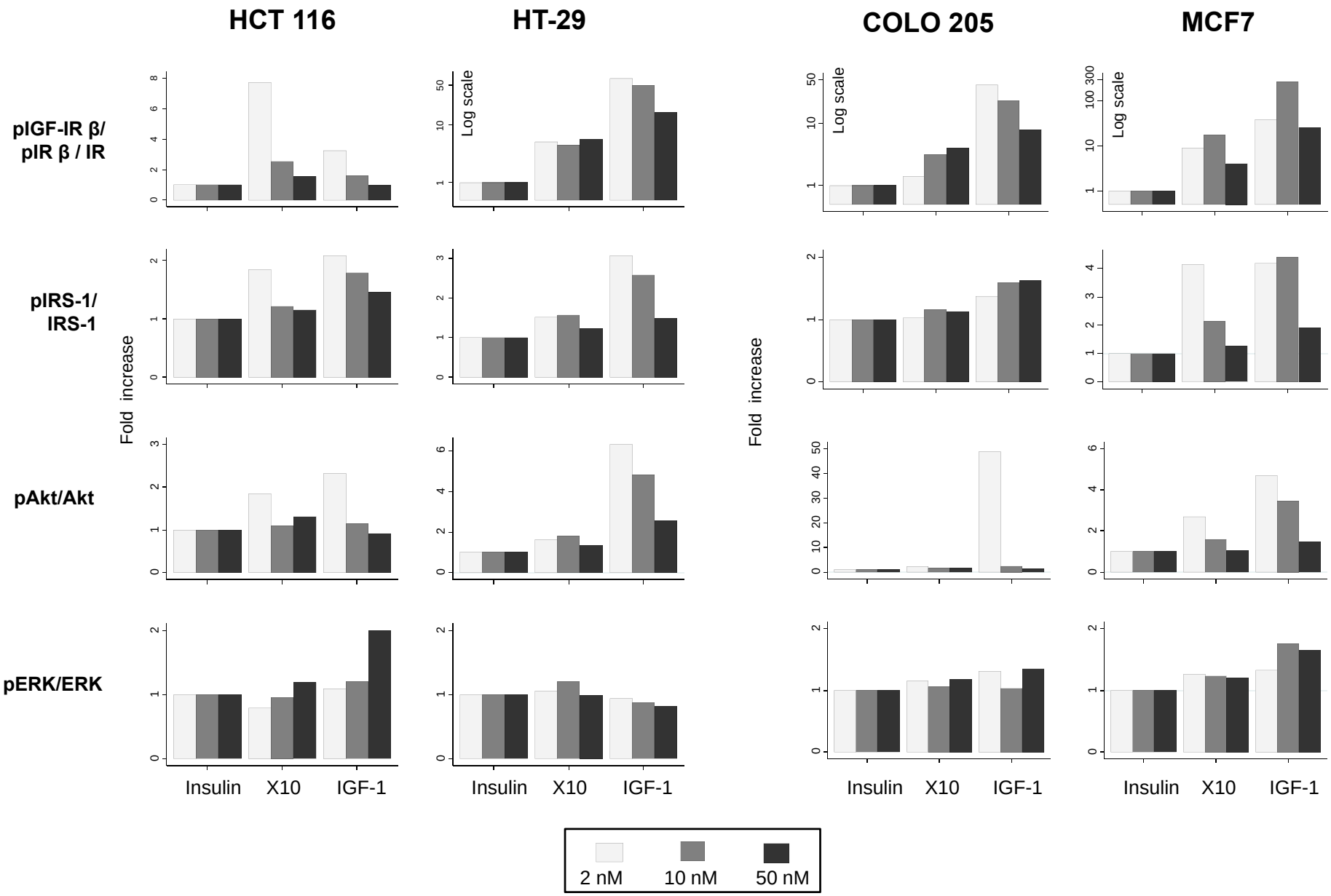
1. Nogueira, V., *et al.* (2008) Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis. *Cancer Cell*, **14**, 458-70.
2. van Gorp, A.G., *et al.* (2006) Chronic protein kinase B (PKB/c-akt) activation leads to apoptosis induced by oxidative stress-mediated Foxo3a transcriptional up-regulation. *Cancer Res*, **66**, 10760-9.



