



Functional analysis of the potential tumour suppressor SIAH3

Funktionelle Analyse des potenziellen Tumorsuppressors SIAH3

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Summary

Of the seven in absentia homolog (SIAH) family, three members have been identified in the human genome. In contrast to the E3 ubiquitin ligase encoding *SIAH1* and *SIAH2*, little is known about the regulation and function of *SIAH3* in cancer development. Therefore, this work focuses on the widely overlooked family member *SIAH3* and its role in tumorigenesis. This study is based on cell culture experiments using human cell lines, followed by RNA and protein analyses as well as the investigation of cellular metabolism. Since *SIAH3* is missing the RING domain, the part that mediates the ubiquitin transfer to substrates, *SIAH3* does not function as an enzymatically active ligase. This work describes that *SIAH3* is frequently epigenetically silenced in different cancer entities, including cutaneous melanoma, lung adenocarcinoma and head and neck cancer. Low *SIAH3* levels correlate with an impaired survival of cancer patients. In addition to its regulation on gene level, *SIAH3* is also controlled on protein level. *SIAH3* interacts with *SIAH1* and *SIAH2*, which in turn mediate proteasomal degradation of *SIAH3*. Functionally, cell culture experiments showed that induced expression of *SIAH3* reduces cell proliferation and induces cell death. Concerning the underlying mechanisms, the results of my work suggest different possibilities. First, *SIAH3* is involved in Hippo signalling, interacting with *LATS2* and activating tumour suppressive target genes like *GADD45A* and *ANKRD1*. Second, *SIAH3* negatively affects cellular metabolism by shifting cells from aerobic oxidative phosphorylation to glycolysis. Microscopy demonstrated that *SIAH3* localises to mitochondria, with the N-terminal S3UNS domain being responsible for translocation. Proteome analyses revealed reduced levels of proteins involved in the respiratory chain complexes I, III, IV as well as the ATP synthase upon *SIAH3* induction, explaining the decline in oxidative phosphorylation. The interactome showed that *SIAH3* binds proteins involved in mitochondrial ribosome biogenesis and translation. Furthermore, *SIAH3* interacts with several ubiquitin ligases, recruiting at least *SIAH1* to the mitochondrion. I also showed that *SIAH3* interacts with mitochondrial importers like *CHCHD4* and *TIMM50*, which opens a third opportunity how *SIAH3* could affect mitochondrial metabolism. These findings expand our understanding of *SIAH3*'s function beyond its known role in PINK1-Parkin mediated mitophagy, suggesting that *SIAH3* acts as an epigenetically controlled tumour suppressor by regulating cellular metabolism through the inhibition of oxidative phosphorylation. And finally, this work demonstrates that the silenced *SIAH3* gene can be reactivated by epigenetic editing, providing a basis for restoring *SIAH3*'s function and potentially adding a small but important piece to the puzzle of cancer therapy.

Zusammenfassung

Das menschliche Genom enthält drei Mitglieder der seven in absentia homolog (SIAH) Familie. Im Gegensatz zu den viel untersuchten E3-Ubiquitin-Ligasen-kodierenden Genen *SIAH1* und *SIAH2* ist über die Regulation und die Funktion von *SIAH3* wenig bekannt. Daher konzentriert sich diese Arbeit auf dieses bislang wenig beachtete Familienmitglied und seine Rolle bei der Tumorentwicklung. Die Untersuchungen basieren auf Zellkultur-Experimenten mit humanen Zelllinien, gefolgt von RNA- und Protein-Analysen sowie der Untersuchung des zellulären Stoffwechsels. Da *SIAH3* die RING-Domäne fehlt, die Domäne, die den Ubiquitin-Transfer auf die Substrate vermittelt, fungiert *SIAH3* nicht als enzymatisch-aktive Ligase. Diese Arbeit beschreibt, dass *SIAH3* in verschiedenen Krebsentitäten, darunter kutane Melanome, Adenokarzinome der Lunge und Kopf-Hals-Karzinome, häufig epigenetisch stillgelegt ist und dass niedrige *SIAH3*-Level mit vermindertem Überleben von Krebspatienten korrelieren. Zusätzlich zu der Regulation auf Genebene wird *SIAH3* auch auf Proteinebene kontrolliert. *SIAH3* interagiert mit *SIAH1* und *SIAH2*, welche ihrerseits den proteasomalen Abbau von *SIAH3* vermitteln. Hinsichtlich der Funktion zeigten Zellkulturexperimente, dass induzierte *SIAH3*-Expression die Zellproliferation hemmt und Zelltod auslöst. Die Ergebnisse meiner Arbeit weisen auf unterschiedliche zugrundeliegende Mechanismen hin. Erstens spielt *SIAH3* im Hippo-Signalweg eine Rolle, in dem es mit *LATS2* interagiert und tumorsuppressive Zielgene wie *GADD34A* und *ANKRD1* aktiviert. Zweitens beeinflusst *SIAH3* den zellulären Stoffwechsel, indem es die Energiegewinnung der Zellen von der aeroben oxidativen Phosphorylierung zur Glykolyse verlagert. Mikroskopische Untersuchungen haben gezeigt, dass *SIAH3* in den Mitochondrien lokalisiert ist, wofür die N-terminale S3UNS-Domäne verantwortlich ist. Die Proteom-Analyse hat verminderte Proteinlevel von Komponenten der Atmungskette (Komplexe I, III und IV) und der ATP-Synthase (Komplex V) nach *SIAH3*-Induktion offenbart, was die Abnahme der oxidativen Phosphorylierung erklären könnte. Weiterhin hat das Interaktionsnetzwerk gezeigt, dass *SIAH3* Proteine bindet, die an der mitochondriellen Ribosomenbiogenese und an der Translation beteiligt sind. *SIAH3* interagiert außerdem mit diversen Ubiquitin-Ligasen und rekrutiert mindestens *SIAH1* zum Mitochondrium. Auch zeigte sich, dass *SIAH3* mitochondriale Importproteinen wie *CHCHD4* und *TIMM50* bindet, was eine dritte Möglichkeit eröffnet, wie *SIAH3* den Stoffwechsel beeinflussen könnte. Diese Erkenntnisse erweitern unser Verständnis über die bekannte Rolle bei der PINK1-Parkin-vermittelten Mitophagie hinaus und deuten darauf hin, dass *SIAH3* als epigenetisch kontrollierter Tumorsuppressor wirkt, in dem er den Stoffwechsel durch die Hemmung der oxidativen Phosphorylierung reguliert. Abschließend zeigt diese Arbeit noch, dass stillgelegtes *SIAH3* durch epigenetisches Editieren reaktiviert werden kann – eine Grundlage für die Wiederherstellung der Funktion von *SIAH3*. Und diese Informationen könnten einen kleinen aber wichtigen Beitrag zur Krebstherapie leisten.

