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Table 1 and 2 are available in the Supplementary Files section.

Kruppel-like-factors 4, 9 and 13 - modeling and modulating at the master regulatory borders of cell fate and cell death decisions in adrenocortical carcinoma

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Abstract

Transcription factors orchestrate and shape cellular identity during development. Krüppel-like factors (KLFs) are involved in stemness, proliferation and apoptosis, while the master regulator steroidogenic factor 1 (SF-1) regulates the differentiation and steroidogenic profile of the developing adrenal gland. The transcriptional regulatory networks governing the heterogeneity of adrenocortical carcinomas (ACC) are incompletely understood. Here, we report the existence of model-dependent differences in the expression of SF-1, KLF9 and KLF13 and their cross-regulation in three ACC in vitro models (TVBF-7, NCI-H295R and MUC-1). We demonstrate that KLF9 and KLF13 are responsive to dexamethasone and angiotensin II, but undergo dosage-dependent repression by SF-1. Combinatorial silencing of these three transcription factors revealed that KLF9/KLF13 counteract SF-1 activity on nuclear receptors (glucocorticoid receptor and androgen receptor) and metabolic genes (IRF1, RARA, and FOXO3) forming a cross-regulatory loop that might modulate steroidogenesis. Transitioning to 3D spheroid cultures uncovered a KLF4-dependent regulation of the DNA damage response, shifting the balance between cell survival and apoptosis. Together, our findings identify a novel transcriptional axis composed of KLF9/KLF13 and SF-1, alongside KLF4-mediated cell cycle regulation, offering potential pathways for target-based therapeutic interventions in ACC.

Introduction

The adrenal gland is a highly plastic and complex organ composed of two distinct tissue layers: the cortex (subdivided into zona glomerulosa, zona fasciculata, zona reticularis) and medulla. In a highly orchestrated manner, these distinct layers secrete specific hormones to mediate systemic endocrine responses to diverse physiological stimuli: the cortex produces mineralocorticoids, glucocorticoids, and androgens, while the medulla synthesizes and secretes catecholamines. The biological activities of glucocorticoids are mediated via both the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Both receptors recognize a spectrum of overlapping ligands, including cortisol, corticosterone, aldosterone, and progesterone, though their binding affinities and downstream biological consequences vary substantially (1). Upon ligand activation, the ubiquitously expressed GR targets a multitude of genomic loci within a given target cell (1). Consequently, fluctuations in circulating, adrenal-derived glucocorticoids exert profound, systemic effects on gene expression, ultimately shaping peripheral physiology and behavior. Given the vital nature of these adrenal-derived hormones, the adrenal cortex undergoes continuous regeneration and repair mediated by distinct stem cell populations to maintain its structural and functional integrity (2).

The described tissue architecture results from tightly controlled multi-cellular arrangements and migratory movements which begin early in the human fetus at around 30 days-post-conception when the adrenogonadal primordium becomes the first time visible as defined structure (3). Shortly after, the expression of the adrenal master transcription factor steroidogenic factor 1 (SF-1, encoded by NR5A1) is initiated and thereafter sustained in an autoregulatory feedback-loop. In parallel, controlled migratory events lead to the formation of the adrenal primordium followed by neural crest cell invasion which are the precursors for the later medullary component (3). Patterning principles and migratory events, as described above, are widely established in developmental processes and such frameworks are known to guide cells during development by providing information about positioning and cell fate (4). From such studies it is known that in the developing *Drosophila* embryo gradients of transcription factor appear in form of a well-controlled cascade (5). In this context it should be mentioned that SF1 is the homolog of Fushi Tarazu Factor I (FTZ-F1), a nuclear receptor that regulates the fushi tarazu homeobox and so-called pair-rule gene in *Drosophila melanogaster* where precisely timed regulation of enhancer activity defines the binary expression pattern of Fushi tarazu in the developing *Drosophila* embryo (6). Thus, for *Drosophila* development it is well appreciated that gap (including Krüppel), pair rule (including

fushi-tarazu) and segment-polarity genes control not only initiation and expression of essential pathways such as Wnt or hedgehog, but also HOX genes, which define specific segment identities and thereby differentiation. Of note, the human Krüppel-like factor family (KLF) is an evolutionarily conserved family of zinc-finger transcription factors that share homology with the *Drosophila melanogaster* protein Krüppel (7). Since their discovery, human Krüppel-like factor (KLF) family of transcription factors has been implicated in a broad spectrum of physiological processes. KLFs govern essential cellular functions such as proliferation, differentiation, apoptosis and pluripotency, and thereby have been linked to cellular reprogramming and regenerative medicine paradigms (7). Notably, KLF9 and KLF13 have been characterized as circadian transcription factors (8-10). KLF9 has also been identified as a critical feedforward regulator downstream of the glucocorticoid receptor (GR), facilitating oscillatory metabolic dynamics across peripheral tissues (11). However, whether these human Krüppel-like-factors play a regulatory role along with the human adrenal master regulator SF-1 remains unknown. Interestingly, emerging evidence indicated recently that the super-enhancer landscape of adrenocortical carcinoma (ACC) could be indeed shaped by an intricate interplay between SF-1/NR5A1 and KLF9 (12). Together with the role of these transcription factors during normal adrenal differentiation, these findings strongly suggest the involvement of an SF-1/KLF-related regulatory network in critical cell-fate decisions.

Materials and Methods

Cell culture conditions

Human ACC cell lines NCI-H295R, MUC-1 and TVBF-7 cells were cultured as previously published (13, 14). Briefly, NCI-H295R were cultured in DMEM-F12, 2% Ultrosor G serum substitute (Sartorius), Insulin (10 mg/ml), transferrin (5.5 mg/ml), and selenium (5 ng/ml) (ITS), penicillin (100 U/ml), streptomycin (100 µg/ml) (all from GIBCO Thermo Fisher Scientific). TVBF-7 were cultured in Advanced DMEM-F12 (GIBCO Thermo Fisher Scientific), 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), 2mM L-Glutamine, 2.5mg/ml Amphotericin B (all from GIBCO Thermo Fisher Scientific). MUC-1 were cultured in Advanced DMEM-F12, 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) (all from GIBCO Thermo Fisher Scientific).

Stimulation experiments and RNA-silencing

Detailed information regarding the RNAseq dataset, culture conditions including specific compounds, cell numbers and incubations times can be found here (12). The si-RNA mediated knockdown experiments were conducted in triplicate. Cells were plated in 6-well plates and transfected with Lipofectamine RNAiMAX Transfection Reagent (13778100, Thermo Fisher Scientific, MA, USA) in Opti-MEM (7001587, Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. ON-TARGET siRNA SMARTPool from Horizon Discovery (Cambridge, UK) were used at a concentration of 40pmol (single transfection) or 20pmol each (double transfection): human KLF9 (L-011223-00-0005), human KLF13 (L-006994-00-0005), human NR5A1 (L-003429-00-0005) and no-target control (D-001810-10-05). Cells were harvested 48 hours after transfection and processed for real-time PCR. For the stimulations experiments with dexamethasone (D) and angiotensin II (A), 1 x 10⁶ cells were seeded in 6-well plates and incubated for 24 hours with the different concentrations: 1mM (1D), 10nM (10D), 100nM (100D) or 50mM (50A). Cells were harvested and processed for real-time PCR as indicated below.

Quantitative real-time PCR

Lysates were homogenized using the QIAshredder (79656, QIAGEN, Netherlands) prior to RNA purification. Subsequently, RNA was extracted using the RNeasy®Mini Kit (79656, QIAGEN, Netherlands) and treated with the DNase kit (TURBO DNA-free™ Kit, Thermo Fisher Scientific, USA) according to manufacturer's instructions. The quality and quantity of the RNA was analyzed using a NanoDrop® spectrophotometer (Thermo Fisher Scientific, USA). Subsequently, 500 ng of total RNA was reverse transcribed using the RevertAid Reverse Transcription Kit (K1691, Thermo Fisher Scientific, US) following the provided random hexamer protocol. The reaction was carried out in the Biometra TRIO (Analytik Jena, Germany) with 10 minutes at 25°C, 60 minutes at 42°C and 10 minutes at 70°C. The cDNA was stored at -20°C until further use. Real-time PCR primer information is provided in Table 1.

Culturing, fixation and paraffin embedding of spheroids

Spheroids were formed by plating 25000 cells per well in Sphericalplates 5D (Kugelmeiers, Erlenbach, Switzerland) and culturing them over 6 days using the same culture conditions as above. On day 7, the spheroids were treated with mitotane for 72 hours. General plating procedure, fixation and paraffin embedding have been previously described in detail (15). Briefly, spheroids were harvested and fixed for 10 minutes at room temperature with 4% paraformaldehyde (Sigma Aldrich) and coated in Histogel (Thermo Fisher Scientific). Spheroids were then dehydrated and processed for paraffin embedding using the Excelsior AS tissue processor (Thermo Fisher Scientific) overnight. Spheroids were embedded using Myr embedding center (Especialidades Médicas MYR) and 4µm sections were prepared (microtome Leica RM2255, Leica Biosystems).

Immunofluorescence

Sections were deparaffinized, subjected to antigen retrieval in sodium citrate buffer, blocked with 10% goat serum and incubated overnight at 4 °C with primary antibody. The following day, secondary antibody conjugated to Alexa-fluorophores was applied for 1 hour at room temperature. In the case of multiple staining, primary antibodies were applied sequentially. Primary and secondary antibodies are listed in Table 2. DAPI (D9542, Sigma Aldrich, USA) was used at final concentration 1µg/mL. Aqueous

non-fluorescing mounting media Hydromount (HS-106, National Diagnostics, USA) was used to coverslip for the preservation of the sections. Images were acquired with a Zeiss Axio Observer 7 equipped with the AxioCam 305 mono, using ZEN 10 software (Carl Zeiss, Oberkochen, Germany)s.

Statistical analysis

All statistical analysis and graphical representation of the data were conducted using GraphPad Prism software (version 10, GraphPad Software, La Jolla, CA, USA). For statistical comparison, a one-way or two-way ANOVA was performed, followed by Dunnett or Bonferroni's post test. Results are presented as mean $\pm$ SEM. Statistical significance was set at $P < 0.05$ and indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) in all figures, if not stated otherwise.