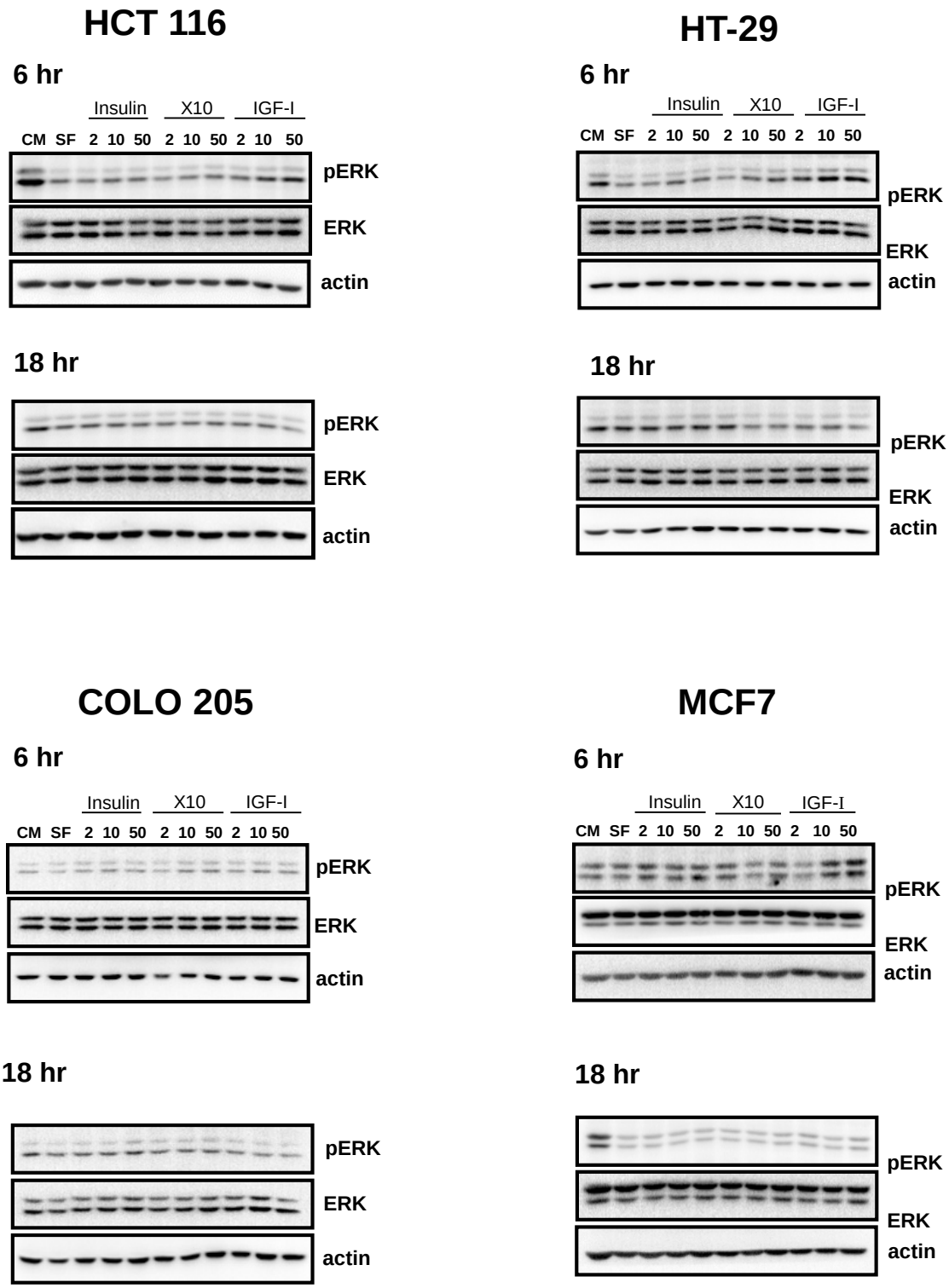
ESM Fig. 1 Dose- and time-dependent activation of the Akt signalling pathway – exemplified by HT-29 and MCF7 cells. Western blots for HT-29 (upper panels) and MCF7 (lower panels) cells after 15 min, 6 hr and 18 hr stimulation with three ligands (2, 10 and 50 nM). Densitometry of the time-related changes (15 min, 6 hr and 18 hr) in pIGF-IR/pIR and in pAkt (Ser 473) after 10 nM stimulation with all three ligands. Details of time-course-dependent target phosphorylation by X10 and IGF-I are described in the Methods. This experiment was performed three times and here is presented one example. CM-complete medium, SF-serum-free medium.