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Abbreviations

This work uses the abbreviations listed below, as well as the units and derived quantities defined by the *Système international d'unités* (SI units). Commonly used abbreviations, such as “etc.”, are not explicitly included, since they are assumed to be generally understood.

abbreviation	meaning
2-DG	2-deoxy-D-glucose
5-aza-dC	5-aza-2'-deoxycytidine
a	antibody against
AB	antibody
ACTB / β -Actin	β -actin
ANKRD1	ankyrin repeat domain 1
BRCA	breast cancer
BS	bisulfite(-treated/converted)
CCN1	cellular communication network factor 1
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary DNA
CDS	coding sequence
CHCHD4	coiled-coil helix coiled-coil helix domain containing 4
COAD	colon adenocarcinoma
CoBRA	combined bisulfite restriction analysis
CpG	cytosine-phosphate-guanine
CTGF	connective tissue growth factor
ctrl.	control
DIMER	dimerisation domain
DME	demethylation enzyme
DMEM	Dulbecco's modified Eagle medium
DNMT	DNA methyltransferase
Dox	doxycycline
ECAR	extracellular acidification rate
ETC	electron transport chain
EZH2	enhancer of zeste homolog 2
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
FCS	fetal calf serum
GADD45A	growth arrest and DNA damage inducible alpha
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBM	glioblastoma multiforme
HNSC	head and neck squamous cell carcinoma
HRP	horseradish peroxidase
IF	immunofluorescence
IP	immunoprecipitation
kd	knockdown

KIR	kidney renal papillary cell carcinoma
KIRC	kidney renal clear cell carcinoma
LATS2	large tumour suppressor kinase 2
LIHC	liver hepatocellular carcinoma
LUAD	lung adenocarcinomas
mRNA	messenger RNA
MS	mass spectrometry
mtDNA	mitochondrial DNA
nickel–nitrilotriacetic acid	Ni-NTA
ns	not significant
OCR	oxygen consumption rate
ORA	overrepresentation analysis
ORF	open reading frame
OXPHOS	oxidative phosphorylation
PARK2	Parkin
PBS	phosphate-buffered saline
PCPG	pheochromocytoma and paraganglioma
PD	pulldown
PHDs	prolyl hydroxylase domain-containing proteins
PINK1	PTEN-induced putative kinase 1
(RT-)qPCR	(reverse transcription) quantitative polymerase chain reaction
RASSF1A	Ras association domain family 1A
RING	really interesting new gene
Rm	RING-domain mutant
ROS	reactive oxygen species
Rot/AA	rotenone and antimycin A
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
S3UNS	SIAH3 unique N-terminal sequence
SBS	substrate binding site
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sgRNA	singe guide RNA
SIAH (SIAH1, SIAH2, SIAH3)	seven in absentia homolog
SINA1	Seven in absentia
siRNA	small interfering RNA
SKCM	skin cutaneous melanoma
SOC	super optimal broth with catabolite repression
STR	short tandem repeat
STUB1 (CHIP)	STIP1 homology and U-box containing protein 1
SZF	SIAH-type zinc finger
TBE	tris-borate-EDTA buffer
TBS	tris-buffered saline
TCA cycle	tricarboxylic acid cycle

TE	Tris-EDTA buffer
Tet	tetracycline
TGS	tumour suppressor gene
THYM	thymoma
TIMM50	translocase of the inner mitochondrial membrane 50
TLS	translation start site
TSS	transcription start site
ubi	ubiquitin
UCEC	uterine corpus endometrial carcinoma
UPS	ubiquitin–proteasome system
VEGF	vascular endothelial growth factor
WB	Western blot
Wt	wild-type
YAP	Yes-associated protein

INTRODUCTION

1 INTRODUCTION

Worldwide, one in five people develops cancer during their lifetime and one in nine men and one in twelve women die from it (Bray *et al.*, 2024). In Germany, every second person develops cancer at some point in their life (Robert Koch-Institut, 2023). These numbers reflect the large impact cancer has on each individual and on society, showing the importance of continued efforts to reduce its burden. Even though cancer is often described as “a genetic disease” (Hanahan & Weinberg, 2011; Vogelstein *et al.*, 2013), the epigenetic aspects of tumorigenesis are increasingly coming into focus, as emphasized by the inclusion of “nonmutational epigenetic reprogramming” to the well accepted hallmarks of cancer by Hanahan and Weinberg (Hanahan, 2022). An unpublished whole methylome analysis of our working group (Illumina Human Methylation 450K BeadChip) comparing nevus cells with melanoma revealed the *SIAH3* (seven in absentia homolog 3) gene as one of the top 50 hypermethylated candidates in melanoma, suggesting it as a putative novel, silenced tumour suppressor. Motivated by this finding, this work investigates the functional and epigenetic characteristics of SIAH3.

1.1 Characteristics of tumours

The term “cancer” goes back to around 400 years BC, when Hippocrates named non-healing swellings and ulcerous formations “karkinos”, Greek for “crab” (Weiss, 2000). The association with a crab refers either to the animal’s aggressive behaviour or to the visual resemblance between the veins radiating from the site of cancer development and the legs of a crab (Panegyres, 2024). Celsus (25 BC – 50 AD) translated this word afterwards into “cancer”, the Latin word for crab. A few hundred years after Hippocrates, Galen used the word “oncos” (greek for swelling) to describe unnatural swellings (Panegyres, 2024), which is now used in many technical terms like “oncology” or “oncogenes”.

Tumorigenesis is a multistep process, during which the cell acquires traits that enable the progressive transformation from a normal human cell into highly malignant derivatives (Hanahan & Weinberg, 2000). Although tumours are very divers and occur in many different organs, they share certain characteristics. And to break down the complexity of the cancer disease, Hanahan & Weinberg defined the so-called “hallmarks of cancer”, functional capabilities that, as they suggest, most if not all cancers have acquired (Hanahan & Weinberg, 2000). Next to “inducing angiogenesis”, “enabling replicative immortality”, “activating invasion and metastasis” and “evading growth suppressors”, two core features of cancer cells are their ability to “resist cell death” and “sustain chronic proliferation” (Hanahan & Weinberg, 2011). In healthy tissues, a homeostasis between proliferation and death is maintained which is out of balance in tumour

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cells. This is, in part, attributable to genetic changes. These alterations can be divided into two groups: gain-of-function mutations from proto-oncogenes result in dominant-acting oncogenes that stimulate cell proliferation and cell division, favouring survival. Tumour suppressors – the second group of genes involved in tumorigenesis – lose their ability to control unrestricted proliferation and initiate DNA repair through loss-of-function mutations (Lee & Muller, 2010). Since mostly one copy of the gene is sufficient, an inactivation of the second allele, a second hit, is needed for a functional loss (two-hit hypothesis) (Knudson, 1971). Besides the classical genetic alterations like point mutations, insertions and deletions or chromosomal translocations of genes or genetic regions, epigenetic changes can also contribute to the formation of oncogenes and the deactivation of tumour suppressors.

1.2 Regulation of gene expression and protein level in carcinogenesis

1.2.1 Epigenetic regulation of gene expression

The word “epigenetics” was coined in the 1940s by Conrad. H. Waddington and refers to a then-new branch of biology that focuses on the links between gene and protein expression, between genotype and phenotype (Waddington, 2012; Peixoto *et al.*, 2020). The so-called “cancer epigenome” possesses a lot of abnormalities that are based on heritable alterations without being manifested in the primary DNA sequence (Jones & Baylin, 2007; Baylin & Jones, 2011). And Adrian Bird defined epigenetic events as “the structural adaption of chromosomal regions so as to register, signal or perpetuate altered activity states”, which explicitly includes chromosomal marks (Bird, 2007). One of these epigenetic modifications is DNA methylation. DNA methylation occurs on cytosines within CpG (cytosine-phosphate-guanine) dinucleotides, where a methyl group is added onto the C5 position of the cytosine to form 5-methylcytosine (5mC). This transfer is mediated by DNA methyltransferases (DNMTs) (Moore *et al.*, 2013). Global loss of DNA methylation (hypomethylation) was the first described epigenetic abnormality in cancers cells (Feinberg & Tycko, 2004). Later on, hypermethylation of CpG islands in the promoter regions of tumour suppressor genes (TSG), which leads to an inactivation of these genes, has been described in many tumours (Esteller, 2002). 5-10 % of the CpG islands in promoters that are unmethylated in normal tissues are abnormally methylated in various cancer genomes (Dawson & Kouzarides, 2012). Two important and frequently described hypermethylated tumour suppressors are for example CDKN2A (cyclin-dependent kinase inhibitor 2A, p16) (Xing *et al.*, 2013) and RASSF1A (Ras association domain family 1A) (Dammann *et al.*, 2003; Dammann *et al.*, 2005). Promoter hypermethylation in tumours can be seen as a characteristic of tumour suppressor genes and since epigenetic modifications represent an early change during cancer development, they are predestined as potential cancer biomarkers (Kulis & Esteller, 2010; Baylin & Jones, 2011).