Results

NR5A1 and KLF9 were recently identified as master regulators of the ACC super enhancer landscape (11). More detailed analysis of this RNAseq dataset revealed that KLF9 is differentially expressed amongst the three pre-clinical ACC models, with highest expression in MUC-1 and lowest in TVBF-7 ((11) and Figure 1). High baseline KLF9 levels correlated with lower SF1 expression in MUC-1, whereas low KLF9 levels corresponded to elevated SF-1 expression in TVBF-7. The therapeutic gold standard mitotane inversely modulated expression of KLF9 and SF-1 in TVBF-7 cells, indicating a possible inverse relationship between these two factors. Since KLF9 belongs to a superfamily of transcription factors, we expanded our analysis to encompass all 18 members. The KLF gene family displayed distinct baseline variations and dynamic drug-dependent alterations, highlighting the plasticity of the transcriptional network of these in vitro ACC models. Intriguingly, although KLF13 can functionally substitute for KLF9 in certain settings, baseline KLF13 expression positively correlated with SF-1 (highest in TVBF-7, lower in NCI-H295R/ MUC-1) rather than with KLF9. Furthermore, unlike SF-1 and KLF9, KLF13 expression was not significantly altered by mitotane in any model, identifying KLF13 as a distinct transcription factor of interest. Finally, we identified KLF4 and KLF6 as the only members of the Krüppel family whose expression was induced by mitotane relative to baseline across all three models. Basal KLF4 expression mirrored that of KLF9 (low in TVBF-7, higher in NCI-H295R and MUC-1), whereas KLF6 expression remained comparable across all models. Thus, we explored further the relevance of KLF9, KLF13 and KLF4 in the three ACC cell lines.

Cell cycle regulation downstream of KLF4

Given the established role of KLF4 in cell cycle progression and the DNA damage response (DDR) and together with the observation of KLF4 induction upon mitotane in our ACC models, we investigated markers of cell cycle and DDR in untreated and mitotane-treated spheroids (Figure 2, 3). KLF4 immunopositivity was strongest in TVBF-7 spheroids and lowest in NCI-H295R and MUC-1 spheroids (Figure 2A), conversely to the results of RNAseq dataset. To understand the regulation of KLF4 at the protein level, we analyzed the expression of Von Hippel-Lindau (VHL), a key E3 ubiquitin ligase that targets KLF4 for proteasomal degradation (16). VHL exhibited a reciprocal expression pattern relative to KLF4 across the three models, suggesting higher turnover of KLF4 protein in MUC-1 and NCI-H295R

than TVBF-7 spheroids (Figure 2A). To evaluate the activity of KLF4, we analyzed its transcriptional targets, p53 and p21, which induce cell cycle arrest to facilitate DNA repair (17-19). MUC-1 spheroids displayed the highest baseline immunopositivity for p53 (Figure 2B), whereas robust p21 signal was evident in both TVBF-7 and MUC-1, but absent in NCI-H295R spheroids (Figure 2C). Therefore, KLF4 might actively regulate cell cycle in the metastasis-derived models, MUC-1 (13) and TVBF-7 (20), but not in the primary-derived NCI-H295R. To understand if KLF4 and its downstream targets disrupt the progression of the cell cycle, we analyzed the subcellular localization of cyclin-dependent kinases (CDK). Although levels of Cyclin D1, a target of KLF4, were unaffected (Figure 2D), CDK6 was predominantly nuclear in TVBF-7 (Figure 3B), whereas CDK4 (Figure 3A) and specifically CDK1/2 (Figure 3C) were enriched in NCI-H295R and MUC-1 spheroids. Furthermore, though mitotane affected transcription of KLF4, the subcellular localization of KLF4 and the other cell cycle components was unchanged by mitotane treatment of the spheroids. However, mitotane induced the accumulation of phospho-H2AX, a hallmark of double-stranded DNA breaks (21) specifically in TVBF-7 spheroids (Figure 3D). In contrast, mitotane increased immunopositivity for cleaved caspase-3 explicitly in NCI-H295R as compared to the metastatic models (Figure 3E). These data suggest that KLF4 might support cell cycle arrest in the metastatic models (TVBF-7 and MUC-1) as a protective mechanism, while NCI-H295R spheroids might preferentially undergo apoptosis in response to cellular stress.

KLFs regulation in the context of steroidogenesis

Aside from cell cycle control, KLFs and glucocorticoids engage in bi-directional cross-talks and more specifically KLF9 has been shown to mediate the effects of GR and cortisone on gene expression and cellular metabolism (22). To explore this interplay in ACC models, we evaluated how dexamethasone regulates KLF9 and KLF13, and the reciprocal regulation of GR by these two KLFs. Increasing concentrations of dexamethasone significantly upregulated KLF9 transcript by 3-fold in MUC-1 but not NCI-H295R cells, while KLF13 levels were unaffected in both models (Figure 4). To extend our data to the regulation of the mineralocorticoid biosynthesis, we also evaluate the effects of angiotensin II on KLF9 and KLF13 expression (Figure 4). Angiotensin II has been additionally shown to regulate both the mineralo- and glucocorticoid pathway in NCI-H295R cells (23). Interestingly, angiotensin II treatment significantly suppressed KLF13 expression specifically in NCI-H295R cells but did not affect KLF9

expression in either cell line. Since SF-1 is the master regulator of steroidogenesis, we assessed its transcription under the different conditions. SF-1 expression was upregulated by dexamethasone in both NCI-H295R and MUC-1 but remained unaffected by angiotensin II. Therefore, KLF9 and KLF13 might be involved in the feedback regulation of steroidogenic transcriptional programs similarly to SF-1.

Next, we explored through a series of siRNA knockdown experiments whether KLF9 and KLF13 regulate expression of GR (NC3C1, Figure 5A) as well as that of another nuclear receptor, the androgen receptor (AR, Figure 5B). In NCI-H295R cells, single and combined knockdown of KLF9 and KLF13 significantly suppressed expression of both GR and AR. However, in TVBF-7, single and combinatorial silencing of KLF9 and KLF13 de-repressed AR without altering GR levels, while expression of both nuclear receptors was largely unchanged in MUC-1. Similar experiments were performed with siRNA directed against SF-1/NR5A1. Expression of GR and AR was significantly induced following knockdown of SF-1 in TVBF-7, while their transcription was unaffected in the other two cell lines. Interestingly, co-transfection of siNR5A1 with either siKLF9 or siKLF13 blunted the effects of SF-1 knockdown on GR and AR, suggesting possible interaction of these transcription factors on promoters of nuclear receptors. The interactions between SF-1/KLF9/KLF13 were not detected to the same extent in NCI-H295R and MUC-1, suggesting that the downstream actions of KLF9 and KLF13 are context- and dosage-dependent.

To better characterize the relationship between KLF9, KLF13 and SF-1, we analyzed their expression under the same siRNA-mediated knockdown conditions. Transcription of both KLF9 (Figure 5C) and KLF13 (Figure 5D) was significantly upregulated upon knockdown of SF-1 in TVBF-7 and MUC-1. The de-repression of KLF9 was most pronounced in the high-SF-1 expressing TVBF-7 cell line, consistent with the gene dosage effect of SF-1. Additionally, we detected evidence of mutual positive regulation between KLF9 and KLF13 in all three cell lines. Specifically, KLF13 might sustain KLF9 expression (evidenced by blunted KLF9 levels in the siNR5A1+siKLF13 condition relative to siNR5A1 alone) and KLF9 reciprocally might maintain KLF13 levels (siNR5A1+siKLF9 vs. siNR5A1 alone). Conversely, single or combined silencing of KLF9 and KLF13 (siKLF9+siKLF13) did not alter SF-1 expression in any of the models tested (Figure 5E). These data indicate that KLF9 and KLF13 are actively repressed by SF-1. Together with the results from hormone treatment and nuclear receptor regulation, we propose

that KLF9 and KLF13 might operate in a positive feedback loop while opposing the effects of SF-1 in the regulation of steroidogenesis.

Regulation of intracellular metabolism

Steroidogenesis is inherently dependent on lipid and cholesterol metabolism, as cholesterol is the precursor for steroid hormones and is stored in lipid droplets (24). We investigated expression of regulators of lipid metabolism recently identified to correlate with KLF9 and SF-1 (11) in response to siRNA knockdown of KLF9, KLF13 and SF-1. Knockdown of SF-1 significantly upregulated expression of IRF1 (Figure 6A), RARA (Figure 6B), and FOXO3 (Figure 6C) in both TVBF-7 and MUC-1. Similarly to the regulation of nuclear receptors, the combination of si-NR5A1 with either si-KLF9 or si-KLF13 blunted the effects of SF-1 single knockdown, suggesting an opposing regulatory action exerted by KLF9 and KLF13. The maximum upregulation of these target genes reached 2- to 3-fold in TVBF-7 compared to a 1.5-fold increase in MUC-1, in line with the higher baseline dosage of SF-1 in TVBF-7. Interestingly, KLF9 knockdown did not affect target genes in any model, whereas KLF13 knockdown significantly induced FOXO3 expression exclusively in MUC-1, underscoring that the functions of KLF9 and KLF13 are not interchangeable. Finally, SREBF2 was upregulated following KLF13 knockdown in TVBF-7 only (Figure 6D). These findings demonstrate that KLF9 and KLF13 might counteract the SF-1 mediated repression of genes involved in intracellular lipid metabolism.

Overall, our data indicated that KLF9 and KLF13 might participate in regulating cholesterol and lipid metabolism alongside steroidogenesis. We therefore investigated whether these regulatory pathways converge on the G0/G1 switch gene 2 (G0S2), a potent promoter of lipid storage and inhibitor of steroidogenesis (25, 26). Analysis of our RNAseq dataset revealed that G0S2 transcript was only detectable and could be dynamically modulated in MUC-1 as compared to the other cell lines (Figure 7A). Knockdown of KLF9 significantly upregulated G0S2 expression (Figure 7B) whereas SF-1 knockdown had the opposite effect (Figure 7C). Given that KLF9 suppresses G0S2 in MUC-1, we next utilized siRNA mediated knockdown of G0S2 to investigate its implications for steroidogenesis (Figure 7D). Silencing of G0S2 resulted in a significant upregulation in the transcription of CYP17A1 (Figure 7E) and HSD3B2 (Figure 7F), the rate-limiting drivers of glucocorticoid and androgen biosynthesis (27,

28). Therefore, by converging on G0S2, this dataset provides further support that KLF9 is involved in the regulation of steroidogenesis.