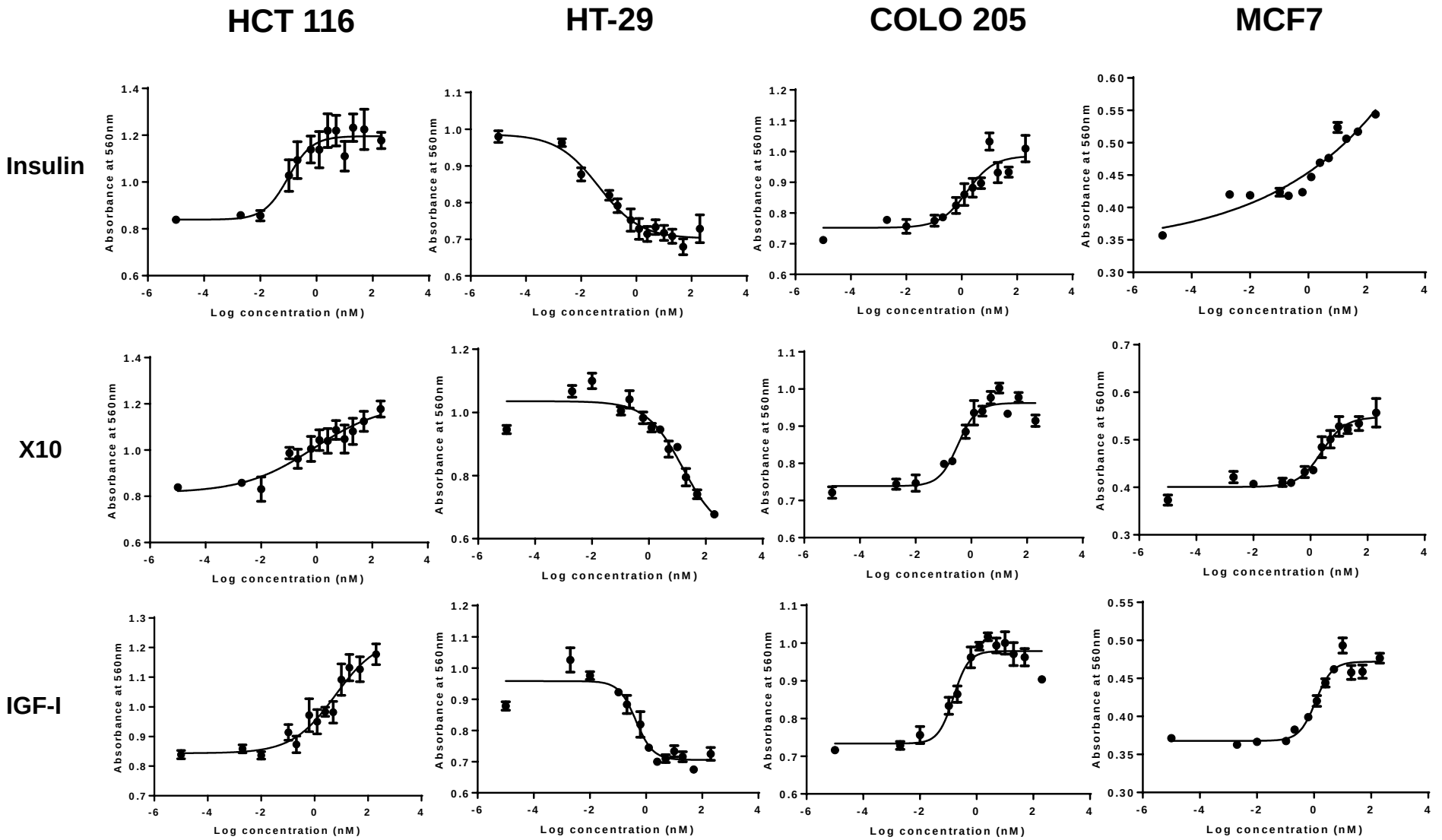
ESM Fig. 2 Effect of short-term stimulation (15 min) with insulin, X10 and IGF-I on activation of IRS-1 and ERK. HCT 116, HT-29, COLO 205 and MCF7 cells were seeded in 6-well plates in complete medium and then starved in serum-free medium for 24 hr. Treatments with insulin, X10 and IGF-I used 3 concentrations (2, 10 and 50 nM). Whole-cell lysates were prepared and proteins were resolved by SDS-PAGE, transferred and immunodetected with the indicated antibodies. This experiment was performed three times and here is presented one example. CM-complete medium, SF-serum-free medium.



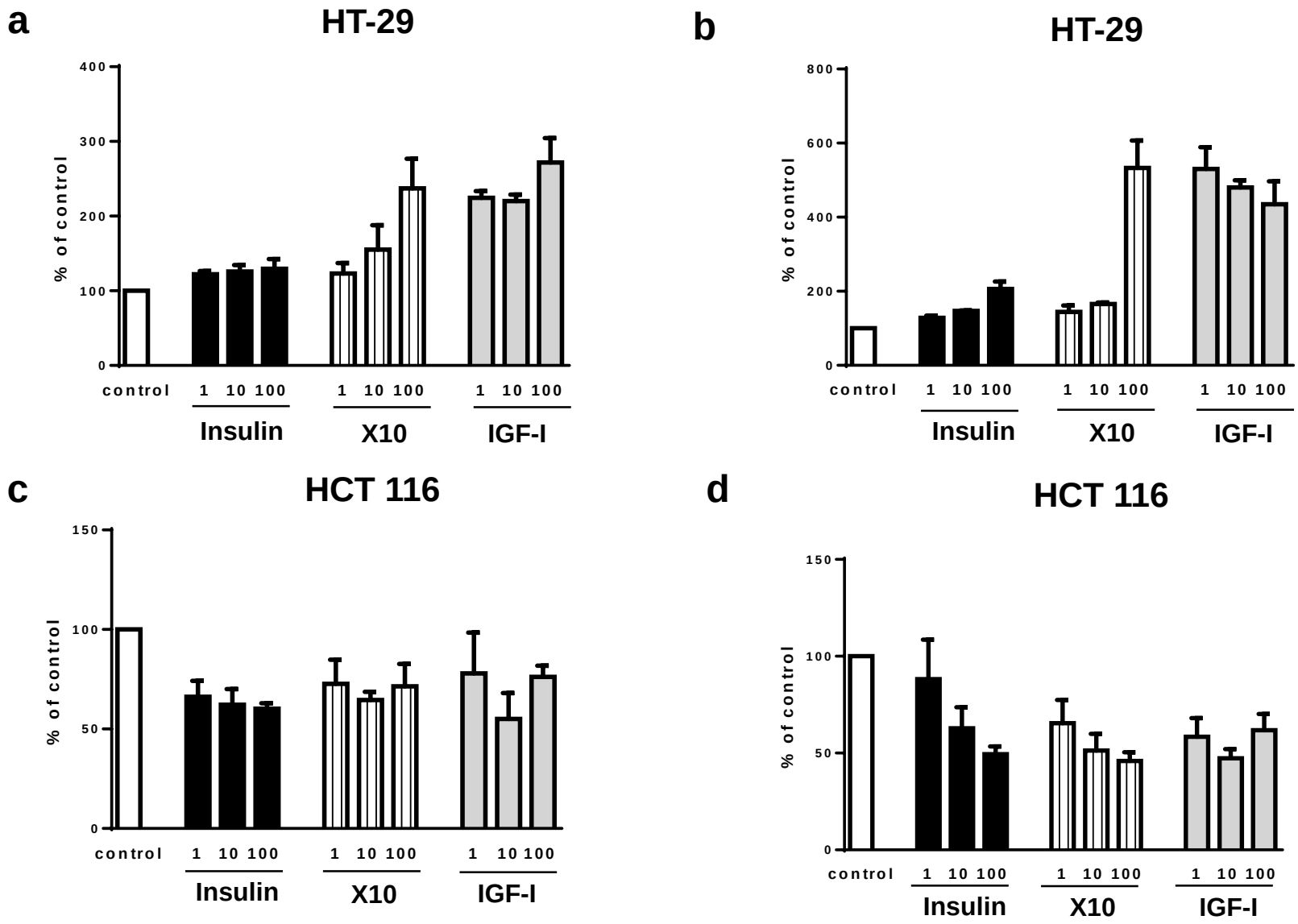
ESM Fig. 3 Densitometric analysis of Western blotting results presented in Fig. 4, ESM Fig. 1 and ESM Fig. 2. The potency of target phosphorylation by X10 and IGF-I after 15 min stimulation was calculated as the fold increase in target phosphorylation over that seen by insulin (the latter set at a value of 1). Note y-axis is logscale for pIGF-IRβ/pIRβ/IR for HT-29, COLO 205 and MCF7 cell lines.