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1.2.2 The ubiquitin-proteasome system

While carcinogenesis is driven by the overexpression of oncogenes and the underexpression of TSG, cancer cells are also characterised by changes in their proteome composition (Brancolini & Iuliano, 2020). Proteostasis is controlled by the activity of E3 ubiquitin ligases (Bernassola *et al.*, 2019) and proteasome inhibitors have been successfully used as drugs in cancer therapy (Manasanch & Orlowski, 2017).

Ubiquitination is a posttranslational modification that marks proteins for proteasomal degradation or non-degradative signalling by attaching ubiquitin, a conserved small protein, to the target protein (Hershko, 1983). In an enzymatic cascade, involving a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3), ubiquitin is covalently bound to the substrate protein's lysine residues. E3 ubiquitin ligases are responsible for the transfer of ubiquitin from an E2 ubiquitin conjugating enzyme to their specific substrates, condemning these proteins for proteasomal degradation (Hershko & Ciechanover, 1998; Lorick *et al.*, 1999). Poly-ubiquitinated proteins are then usually degraded by the 26S proteasome system (Hershko & Ciechanover, 1998) (Fig. 1). This ubiquitin–proteasome system (UPS) is responsible for 80-90 % of cellular proteolysis and 10-20 % of autophagy (Kwon & Ciechanover, 2017), showing the importance of this mechanism for protein homeostasis and the abolishment of damaged or incorrect proteins.

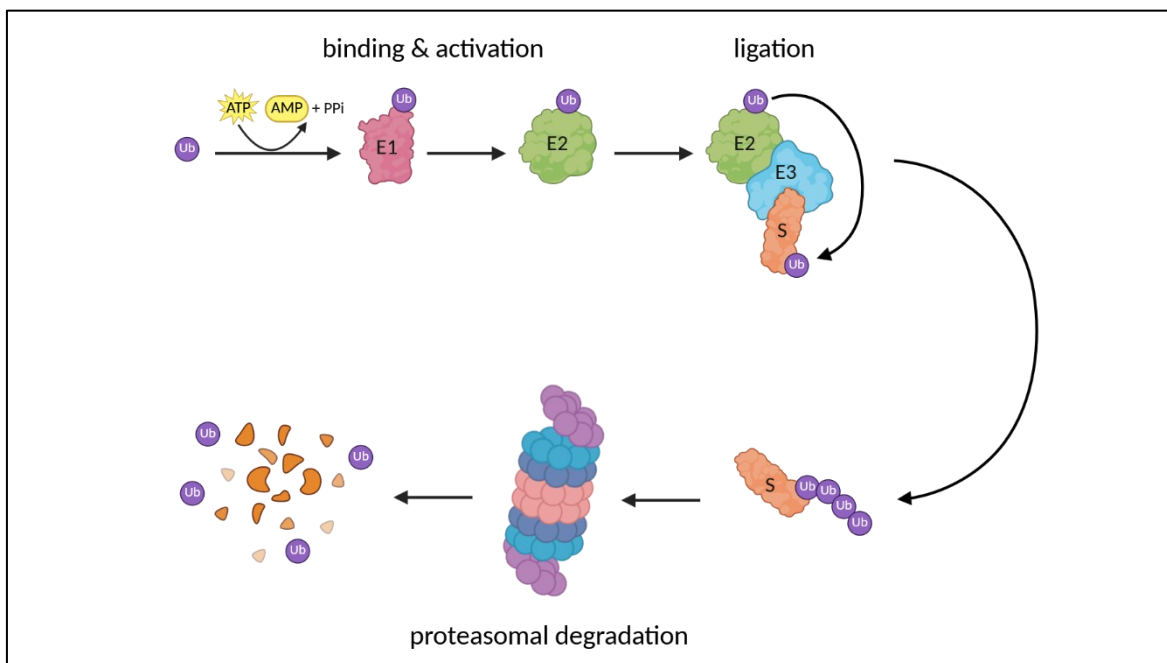


Fig. 1: The ubiquitin–proteasome system (UPS)

First, ubiquitin (ub) is activated in an ATP-dependent reaction by attaching it to a ubiquitin activating enzyme (E1). It is then transferred to a ubiquitin conjugating enzyme (E2). A ubiquitin ligase (E3) mediates the transfer of the ubiquitin to the substrate protein (S), thus being responsible for the substrate specificity. The polyubiquitinated protein is then degraded by the 26S proteasome.

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Ubiquitination can occur as monoubiquitination (a single ubiquitin attached), multimonoubiquitination (multiple ubiquitin proteins are each attached to different sites on the same substrate protein) or polyubiquitination (ubiquitin proteins form a chain that is attached to the substrate protein), with K48-linked polyubiquitination being the main connection type in cells. Whereas monoubiquitination was shown to influence growth, metastasis, radiation resistance and immune escape of cancer cells by affecting DNA repair and gene transcription, K48-polyubiquitination is involved in different facets of cancer by promoting the degradation of corresponding proteins (Liu *et al.*, 2024). E3 ubiquitin ligases and deubiquitinating enzymes play important roles in the regulation of all hallmarks of cancer. Concerning the hallmark “evasion of growth suppressors”, for instance, E3 ligase MDM2 and the deubiquitinating enzyme USP7 regulate the stability of the tumour suppressor p53 (Harakandi *et al.*, 2021; Liu *et al.*, 2024). Regarding “inducing angiogenesis”, HIF1, a central regulator of cellular adaption to hypoxia, is restricted by ubiquitination through the Hippel-Lindau protein (pVHL), an E3 ligase. Their binding is primed through the hydroxylation of HIF1 α by PHDs (prolyl hydroxylase domain-containing proteins). Another E3 ubiquitin ligase, SIAH2 (seven in absentia homologue 2), in turn, targets PHD3 for degradation (Nakayama *et al.*, 2004). Even when it comes to “nonmutational epigenetic reprogramming”, the UPS is involved, as with the E3 ligase RNF180 ubiquitinating DNMT1 (Liu *et al.*, 2024). Thus, even these two different regulatory mechanisms – DNA methylation and protein degradation – are not always separable, reflecting the complexity of cancer’s molecular biology.

1.3 The role of energy metabolism in cancer and other diseases

Next to the already described characteristics of cancer, including genetic and epigenetic changes as well as changes in the proteome composition, metabolic rewiring also characterises the transformation of normal cells to tumour cells (Hanahan, 2022). The most well-known effect of cancer energy metabolism is the so-called “Warburg effect” (aerobic glycolysis), which is characterised by an increase in glycolysis even in the presence of oxygen (Warburg & Minami, 1923; Warburg, 1925).

Being the “powerplant” of the cell, mitochondria are the main site of the energy production. A schematic overview of the cellular respiration is shown in Fig. 2A. In the first step, the glycolysis, glucose is converted to pyruvate. Under aerobic conditions, pyruvate is decarboxylated to acetyl-coA that is then transferred into the mitochondria, entering the tricarboxylic acid cycle (TCA cycle). During the final step, the oxidative phosphorylation (OXPHOS), mitochondria generate energy in form of ATP (Sherratt, 1991). Under anaerobic conditions, instead of following the energy efficient metabolic pathway inside the mitochondria, pyruvate is metabolised through fermentation,

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forming lactate. Thus, the only produced ATP is generated during glycolysis. Fig. 2B compares the energy yield of aerobic and anaerobic energy production.