SF-1 / KLF / HOX axis

From an evolutionary perspective, the interactions between SF-1 and KLFs are reminiscent of the embryonic patterning in *Drosophila melanogaster* involving FTZ-F1 (the ortholog of SF-1/NR5A1), fushi tarazu (Ftz, the orthologue for Hox) and Krüppel (29, 30). We previously demonstrated that the central HOX cluster members, specifically HOXA7 and HOXB7, exhibit a highly dynamic and specific responsiveness to forskolin/cAMP signaling across ACC models (31). However, the upstream transcriptional machinery driving this responsiveness remained uncharacterized. Therefore, we used siRNA-mediated knockdown to understand the transcriptional regulation of HOXA7. While KLF9 did not affect HOXA7 levels (data not shown), knockdown of SF-1 caused a significant increase in HOXA7 expression in TVBF-7 and MUC-1. These preliminary results suggest that KLF9, KLF13 and HOXA7 undergo repression by SF-1 in favor of steroidogenesis in ACC models.

Discussion

Krüppel-like factors have been implicated in the control of a vast array of biological processes ranging from cell fate to cellular metabolism (7). Depending on the cellular context and recruitment of co-factors, KLFs can act as either transcriptional activators or repressors. Although the role of KLFs in ACC is largely unexplored, previous reports have identified KLF4, KLF9 and KLF13 as repressors of steroidogenic enzymes, with KLF9 specifically characterized as a direct target of GR (32, 33). Here, we show that these three KLFs participate in the regulation of steroidogenesis and cell cycle in three ACC cell lines. Specifically, our knockdown experiments in TVBF-7 and MUC-1 revealed a coordinated, inverse transcriptional feedback loop: silencing of the lineage-specific master regulator SF-1 (34, 35) triggered a robust upregulation of KLF9 and KLF13. Therefore, we provide here a functional foundation for our characterization of the ACC super-enhancer landscape, which identified SF1 and KLF9 as the core antagonistic drivers balancing the "high steroid" versus "low steroid" phenotypes (12). Furthermore, the functional pairing of a lineage regulator with organ-specific members of the Krüppel family provides a mechanism by which adult tissues preserve their discrete identities (36-38). This same master regulator/KLF pattern is conserved across other organs, such as, for example, between PDX1 and KLF10/KLF11 in the pancreas (39). Crucially, KLF10 and KLF11 are paralogs of KLF9/KLF13 that coordinate glucose/energy-responsive transcriptional networks, acting in similar fashion to the regulation of the steroidogenic axis as we show here for KLF9/KLF13 (40, 41).

Important insight into the role of KLFs in ACC emerges from the antagonism between KLF9/KLF13 and SF-1. We show that expression of KLF9 and KLF13, alongside that of the lipid metabolic transcription factors, is actively suppressed by SF-1. Since KLF9 and KLF13 conversely blunt the effects of SF-1 on gene promoters, one possibility is that SF-1 actively suppresses these KLFs to maintain its transactivation of steroidogenesis. Consistent with published reports (42-44), this transcriptional repression is dependent on SF-1 gene dosage as the most profound de-repression occurred in TVBF-7 cells, the model with the highest baseline SF-1 expression. Accordingly, KLF9, KLF13 along with the key regulators of lipid metabolism (IRF1, RARA, and FOXO3) were significantly de-repressed by 2- to 3-fold in TVBF-7 cells upon SF-1 knockdown, compared to a modest 1.5-fold upregulation in MUC-1 cells. While KLF13 can compensate for KLF9 in certain oncogenic contexts (45), our combinatorial

siRNA experiments suggest that they are not redundant in ACC. Instead, KLF9 and KLF13 appear to positively influence each other's expression in the three ACC cell lines. Since a highly active steroidogenic phenotype is associated with aggressive ACC (46), understanding the functional consequences of altering the ratio of KLF9/KLF13 to SF-1 may uncover novel therapeutic vulnerabilities.

KLFs are fine-tuners of transcriptional regulation that integrate multiple hormonal inputs to coordinate a robust cellular response (33). Their relationship with nuclear receptors is complex and bidirectional, as they can act downstream to mediate their effects, directly modulate expression of the receptors, or be regulated by the nuclear receptors themselves (22). For example, dexamethasone can stimulate KLF9 expression, while KLF9 subsequently inhibits glycolysis downstream of the cortisol/GR axis to maintain redox homeostasis and cell survival (11, 47). In alignment, we show that dexamethasone stimulates KLF9 expression in MUC-1, while both KLF9 and KLF13 maintain expression of GR and AR. Glucocorticoids are well-documented drivers of immunosuppression within the tumor microenvironment (48). In ACC, a low steroidogenic phenotype correlates with significantly higher levels of immune infiltration and improved patient survival (49). By modulating expression of nuclear receptors and antagonizing SF-1 activity, KLF9 and KLF13 might negatively regulate steroidogenesis, potentially offering a therapeutic strategy to reprogram the classical "immune cold" phenotype of aggressive ACC (50).

The cross-regulatory interaction between KLF9/KLF13 and SF-1 extends beyond steroidogenesis to encompass the lipid metabolic network. IRF1 is a driver of de novo lipid synthesis (51), RARA is a regulator of intracellular lipid droplet dynamics (52, 53) and FOXO3 mitigates oxidative stress and regulates glucose/lipid flux (54). By opposing the SF-1-mediated suppression of these genes, KLF9 and KLF13 might shift cellular metabolism away from steroidogenesis and towards lipid storage. Interestingly, KLF13 was the only transcription factor that inhibits SREBF2 (sterol-regulatory element-binding transcription factor 2), the master regulator of cholesterol biosynthesis (55, 56). The strongest interaction between KLF9/KLF13 and SF-1 was observed in TVBF-7, in line with their steroidogenic profile and in contrast to MUC-1 which are characterized by elevated triacylglycerols and reduced

cholesterol esters (14, 57, 58). Consistent with a central role for lipid metabolism in MUC-1, we show that G0S2 expression was specifically restricted to this cell line. G0S2 inhibits intracellular lipid catabolism and represses steroid biosynthesis enzymes to promote accumulation of lipid droplets (25, 26). Accordingly, we observed a marked upregulation of CYP17a1 and HSD3B1 following si-RNA mediated knockdown of G0S2. Conversely, G0S2 was undetectable in TVBF-7 and NCI-H295R, in line with their C1A classification and reflecting the hypermethylation of G0S2 in recurrent and aggressive ACC (12, 59). The suppression of G0S2 by KLF9 in MUC-1 might represent a potential mechanism by which these cells transition toward a more aggressive phenotype, as underscored by their capacity to secrete cortisol and metastasize in vivo (13, 60). In line with these findings, although MUC-1 is classified as C1B molecular subtype, treatment with the steroidogenic inducer forskolin reprograms these cells towards a C1A phenotype in vitro (12). Given that altered lipid metabolism is increasingly recognized as a key driver of tumor cell survival, migration, and metastasis (61, 62), further studies are highly warranted to delineate the plasticity of these metabolic pathways in ACC.

Beyond the regulation of cellular metabolism and steroidogenesis by KLF9 and KLF13, we also provide evidence that KLF4 and its downstream targets might be involved in cell cycle progression in the three ACC cell lines. KLF4 regulates cell cycle and stemness, by inducing p21-dependent p53 activation and suppressing cyclin D1 to prompt cell cycle arrest (17-19, 63-65). In our dataset, KLF4 emerged as one of two KLF members positively regulated by mitotane, with protein levels highest in TVBF-7 compared to NCI-H295R and MUC-1 spheroids. Although KLF4 transcript increased in response to mitotane potentially as a protective response across all three lines, this upregulation was not reflected in our immunomicrographs. This discrepancy is likely explained by the rapid, ubiquitin-mediated turnover of KLF4 protein (66), a hypothesis supported by the high expression of the Von Hippel-Lindau tumor suppressor (VHL) and an elevated KLF4 RNA-to-protein ratio in MUC-1 cells. VHL targets specific proteins for proteasomal degradation and directly modulates steady-state KLF4 levels; notably, VHL loss is known to induce cell cycle arrest and senescence downstream of p21/p27 (16, 67-69). Consistent with the oncogenic potential of KLF4 and the survival-promoting role of the DNA damage response (70, 71), p21 and p53 were stabilized in TVBF-7 and MUC-1 while apoptosis was initiated in NCI-H295R spheroids by mitotane. These results are in line with previous findings that MUC-1 are resistant to mitotane and sustain proliferation (13, 58). Since nuclear CDK1/2 predominated in MUC-1

while nuclear CDK6 was specific to TVBF-7 spheroids, MUC-1 cells may attempt to bypass cell cycle arrest induced downstream of KLF4. Although additional experiments are necessary to fully understand the role of KLF4 in this regard, CDK1/2 can substitute for earlier CDKs to drive cell cycle progression (72, 73). Additionally, KLF4 can modulate the epithelial-mesenchymal transition (EMT) via p53-dependent mechanisms (70). Consequently, TVBF-7 and MUC-1 might employ distinct mechanisms to maintain proliferation and resist mitotane, whereas NCI-H295R spheroids shift toward apoptosis in the absence of KLF4 activation. Although KLF4 can function as a tumor suppressor or oncogene in nuclear receptor-driven tumors (45), activation of KLF4 might have a protective effect in ACC.

KLFs are core components of human super-enhancer landscapes, where they establish and maintain somatic cell identity (74). Furthermore, HOX activity is required to initiate SF-1 transcription during adrenal development (75). By integrating HoxA7 into our analysis and identifying the asymmetric relationship between SF-1 and KLF9 / KLF13 / HOXA7, we show that an adult endocrine organ continues to utilize highly conserved, ancestral circuits to safeguard cellular identity. This framework provides mechanistic context to our previous findings. While we previously demonstrated that forskolin, a potent stimulator of ACTH/cAMP signaling and SF-1, repressed HOXA7/HOXB7 across ACC models, its transcriptional regulation was unknown (31). By now demonstrating that HOXA7 which, together with HOXB7, is an evolutionary close relative of the well-known drosophila fushi tarazu gene, is repressed by SF-1, we show that adrenocortical cells might still exploit this highly conserved frameworks. We propose that the SF-1 / KLF9 / KLF13 / HOX axis might govern the balance between undifferentiated progenitor and steroidogenic/differentiated states, offering a promising therapeutic rationale for targeting aggressive, de-differentiated carcinomas.