ESM Fig. 4 Effect of long-term stimulations (6 hr and 18 hr) with insulin, X10 and IGF-I on activation of the ERK pathway. HCT 116, HT-29, COLO 205 and MCF7 cells were seeded in 6-well plates in complete medium and then starved in serum-free medium for 24 hr. Treatments with insulin, X10 and IGF-I for both time points used 3 concentrations (2, 10 and 50 nM). Whole-cell lysates were prepared and proteins were resolved by SDS-PAGE, transferred and immunodetected with the indicated antibodies. This experiment was performed three times and here is presented one example. CM-complete medium, SF-serum-free medium.



ESM Fig. 5 Dose-response curves for mitogenic activity of insulin, X10 and IGF-I in HCT 116, HT-29, COLO 205 and MCF7 cells determined by the MTT assay. Cells were seeded in 96-well plates in medium containing 0.5% FCS and 0.5% BSA. After 24 hr the agents (insulin, X10 and IGF-I) were added in the same medium in which the cells were seeded. 12 different concentrations of each agent were used (final concentrations were 0.001, 0.005, 0.05, 0.1, 0.3125, 0.75, 1.25, 2.5, 5, 10, 25 and 100 nM). The experiment lasted for 5 days and the agents were replaced every 48 hr. After 96 hr of incubation, MTT reagent was added to each well and incubated for a further 24 hr. After solubilisation of formazan product the absorbance was read at 560 nm. Each point in the figure represents the mean ± standard deviation of a triplicate determination. The experiment was performed three times.



ESM Fig. 6 Determination of caspase-3 and -7 activities after stimulation with insulin, X10 and IGF-I in HT-29 (panels a and b) and HCT 116 (panels c and d) cells. Cells were plated in 96-well plates in complete medium and allowed to adhere for 24 hr. For a further 24 hr the cells were maintained in serum-reduced (0.1% FCS) medium. All the cells were control treated or treated with 3 concentrations (1, 10 and 100 nM) of insulin, X10 and IGF-I for either 24 (panels **a** and **c**) or 48 hr (panels **b** and **d**). Caspase Glo 3/7 reagent was added at the end of each incubation time for one hour and luminescence was then measured. Each bar on the graph represents the mean ± standard deviation of a triplicate determination. The experiment was performed three times.