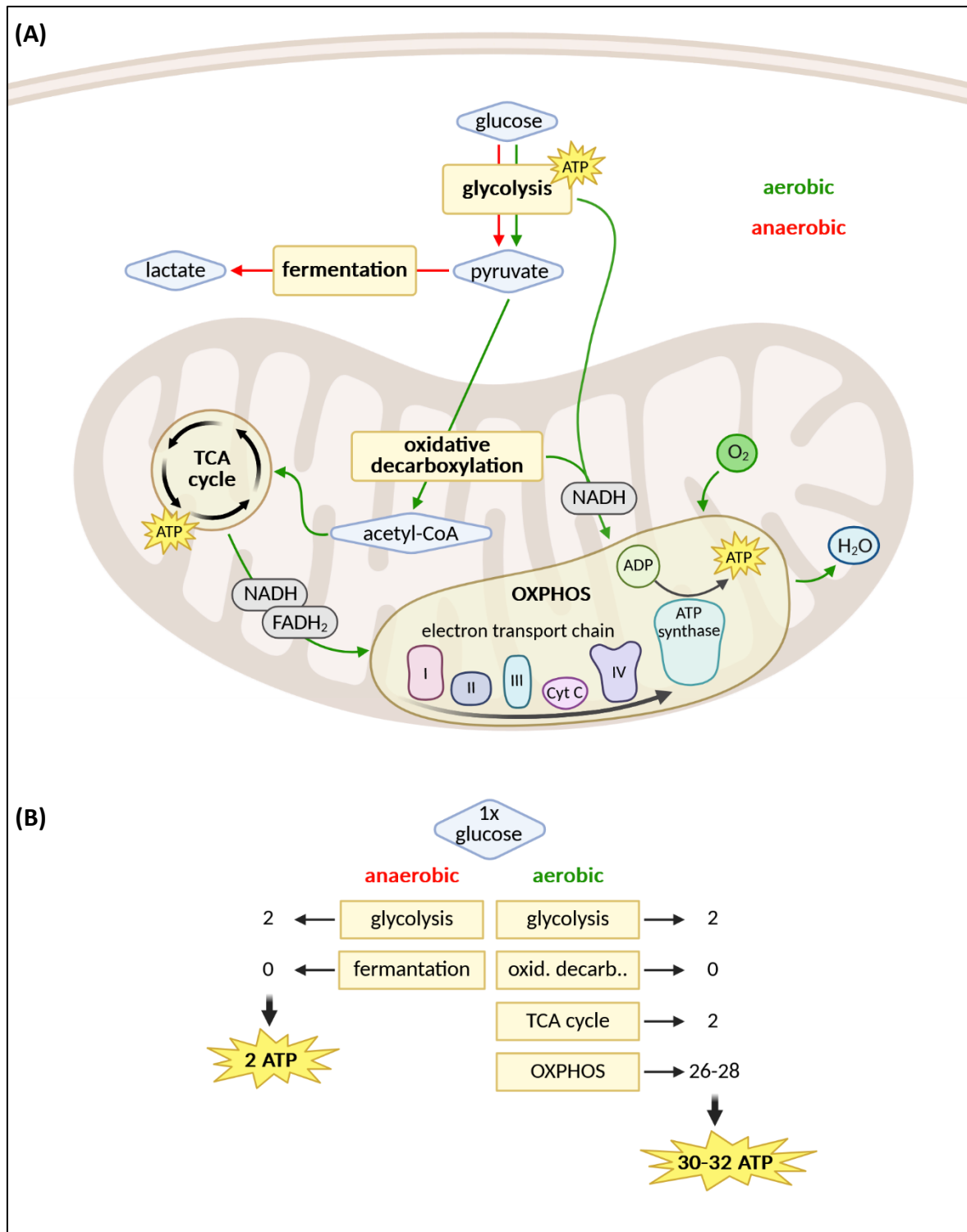


Fig. 2: Cellular respiration and energy production

(A) Schematic overview of cellular respiration showing the main steps and metabolic products. Green arrows depict the pathway of the aerobic energy production, red marks the anaerobic pathway. **(B)** ATP yield of the aerobic and anaerobic energy production pathways. The figure was created with BioRender.com.

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Explaining his observation, that tumour samples take up glucose and convert it to lactate even when there is enough oxygen available to convert it to CO₂ via OXPHOS, Warburg later proposed that dysfunctional mitochondria are the reason for the shift to aerobic glycolysis (Warburg, 1956). And even though this hypothesis has been questioned (Vaupel & Multhoff, 2021), mitochondria are still involved in tumorigenesis through other mechanisms. Reactive oxygen species (ROS) accumulation due to leakage of the electron transport chain (ETC) can induce the oxidation of macromolecules (e.g. proteins, DNA, lipids) which can contribute to tumorigenesis, for example via the PHD-HIF signalling pathway (Badrinath & Yoo, 2018). Furthermore, mitochondria are also directly involved in apoptosis. Permeabilisation of the mitochondrial outer membrane leads to cytochrome c release that activates caspases which promote apoptosis (Jiang & Wang, 2004; Bock & Tait, 2020).

Mitochondria also play a critical role in neurodegenerative diseases. Mitochondrial abnormalities have been described in Parkinson's, Alzheimer's and Huntington's disease as well as in amyotrophic lateral sclerosis (Filosto *et al.*, 2011). Since neurons possess a high amount of mitochondria, they are especially vulnerable to energy-dependent defects caused by mitochondrial abnormalities (Filosto *et al.*, 2011). Another pathway involved in neurodegeneration is mitophagy, the recycling of (damaged) mitochondria by autophagy. Loss of mitophagy leads to an accumulation of defect mitochondria, which is a trait of aging and neurodegeneration (Fivenson *et al.*, 2017; Killackey *et al.*, 2020). The best studied initiators of this pathway are PTEN-induced putative kinase 1 (PINK1) and Parkin (PARK2) (Truban *et al.*, 2017), that are suggested to be inhibited by SIAH3 (Hasson *et al.*, 2013; Yoon *et al.*, 2021).

1.4 The SIAH family

SIAH3, the potential tumour suppressor that is the focus of this study, is the third member of the SIAH protein family. This family consists of 3 human homologs of *Drosophila's* SINA (Seven in absentia) (Pepper *et al.*, 2017). SINA was first identified playing a role in photoreceptor cell development by acting downstream of Ras. A loss of function mutation of SINA led to a loss of the photoreceptor R7 due to defects in neuronal cell differentiation (Carthew & Rubin, 1990; Zipursky & Rubin, 1994). In humans, two homologs were described initially: SIAH1 and SIAH2 (Hu & Chung *et al.*, 1997). Later, thanks to phylogenetic analyses, SIAH3 was characterised as a third human homolog (Pepper *et al.*, 2017).

SIAH1, 2 and 3 are structurally closely related. Near the C-terminus, all three proteins contain a SIAH-type zinc finger (SZF), a substrate binding site (SBS) and a dimerisation domain (DIMER), together forming the substrate binding or SINA domain (Pepper *et al.*, 2017). While SIAH1 and 2 possess a dual zinc finger motif, SIAH3 has only a single motif. SIAH1 and SIAH2 further contain a RING (Really Interesting New Gene) domain enabling them to function as ubiquitin E3 ligases that

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regulate the degradation of substrate proteins by the UPS (Grishina *et al.*, 2012; Pepper *et al.*, 2017). In contrast, SIAH3 lacks this domain (Fig. 3), suggesting it to not function as an enzymatically active E3 ubiquitin ligase (Pepper *et al.*, 2017). Instead, SIAH3 harbours a histidine-rich region (Poly-His) and an evolutionary highly conserved SIAH3-unique N-terminal sequence (S3UNS) (Pepper *et al.*, 2017; Zhang *et al.*, 2017). A schematic overview of the protein domain structure of SIAH1, 2 and 3 is shown in Fig. 3.