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Declaration of Interests

The authors declare no competing interests

Figure Legends

Fig. 1. Transcription analysis of SF-1 and KLF family based on RNAseq dataset. TVBF-7, NCI-H295 and MUC-1 are colored in orange, green and blue, respectively. Drug treatments are depicted from left to right for each model as untreated controls (C), JQ1, BET inhibitor (J), THZ-1, CDK7 inhibitor (T), mitotane (M), T+M, forskolin (F), F+M, F+T. Statistical analysis using ANOVA with Dunnett's multiple comparison to control. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Fig. 2. Subcellular localization of KLF4 and its targets. Representative immunomicrographs for double staining for KLF4 and VHL (A), p53 (B), p21 (C) and cyclin D1 (D). Nuclei were counterstained with DAPI. Images are from untreated and mitotane-treated spheroids from TVBF-7, NCI-H295R and MUC-1. Scale bar 20um.

Fig. 3. Subcellular localization of cell cycle proteins. Representative immunomicrographs of CDK4 (A), CDK6 (B), CDK1/2 (C), phospho-H2AX (D) and cleaved caspase-3 (E). Nuclei were counterstained with DAPI. Images are from untreated and mitotane-treated spheroids from TVBF-7, NCI-H295R and MUC-1. Scale bar 20um.

Fig.4. siRNA-mediated silencing in ACC models. Cells were treated with single or a combination of siRNAs against KLF9, KLF13 and NR5A1. Relative expression of NR3C1 (A) and AR (B), KLF9 (C), KLF13 (D) and NR5A1 (E), normalized to GAPDH in TVBF-7 (orange), NCI-H295 (green), and MUC-1 (blue) cells.

Fig. 5. Regulation of gene expression by dexamethasone and angiotensin II. NCI-H295R (A) and MUC-1 (B) cells were treated with increasing concentrations of dexamethasone (D) and angiotensin II (A). In the x-axis the concentrations are as follows: 1mM (1D), 10nM (10D), 100nM (100D) and 50mM (50A). Relative expressions of KLF9, KLF13 and NR5A1 were measured by real-time RT-PCR and normalized to GAPDH. Statistical analysis using ANOVA with Dunnett's multiple comparison to control. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Fig.6. siRNA-mediated silencing of genes involved in cellular metabolism in TVBF-7, NCI-H295 and MUC-1. Cells were treated with single or combination of siRNAs against KLF9, KLF13 and NR5A1. Gene expression was measured by real-time RT-PCR for IRF1 (A), RARA (B), FOXO3 (C) and SREBF2

(D). Results were normalized to GAPDH. Statistical analysis using ANOVA with Dunnett's multiple comparison to control. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Fig. 7. Gene expression analysis from RNAseq dataset for G0S2 in TVBF-7, NCI-H295 and MUC-1 (A). Relative expression was measured as indicated by real-time RT-PCR following treatment with siRNAs against KLF9 (B), SF-1 (C) and G0S2 (D-F). Statistical analysis using ANOVA with Dunnett's multiple comparison to control. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Fig. 8. siRNA-mediated silencing of Hox genes. Cells were treated with siRNA against NR5A1 and relative expression of HOXA7 was normalized to GAPDH in TVBF-7 (A), NCI-H295R (B) and MUC-1 (C). Statistical analysis using Ttest. * $p < 0.05$, ** $p < 0.01$.

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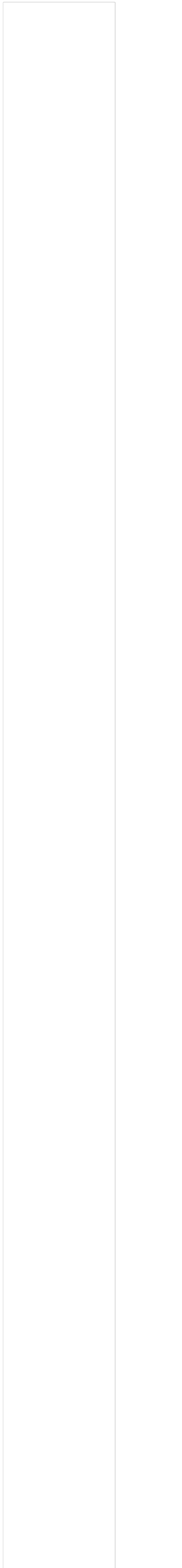
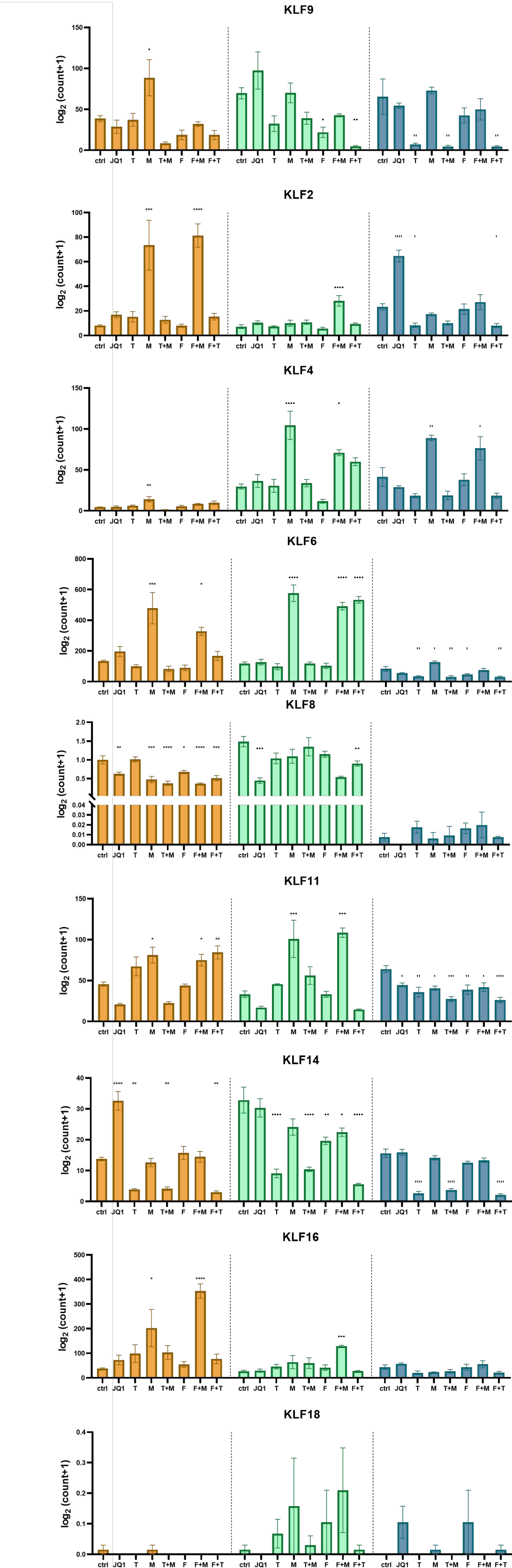
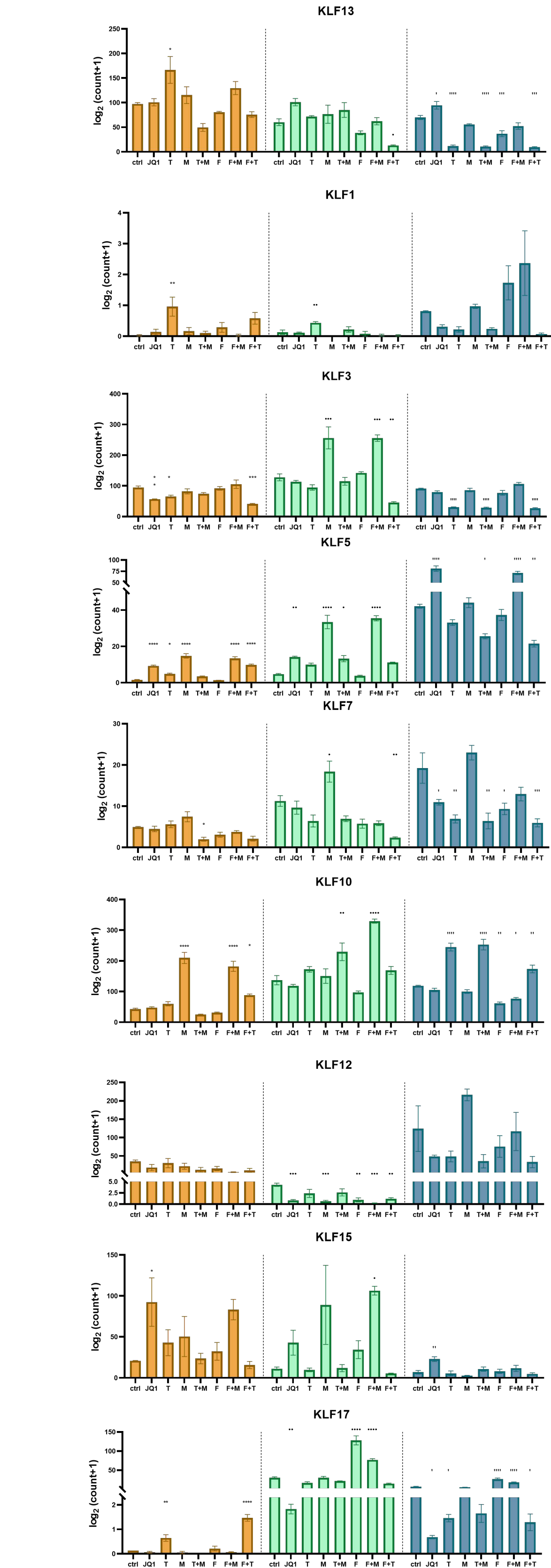
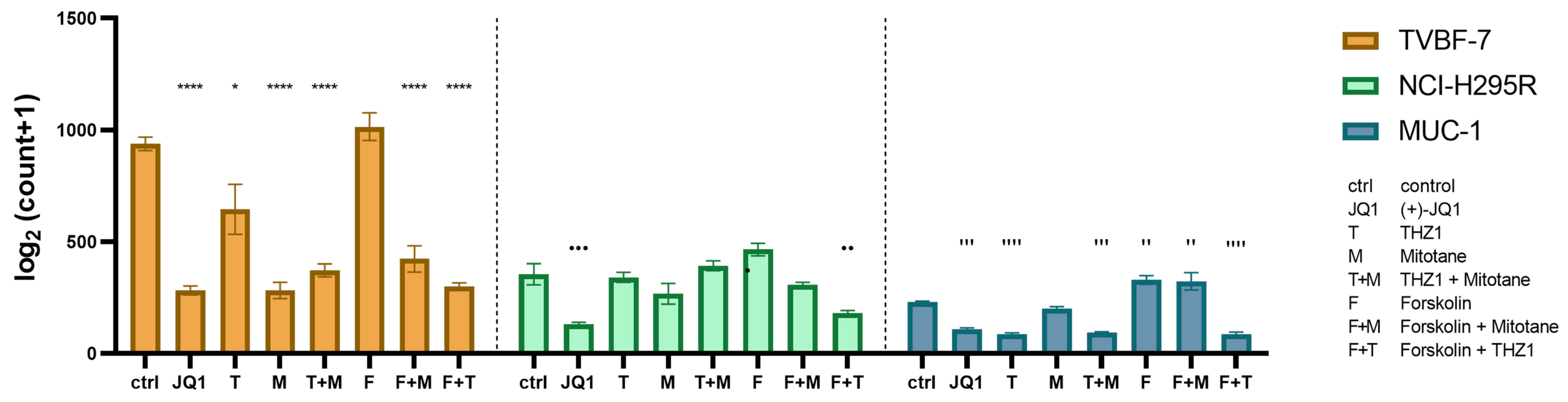
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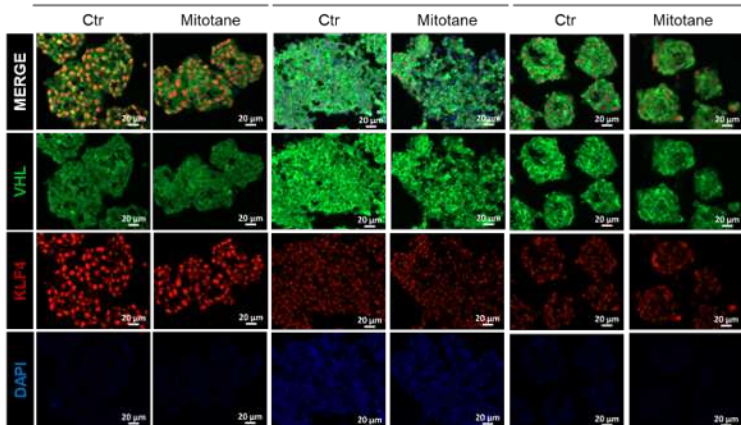
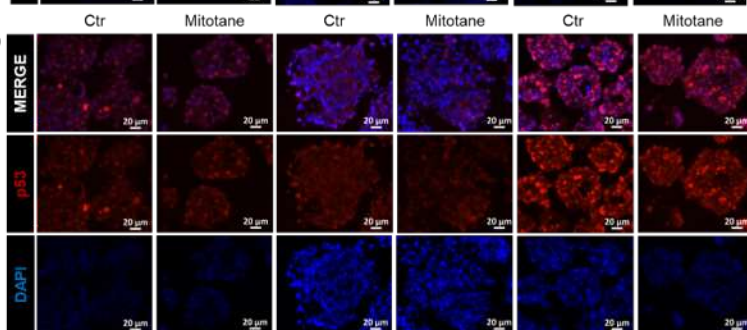
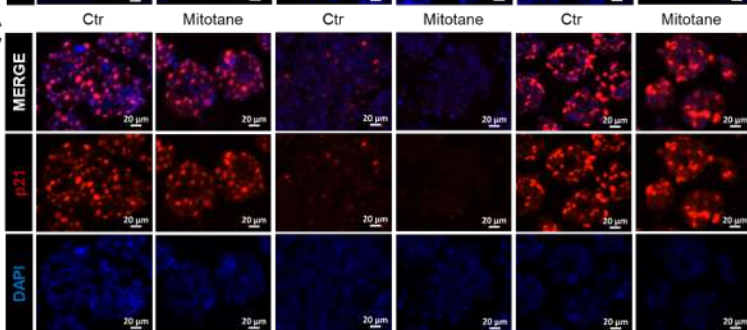
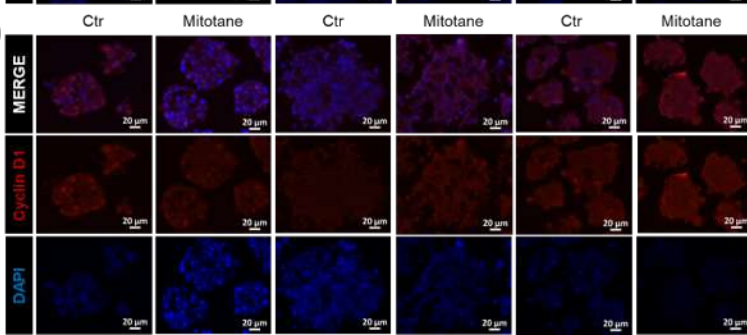
NR5A1



TVBF-7

NCI-H295R

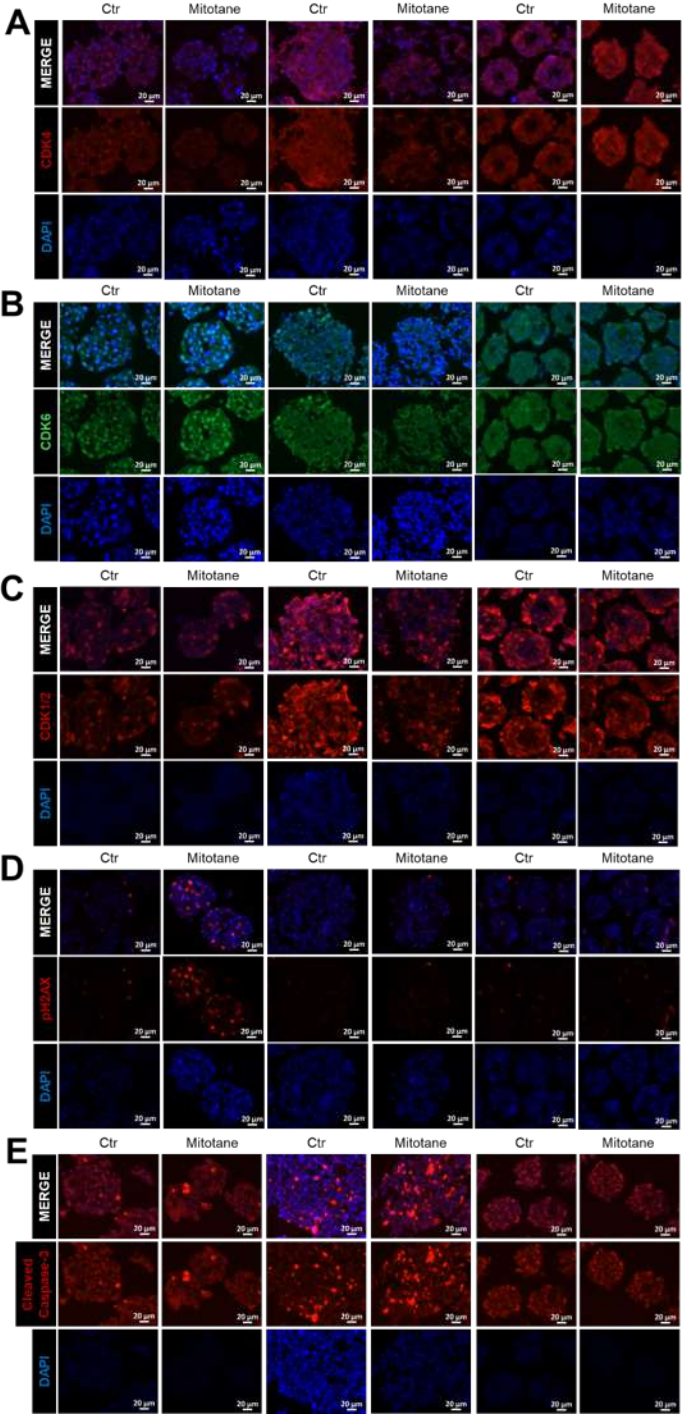
MUC-1

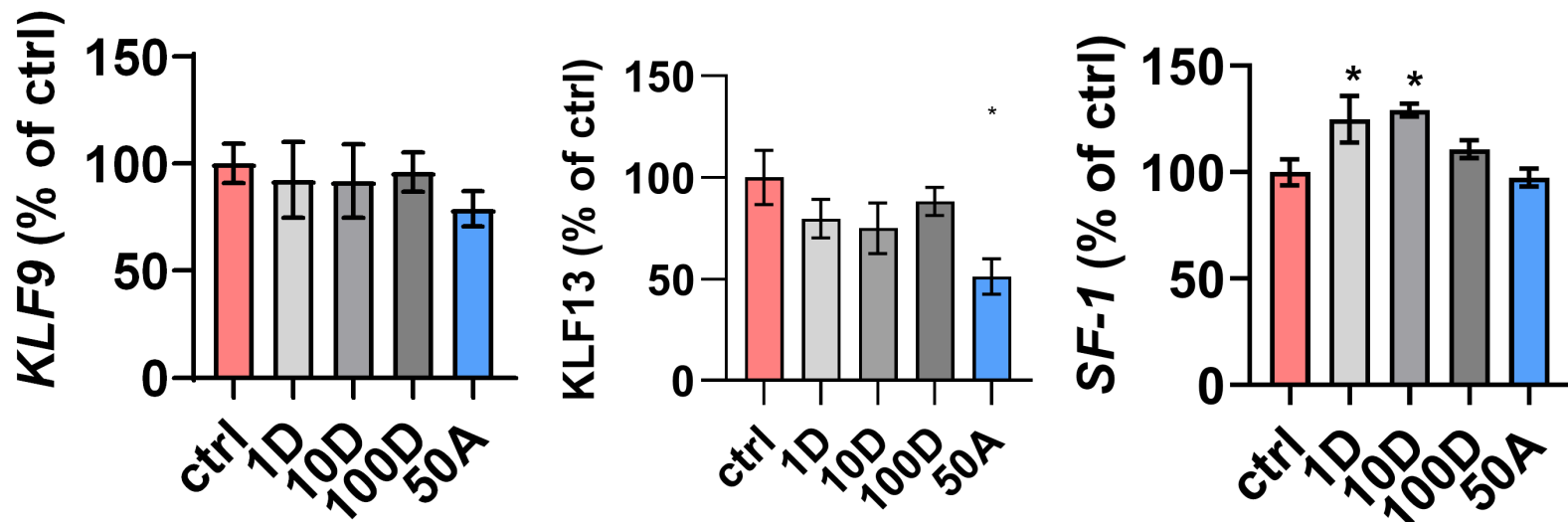
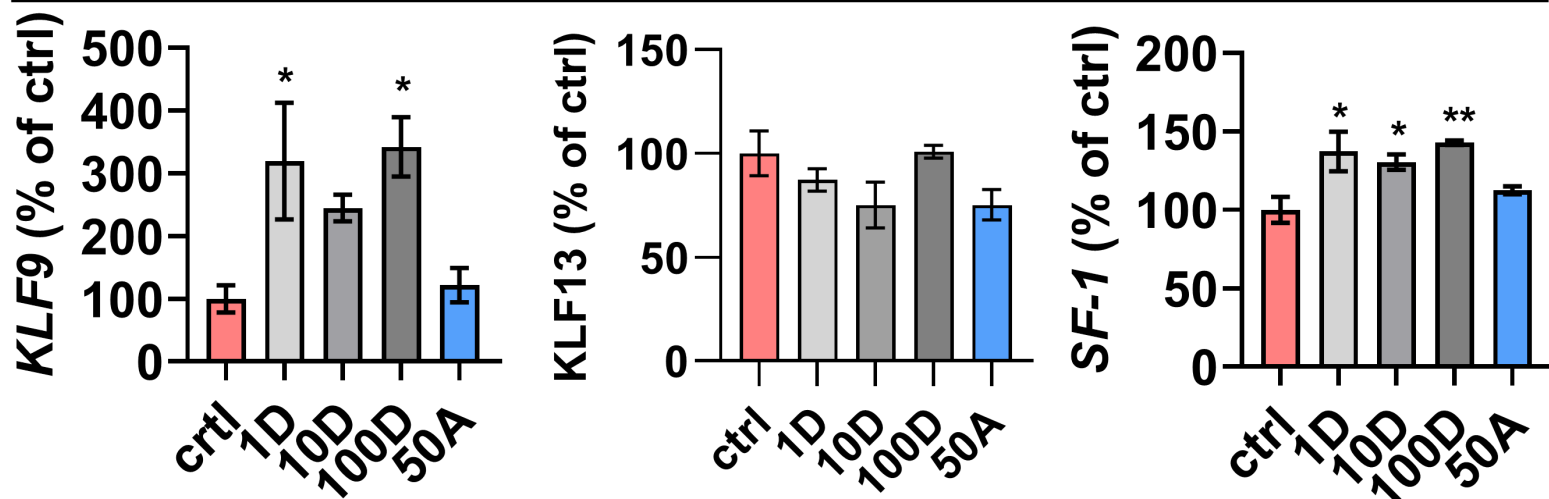
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TVBF-7

NCI-H295R

MUC-1



A**NCI-H295R****B****MUC-1**

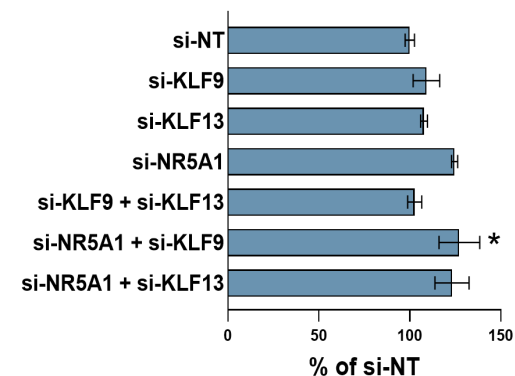
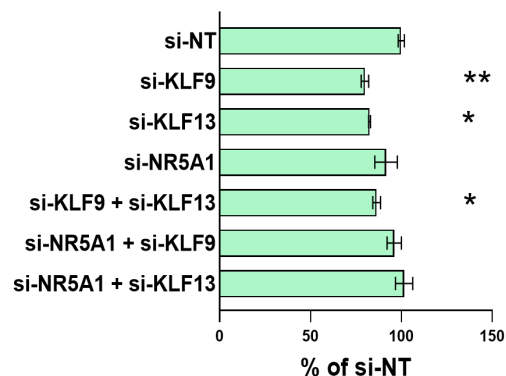
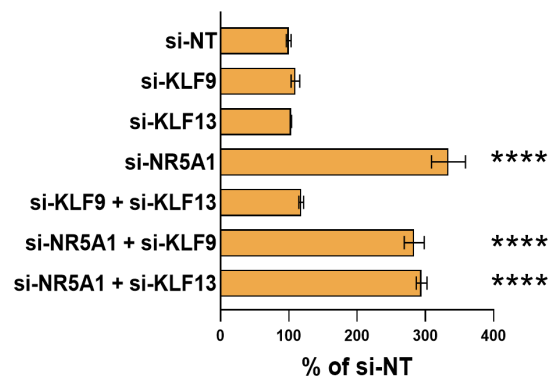
A

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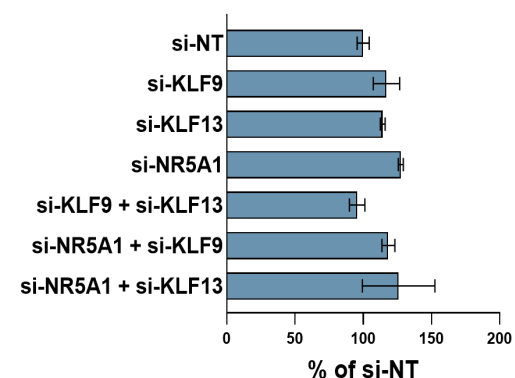