1.4.1 The E3 ubiquitin ligases SIAH1 and SIAH2

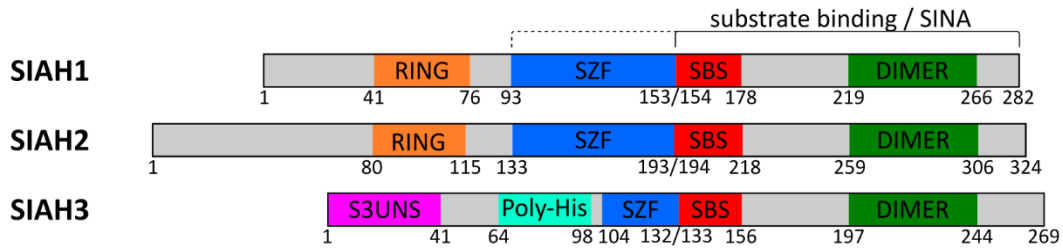


Fig. 3: Domain structure of SIAH1, SIAH2 and SIAH3

Comparison of the domain structure of the three human SIAH proteins. The domains are coloured equally. RING = Really Interesting New Gene, SZF = SIAH-type zinc finger, SBS = substrate binding site, DIMER = dimerisation domain, S3UNS = SIAH3-unique N-terminal sequence, Poly-His = histidine rich region. SBS and DIMER (and SZF, depending on the literature) form the substrate binding or SINA domain. The numbers refer to the respective amino acid positions. The figure was built after Pepper *et al.* (2017) and Zhang *et al.* (2017).

SIAH1 and SIAH2 are well-known actors in numerous biological pathways and diseases, e.g. hypoxia and inflammation or cancer and neurodegeneration (Calzado *et al.*, 2009; Schmitz *et al.*, 2022; Zhang *et al.*, 2022). Being RING E3 ubiquitin ligases, they play critical roles in the UPS by mediating substrate specificity for proteasomal degradation (Hu & Zhang *et al.*, 1997; Hu & Fearon, 1999; Polekhina *et al.*, 2002; Germani *et al.*, 2003). Due to their diverse substrates, SIAH1 and SIAH2 execute a lot of different functions. For example, they play a role in DNA damage and cellular senescence by regulating HIPK2 and TRF2 (Winter *et al.*, 2008; Fujita *et al.*, 2010; Qi *et al.*, 2013) and during hypoxia by ubiquitinating PHD1/3 which leads to a stabilisation of HIF1 α and thus to an increase in hypoxia-responsive genes (Nakayama *et al.*, 2004; Nakayama *et al.*, 2009; Qi *et al.*, 2013). DNA damage and hypoxia are only two examples of signalling pathways through which the SIAHs are involved in tumorigenesis but their precise roles in tumorigenesis are diverse and not fully understood yet. SIAH1 and 2 have been shown to act as both oncogenic and tumour suppressive across multiple patient cohorts and animal cancer models (Wong & Möller, 2013). Except for glioblastoma, where it exerts an oncogenic function (He *et al.*, 2019), SIAH1 has been described to have a protective role in multiple cancer entities (Wong & Möller, 2013; Zhang *et al.*, 2022) like breast (Wen, Yang, Song, Li & Yao *et al.*, 2010; Wen, Yang, Song, Li, Zhu & Wang, 2010; Knauer *et al.*, 2015) and colorectal cancer (Xiao *et al.*, 2020), leukemia (Krämer *et al.*, 2013), pancreatic carcinogenesis (Wu *et al.*, 2018) and osteosarcoma (Han *et al.*, 2019). In hepatocellular

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carcinoma SIAH1 seems to be able to perform both functions (Zhang *et al.*, 2022): being helpful as tumour suppressor (Yoshibayashi *et al.*, 2007) or being unfavourable as an oncogene (Brauckhoff *et al.*, 2011). In contrast, SIAH2 has mainly been associated with tumour progression (Li *et al.*, 2022), for example in breast cancer (Chan *et al.*, 2011), lung cancer (Moreno *et al.*, 2015) or in melanoma (Scortegagna *et al.*, 2020). Some studies, however, also showed an anti-tumour effect of SIAH2. In leukemia, for example, activation of SIAH2 by hyperthermia could promote SIAH2-dependent degradation of oncogenic fusion proteins (Maimaitiyiming *et al.*, 2021). In lung cancer, additionally to its tumour promoting function, SIAH2 can have an anti-tumour effect by degrading TYK2 and thereby suppressing the cancer driver STAT3 (Müller *et al.*, 2014). Their exact function depends on the specific tissue, the presence of substrates, their subcellular localisation and posttranslational modifications and the cellular microenvironment (Zhang *et al.*, 2022). Next to the described ambiguous role in cancer, SIAH1 and 2 play an important role in neuronal development and neurodegenerative diseases e.g. by regulating the exit of cerebellar granule neurons from the germinal zone and the formation of Lewy bodies that are characteristic for Parkinson's disease (Liani *et al.*, 2004; Famulski *et al.*, 2010).

1.4.2 SIAH3 – the extraordinary family member

A search on NCBI's PubMed with the keywords SIAH1 and SIAH2 yielded 367 and 271 publications, respectively (03/2026). In contrast, systematic research on SIAH3 is still rare. There are only 20 publications that mention SIAH3. The vast amount of the existing literature only refers to SIAH3 as one of the identified targets within genome-, transcriptome- or methylome-wide analyses. But these studies suggest that SIAH3 plays a role in processes like wound healing (Wang & Tatakis, 2017) and reproduction (Mohanty *et al.*, 2015; Wang *et al.*, 2024), in different cancers as prostate (Robbins *et al.*, 2011) and pancreatic cancer (López de Maturana *et al.*, 2021; Zohar *et al.*, 2025) as well as in retinoblastoma (Schieffer *et al.*, 2021), but also in brain aging and neurodegeneration (Li & Chu *et al.*, 2019; Ichise *et al.*, 2021; Wiegand *et al.*, 2021; Abd Elghani *et al.*, 2022; Cochran *et al.*, 2023; Abraham *et al.*, 2024). Fig. 4 summarises the areas in which SIAH3 plays a role. The corresponding published findings, like SNPs (single nucleotide polymorphisms) or hypomethylation, are also included.

1.4.2.1 The role of SIAH3 in mitochondrial homeostasis

Despite evidence of its involvement in several diseases, knowledge about the molecular function of SIAH3 is scarce, with most available information being linked to neurodegenerative diseases. SIAH3 has been shown to inhibit the Parkinson's disease-associated protein PINK1 (Hasson *et al.*, 2013; Yoon *et al.*, 2021; Abd Elghani *et al.*, 2022). It localises to mitochondria where it inhibits PINK1 accumulation, resulting in reducing Parkin translocation to damaged mitochondria and consequently the negative regulation of mitophagy (Hasson *et al.*, 2013) (see Fig. 4). SIAH3 was also

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shown to directly interact with PINK1 (Yoon *et al.*, 2021; Abd Elghani *et al.*, 2022). Abd Elghani and colleagues found SIAH3 aggregating with PINK1 in the mitochondria and inactivating it, leading to mitochondrial depolarisation and neuronal death (Abd Elghani *et al.*, 2022). Moreover, Abraham *et al.* (2024) showed that (mouse) Siah3 is transcriptionally induced by nerve growth factor deprivation, that it interacts with Parkin, negatively regulates mitophagy and thereby positively regulates axonal degeneration upon trophic deprivation upstream of caspase-3 activation. In addition to this neurological context, Yoon *et al.* described SIAH3 to be highly expressed in the renal cortex during chronic kidney disease, again interfering with the accumulation of PINK1 (Yoon *et al.*, 2021). These studies indicate that SIAH3 is involved in the regulation of mitochondrial quality control.