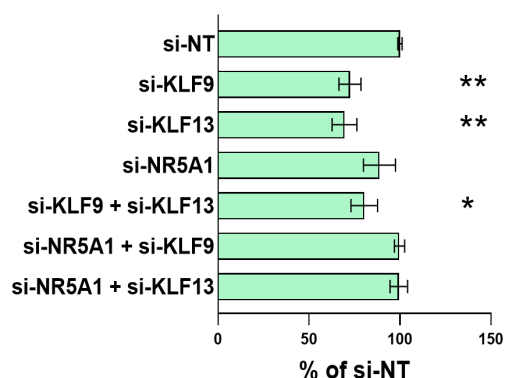
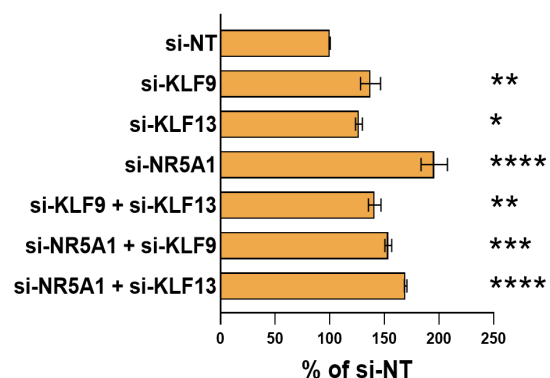
MUC-1

NR3C1



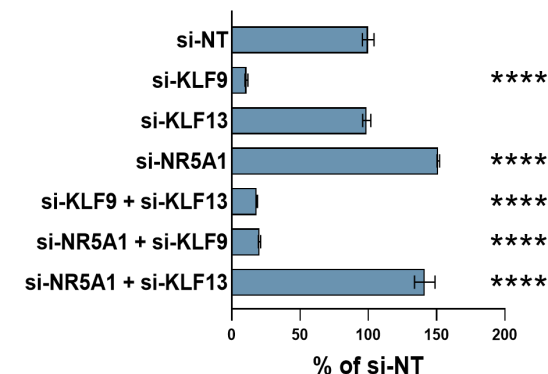
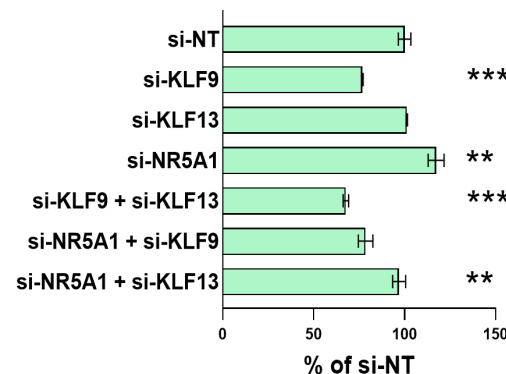
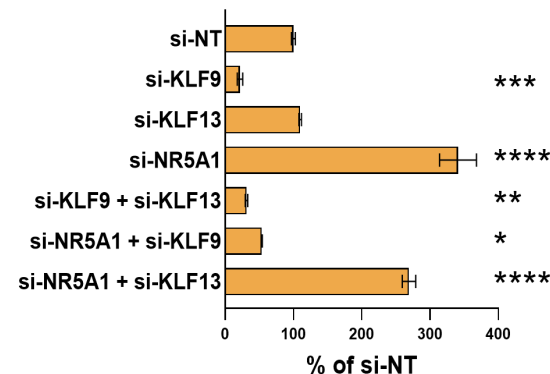
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AR



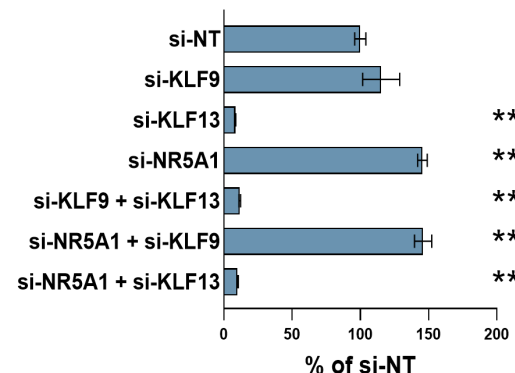
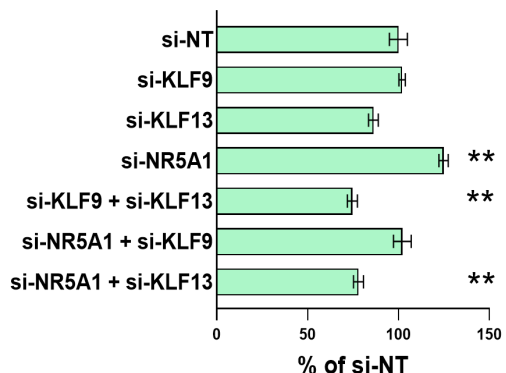
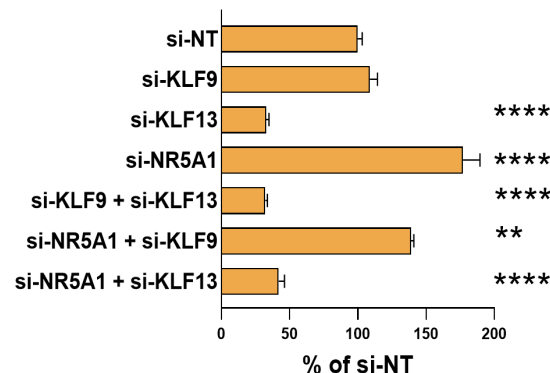
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KLF9