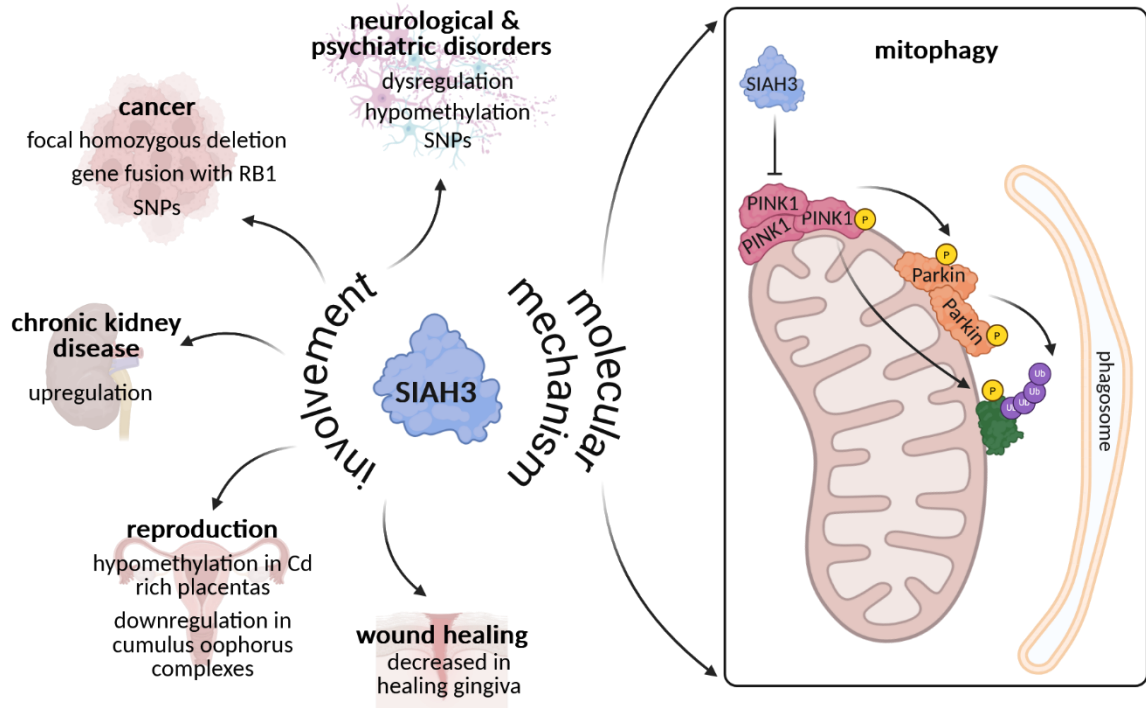


Fig. 4: The known functions of SIAH3

Published findings of SNPs, a genetic deletion or fusion within the SIAH3 region, as well as deregulation of expression and both hypo- and hypermethylation, show an involvement of the SIAH3 in several diseases and developmental processes. On a molecular level, SIAH3 (blue) plays a role in mitochondrial homeostasis by inhibiting PINK1 (magenta) accumulation on the mitochondrial membrane. Normally, PINK1 recruits and phosphorylates the ubiquitin ligase Parkin (orange) as well as other mitochondrial proteins. Activated Parkin ubiquitinates proteins on the mitochondrial outer membrane, such as VDAC1 and Mitofusin (green), which in turn leads to the recruitment of the autophagosome and the subsequent degradation of the mitochondrion. SIAH3 was also shown to interact with PINK1 and Parkin. ub = ubiquitin.

The figure was created with BioRender.com.

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1.4.2.2 *SIAH3* is hypermethylated in melanoma cell lines

Under healthy conditions *SIAH3* is predominantly weakly expressed, showing the highest expression in brain and fallopian tube (Fig. 5). But there are a few other tissues, in which *SIAH3* is expressed, even when it is on low levels, like in lung and skin.

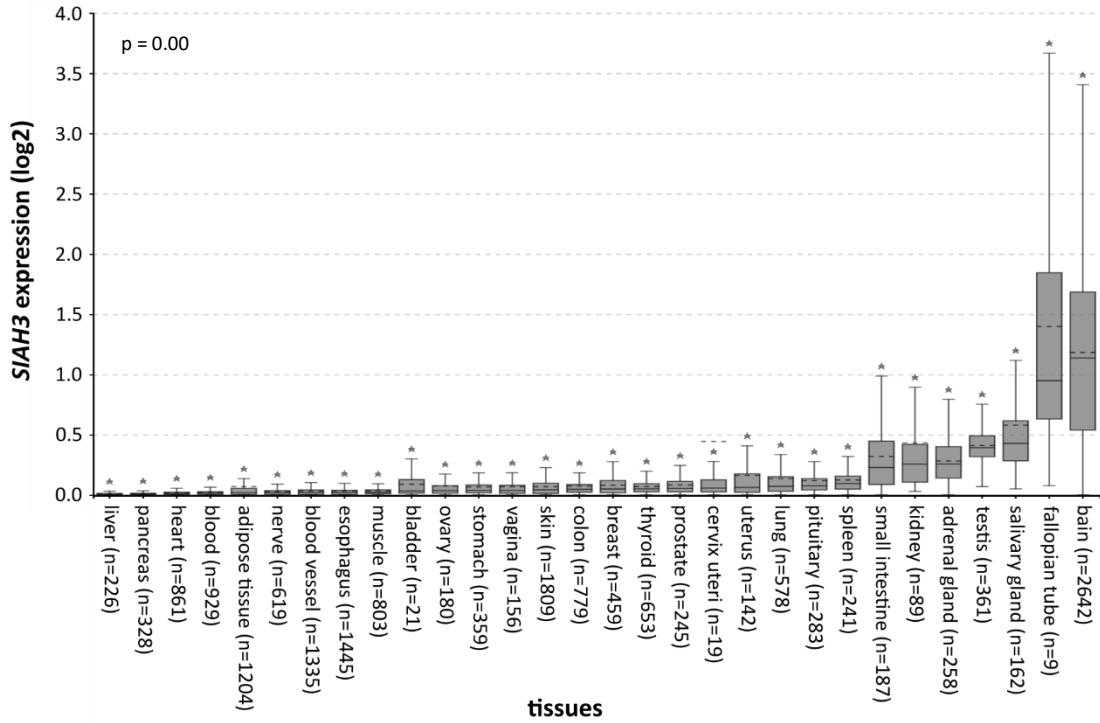


Fig. 5: Expression level of *SIAH3* in healthy tissues

Expression level of *SIAH3* (log2) in 30 different tissues. Used dataset: Normal Tissues GTEx v8 Prot-Coding, $n = 17382$, $p = 0.00$ (Anova). The stars represent high extreme values. The analysis was performed using R2.

One of the important mechanisms for the regulation of gene expression is – as mentioned above – DNA methylation. In literature, the methylation status of *SIAH3* is rarely described. Mohanty *et al.* (2015) published a hypomethylation of *SIAH3* in cadmium rich placentas of female infants and Wiegand *et al.* (2021) found a reduced DNA methylation level for *SIAH3* in persons with early life adversity. While these two studies described CpG sites within the first and second *SIAH3* exon, respectively, neither an analysis of the regulatory promoter region nor an assessment of whether differential methylation influences expression has been reported to date. However, after being identified as a potential hypermethylated TSG in melanoma, *SIAH3* came under investigation in our laboratory. Sara Walesch and Janine Moyer analysed the *SIAH3* methylation and gene expression status of *SIAH3* in several cell lines of different entities and in HUVEC cells. The *SIAH3* gene is located on chromosome 13 and regulated by a CpG rich promoter. For the methylation analysis a 156 bp PCR fragment around the transcription start site (TSS) was amplified and five CpG sites were analysed by quantitative bisulfite pyrosequencing (Fig. 6A). *SIAH3* mRNA level was found to be

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highest in C918 cells, followed by HUVECs, whereas A-549, MDA-MB-231 and HEK293T showed only very low or no expression (Fig. 6B). Conversely, C918 and HUVEC were unmethylated (< 20 %), while A-549 was partially (44 %) and MDA-MB-231 and HEK293T were fully methylated (> 90 %) (Fig. 6C), showing an inverse correlation of DNA methylation and gene expression.