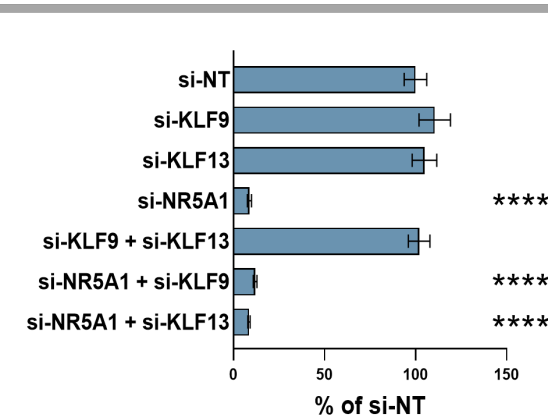
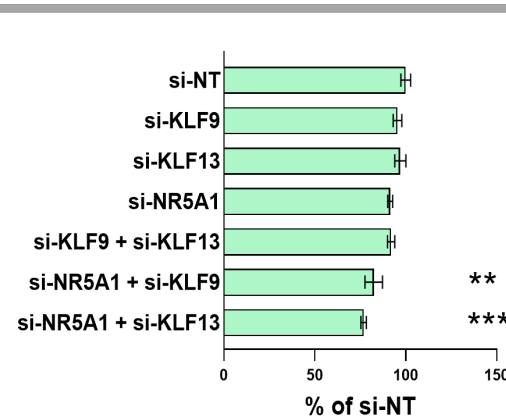
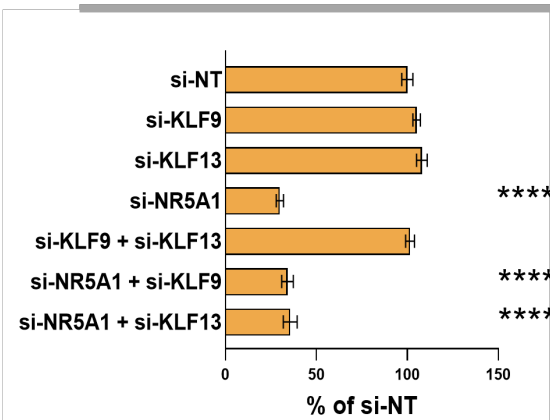
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KLF13



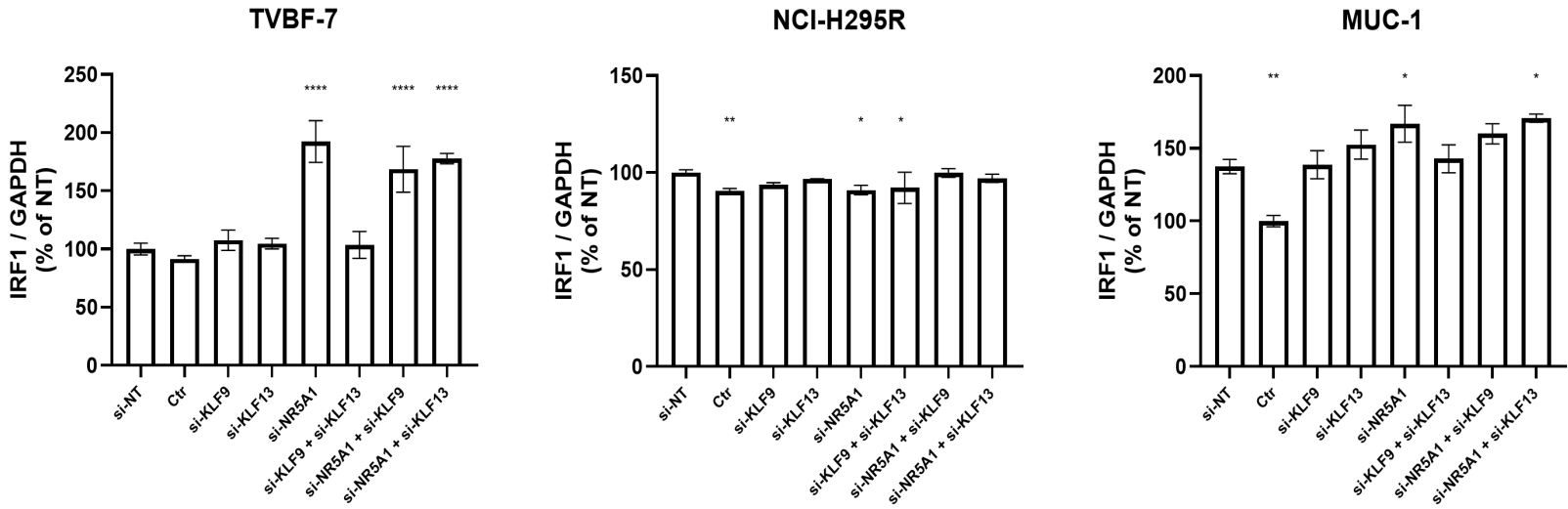
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NR5A1



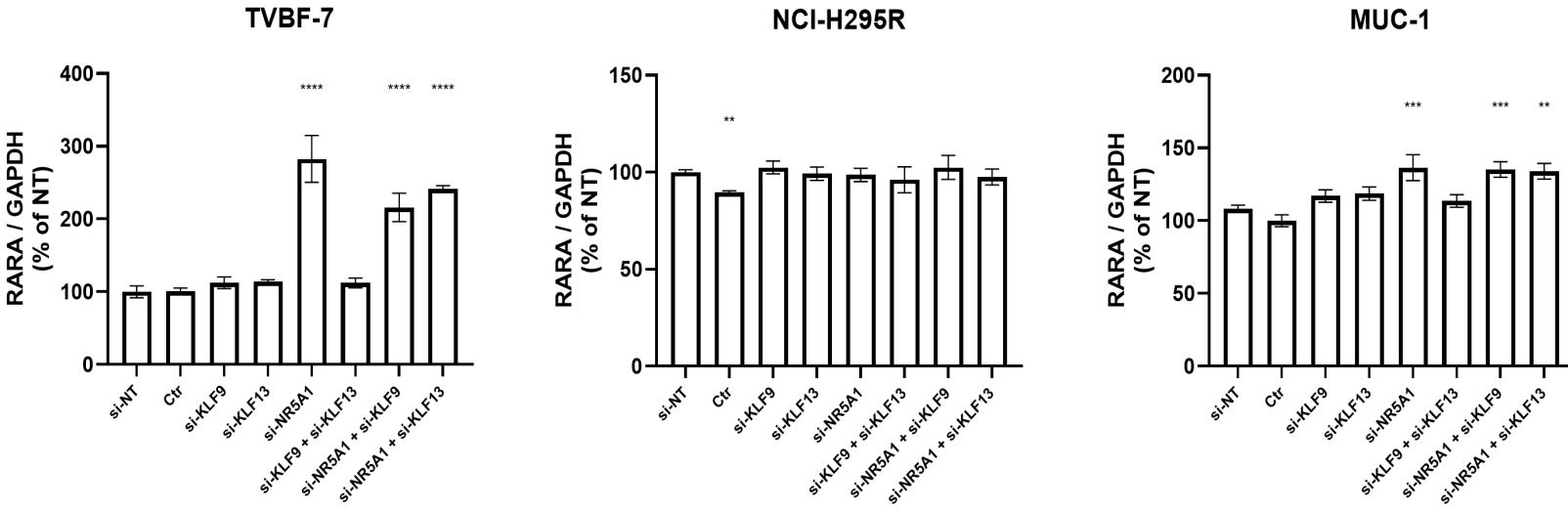
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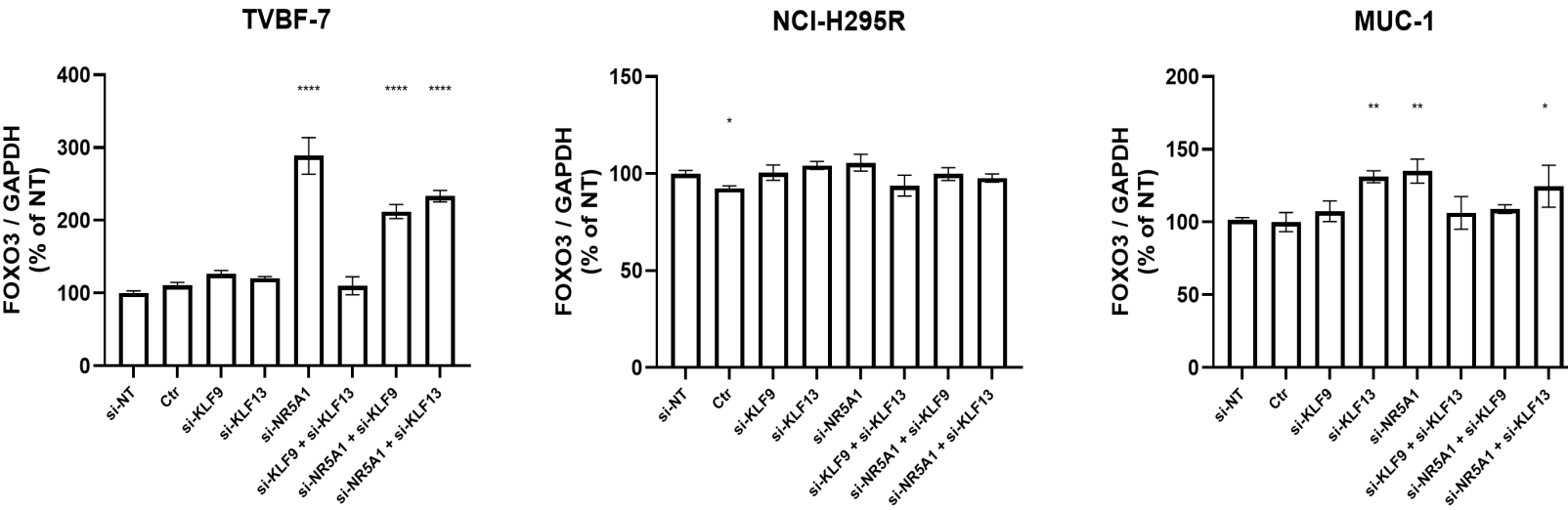
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RARA