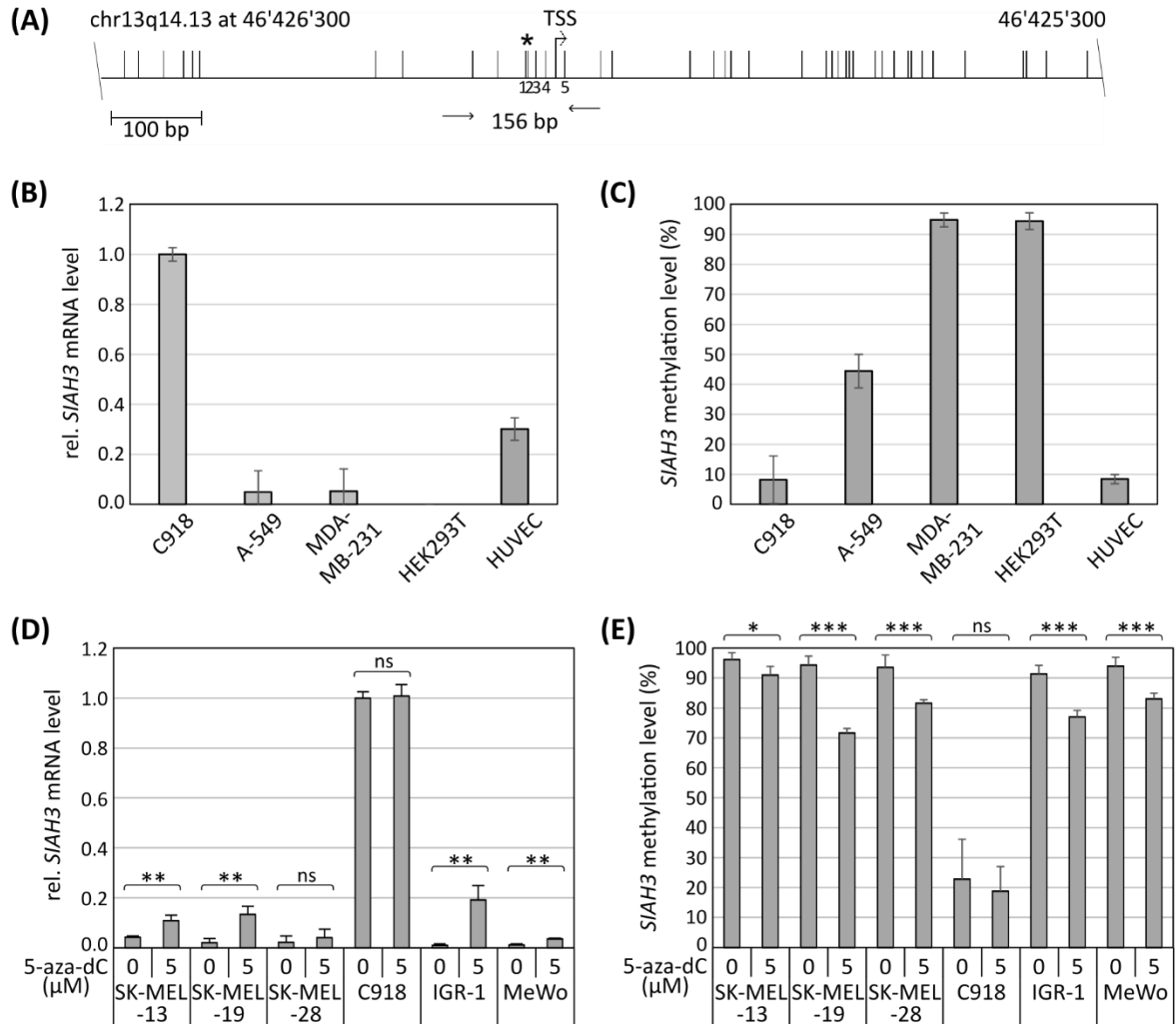


Fig. 6: Expression and promoter methylation of *SIAH3* in cancer cell lines

(A) Schematic overview of the *SIAH3* promoter located on 13q14.13. The CpG sites are depicted as vertical lines. The 156 bp fragment for methylation analysis is indicated and the 5 pyrosequenced CpGs are numbered. The CpG site 1 (annotated as cg26667946), that is also part of a *Bst*UI restriction site, is marked with an asterisk. TSS = transcriptional start site. **(B)** *SIAH3* expression in C918, A-549, MDA-MB-231, HEK293T and HUVEC. *SIAH3* and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels were analysed by RT-qPCR (reverse transcription quantitative PCR). *SIAH3* expression was normalised to *GAPDH*. C918 was set to 1. **(C)** Methylation of *SIAH3* in C918, A-549, MDA-MB-231, HEK293T and HUVEC. Genomic DNA was isolated and methylation of *SIAH3* was quantified by bisulfite pyrosequencing. Mean methylation levels (%) of 5 CpGs were plotted. **(D)** *SIAH3* expression in 6 melanoma cell lines. SK-MEL-13, SK-MEL-19, SK-MEL-28, C918, IGR-1 and MeWo were treated with 5 μ M 5-aza-dC for 4 days and RNA was isolated. *SIAH3* and *GAPDH* mRNA levels were analysed by RT-qPCR. *SIAH3* expression was normalised to *GAPDH*. 0 μ M C918 was set to 1. **(E)** Hypermethylation of *SIAH3* in 6 melanoma cell lines. Cell lines were treated with 5 μ M 5-aza-dC for 4 days and *SIAH3* methylation was compared to mock-treated cells (0 μ M). Genomic DNA was isolated and methylation of *SIAH3* was quantified by bisulfite pyrosequencing. Mean methylation levels (%) of the 5 CpGs were plotted. */**/** indicate the significance levels (Student's t-test). Experiments were conducted by S. Walesch and J. Moyer. Figure adapted from Deutschmeyer *et al.*, 2025.

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Analysis of melanoma cell lines revealed that five out of six did not express *SIAH3* and were fully methylated. Only C918, displaying reduced methylation (< 25 %), expressed *SIAH3* (Fig. 6D-E). Treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) yielded an induction of *SIAH3* expression in the cell lines SK-MEL-13, SK-MEL-19, SK-MEL 28 and IGR-1 and resulted in a demethylation of the *SIAH3* promoter of 5-20 % in these five cells lines, revealing the functional relevance of DNA methylation. These preliminary findings suggest that *SIAH3* is epigenetically regulated and silenced in cancer cell lines by promoter methylation.

1.5 Aim of this study

This work focuses on *SIAH3*, the third member of the *SIAH* family. While *SIAH1* and *SIAH2* are well studied players in tumorigenesis as well as in neurodegenerative diseases, *SIAH3* is barely investigated, even though there are hints of its relevance. To overcome this shortage, I explored its epigenetic regulation in cancer and the effect of *SIAH3* on cell proliferation. I investigated its influence on the transcriptome as well as on the proteome. Furthermore, I analysed its interaction with protein binding partners and *SIAH3*'s impact on cell metabolism. With these experiments I will answer the following questions: Is *SIAH3* silenced in tumours through DNA methylation? Do other regulatory mechanisms exist? Which effect does *SIAH3* have on tumour growth? Where is it localised and in which molecular pathways is *SIAH3* involved? And finally: Is it possible to restore inactivated *SIAH3*? This together aims to characterise the potential tumour suppressor *SIAH3* epigenetically and functionally. The insights gained should contribute to the understanding of cancer pathogenesis and may help to move one step closer to a better treatment of cancer patients in future.

2 MATERIALS and METHODS

2.1 Materials

All used materials are described in this chapter. Tab. 1 lists the companies from which the materials were obtained. Tab. 2-8 provide detailed information on all chemicals and reagents used, including antibiotics, enzymes, molecular weight standards, transfection reagents and inhibitors. Antibodies, plasmids, primers and sgRNAs are describes in Tab. 9-17 and Tab. 18-20 contain the information on the used organisms and tissue samples. Recipes for media, buffers and additional solutions are presented in Tab. 21-26. And Kits, instruments as well as software and used online tools are summarised in Tab. 27-29.