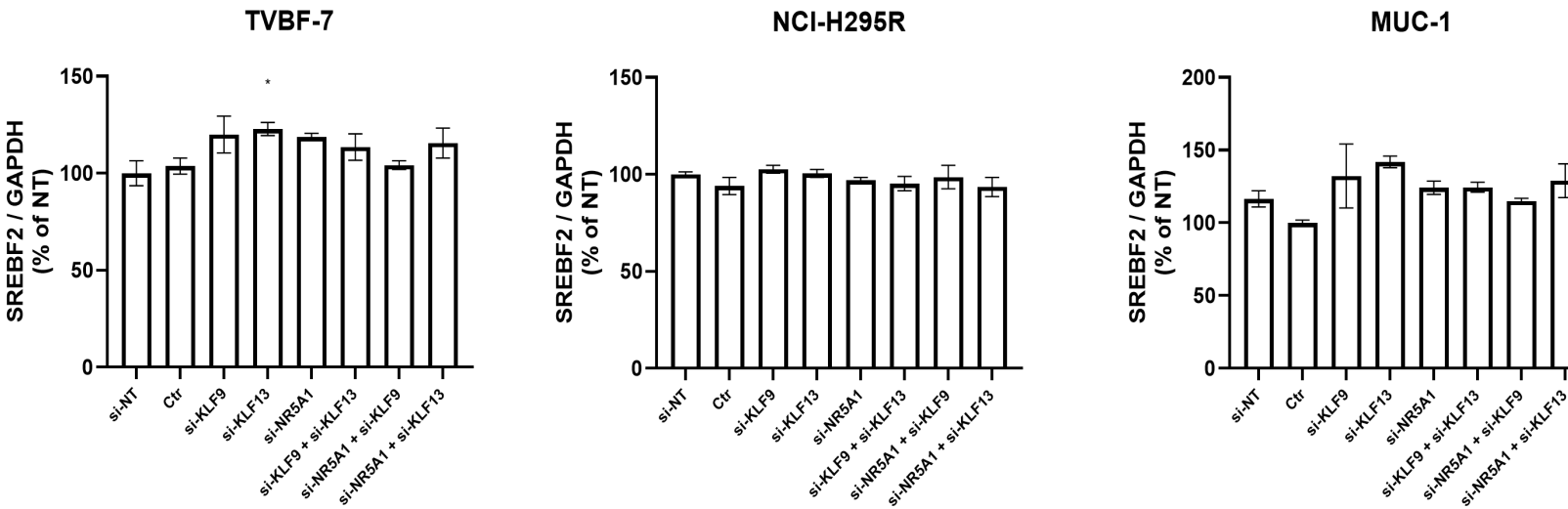
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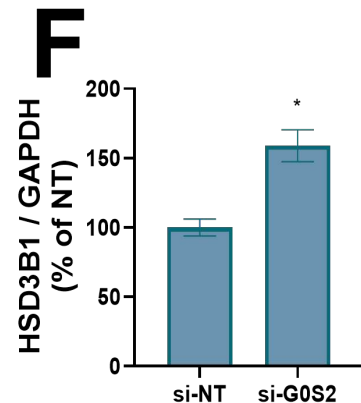
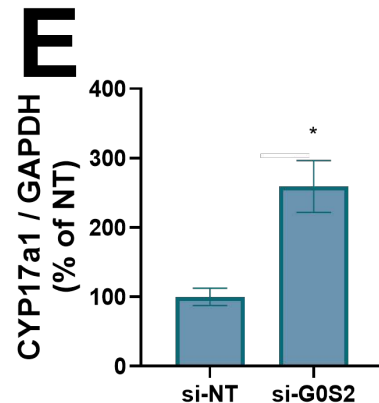
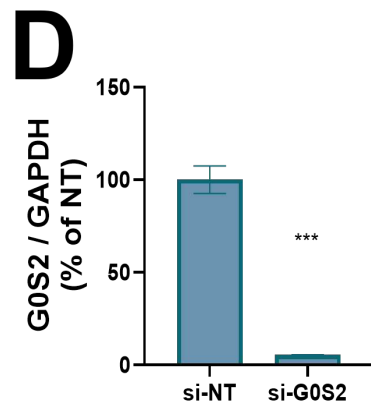
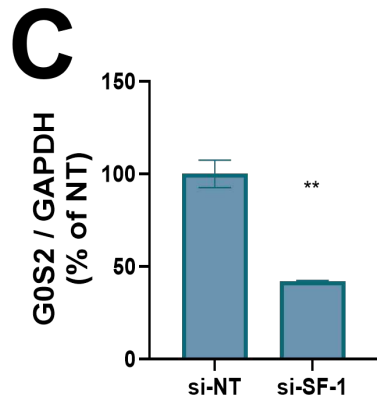
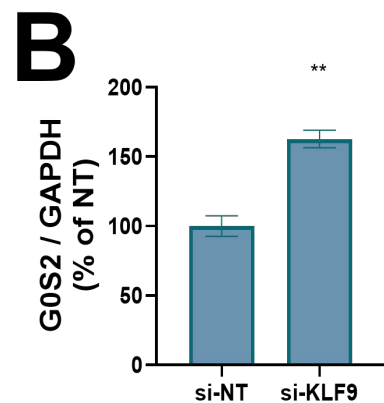
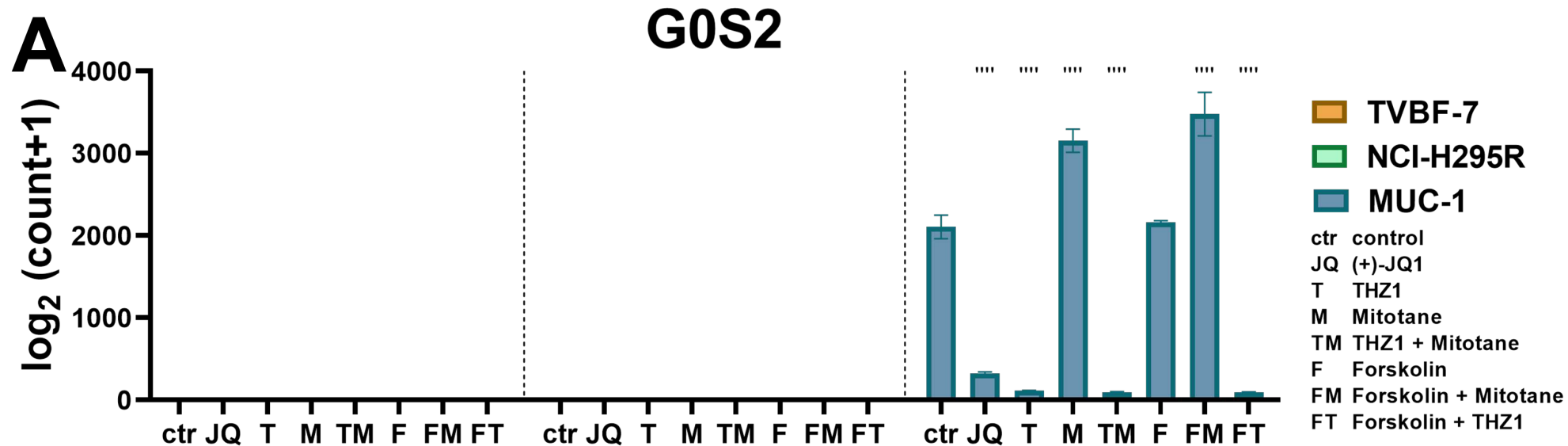
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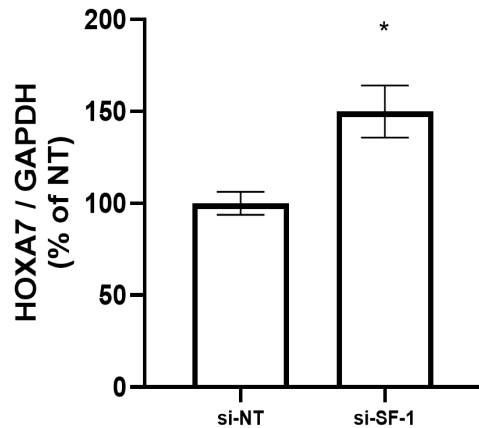
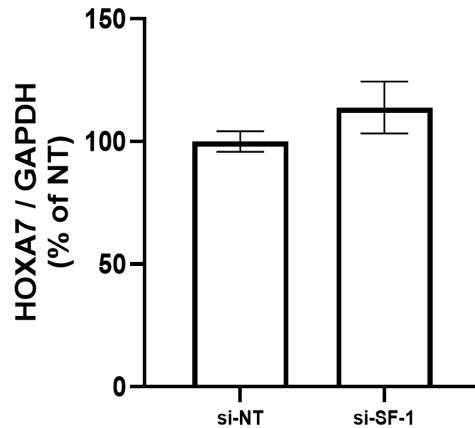
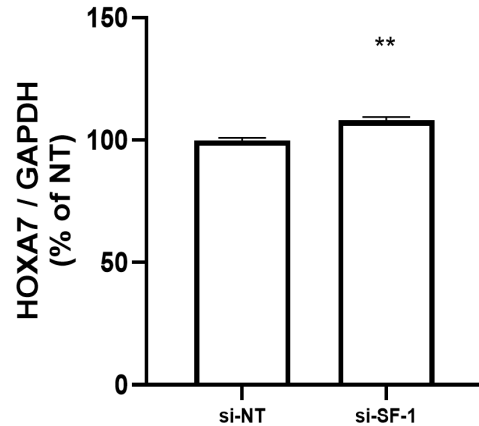


D

SREBF2





A**TVBF-7****B****NCI-H295R****C****MUC-1**

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.xlsx](#)
- [Table2.xlsx](#)