Tab. 1: List of companies and their headquarters location.

company	location
Abcam	Cambridge, Great Britain
Addgene	Watertown, MA, USA
Agilent Technologies	Santa Clara, CA, USA
Analytik Jena GmbH & Co. KG (former UVP)	Jena, Germany
AppliChem GmbH	Darmstadt, Germany
Aurox Ltd	Thame, UK
Aviva Systems Biology Corporation	San Diego, CA, USA
Bemis Company	Neenah, WI, USA
Berthold Detection Systems GmbH	Pforzheim, Germany
BioCat GmbH (transOMIC)	Heidelberg, Germany
BIOZOL Diagnostica Vertrieb GmbH	Hamburg, Germany
Biozym Scientific GmbH	Hessisch Oldendorf, Germany
Bio-Rad Laboratories	Hercules, CA, USA
BD Biosciences (Becton, Dickinson and Company)	Franklin Lakes, NJ, USA
Carl Roth GmbH & Co. KG	Karlsruhe, Germany
Carl Zeiss AG	Oberkochen, Germany
Capricorn Scientific GmbH	Ebsdorfergrund, Germany
Cell Signaling Technology	Danvers, MA, USA
ChromoTek GmbH	Planegg/Martinsried, Germany
Developmental Studies Hybridoma Bank (DSHB)	Iowa City, IA, USA
Dharmacon	Lafayette, CO, USA
Eppendorf SE	Hamburg, Germany
GE Healthcare Life Science / Bio-Science AB	Uppsala, Sweden
Hahnemühle FineArt GmbH	Dassel, Germany
Jackson ImmunoResearch Laboratories	West Grove, PA, USA
Life Technologies GmbH / Invitrogen (e.g. Gibco™), now Thermo Fisher Scientific	Darmstadt, Germany

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MACHEREY-NAGEL GmbH & Co. KG	Düren, Germany
Merck KGaA.	Darmstadt, Germany
Microsynth Seqlab GmbH	Göttingen, Germany
MOTIC Deutschland GmbH	Wetzlar, Germany
New England BioLabs GmbH	Frankfurt am Main, Germany
NIPPON Genetics EUROPE GmbH	Düren, Germany
PerkinElmer	Waltham, MA, USA
PEQLAB / VWR Life Science	Erlangen, Germany / Radnor, PA, USA
Promega GmbH	Walldorf, Germany
PromoCell GmbH	Heidelberg, Germany
QIAGEN (Corbett Research)	Venlo, Netherlands / Hilden, Germany
Roche Diagnostics GmbH (Roche Applied Science)	Penzberg, Germany
Santa Cruz Biotechnology	Dallas, TX, USA
Sarstedt AG & Co. KG	Nümbrecht, Germany
Serva Electrophoresis GmbH	Heidelberg, Germany
Sigma-Aldrich Chemie GmbH	Taufkirchen, Germany
Stratagene, now Agilent Technologies	La Jolla, CA, USA
Strattec Molecular GmbH	Berlin, Germany
Takara Bio USA (Clontech)	San Jose, CA, USA
Tecan Deutschland GmbH	Crailsheim, Germany
Thermo Fisher Scientific	Waltham, MA, USA / Dreieich, Germany

2.1.1 Chemicals

Tab. 2: Used chemicals and materials and the company from which they were obtained.

chemical	company
4',6-Diamidin-2-phenylindol (DAPI)	Serva Electrophoresis
agar-agar	Merck
LE agarose	Biozym Scientific
ammonium acetate	Carl Roth
ammonium persulfate (APS)	Serva Electrophoresis
ammonium sulphate (NH ₄) ₂ SO ₄	Carl Roth
Anti-FLAG® M2 Affinity Gel	Sigma-Aldrich
Bacto Tryptone	Becton, Dickinson and Company
betaine	Sigma-Aldrich
Blotting Grade Blocker, Non-Fat Dry Milk	Carl Roth
blotting paper (1.5 mm thickness)	Hahnemühle FineArt
boric acid	Carl Roth
bovine serum albumin (BSA)	Thermo Fisher Scientific
bromophenol blue	Carl Roth
β-mercaptoethanol	Sigma-Aldrich
chloroform	Carl Roth

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cOmplete™ tablettes, EASYpack, Protease Inhibitor Cocktail (EDTA-free version for MS)	Roche
Coomassie Brilliant Blue G250	Serva Electrophoresis
DEPC H ₂ O	Carl Roth
dimethyl sulfoxide (DMSO)	Carl Roth
disodium hydrogen phosphate (Na ₂ HPO ₄)	Carl Roth
dNTPs (dATP, dTTP, dGTP, dCTP)	Thermo Fisher Scientific
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific (GIBCO™) / Capricorn Scientific
EDTA	Carl Roth
ethanol	Merck
ethidium bromide	Carl Roth
FACS tube 5 mL, 75 x 12 mm, PS	Sarstedt
fetal bovine serum (FBS)	Thermo Fisher Scientific (GIBCO™)
formaldehyde (37 %)	Sigma-Aldrich
formamide	Carl Roth
glucose	Carl Roth
Glutathione Sepharose™ 4B	GE Healthcare Bio-Science
glycerol	Carl Roth
glycine	Carl Roth
glycogen	Thermo Fisher Scientific
guanidine hydrochloride (Gu-HCL)	Carl Roth
Immobilon Western Chemiluminescent HRP substrate	Merck (Millipore)
HEPES	Carl Roth
hydrochloric acid (HCl)	Carl Roth
hydroquinone	Merck
Immobilon-P membrane, PVDF, 0,45 µm	Merck (Millipore)
isopropyl alcohol	Merck
isopropyl β-D-thiogalactopyranoside (IPTG)	Merck
imidazole	Sigma-Aldrich
magnesium chloride (MgCl ₂)	Merck
methanol	Merck
Midori Green Xtra	NIPPON Genetics
MOWIOL™ 4-88 reagent	Merck (Calbiochem / Millipore)
Ni-NTA agarose beads	QIAGEN
N,N,N',N'-tetramethylethylenediamine (TEMED)	Carl Roth
NP-40	AppliChem
Opti-MEM®	Thermo Fisher Scientific (GIBCO™)
Parafilm M	Bemis Company
phenol	Carl Roth
phenol/chloroform/isoamyl alcohol	Carl Roth
Poly-L lysine solution (0,01 %)	Sigma-Aldrich
potassium chloride (KCl)	Carl Roth

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potassium dihydrogen phosphate (KH ₂ PO ₄)	Carl Roth
propidium iodide	Sigma-Aldrich
PyroMark Annealing Buffer	QIAGEN
PyroMark Binding Buffer	QIAGEN
PyroMark Control Oligo	QIAGEN
PyroMark Denaturation Solution	QIAGEN
PyroMark Gold Q24 Reagents	QIAGEN
PyroMark Wash Buffer	QIAGEN
PyroMark Q24 Cartridge	QIAGEN
PyroMark Q24 Plate	QIAGEN
PyroMark Vacuum Prep Filter Probe	QIAGEN
Ribolock (40 U/μl)	Thermo Fisher Scientific
Rotiphorese® Gel 30 (37,5:1)	Carl Roth
RPMI	Thermo Fisher Scientific (GIBCO™)
sodium dodecyl sulfate (SDS)	Carl Roth
sodium acetate (NaAC)	Merck
sodium chloride (NaCl)	Carl Roth
sodium deoxycholate	Sigma-Aldrich
sodium hydrogen phosphate (NaH ₂ PO ₄)	Carl Roth
sodium hydroxide (NaOH)	Carl Roth
sodium metabisulfite	Sigma-Aldrich
SYBR® Select Master Mix	Thermo Fisher Scientific
tris(hydroxymethyl)aminomethan (tris)	Carl Roth
Triton X100	Carl Roth
TRIzol™ Reagent	Thermo Fisher Scientific
Trypan Blue solution	Sigma-Aldrich
TrypLE™ Express (Trypsin)	Thermo Fisher Scientific (GIBCO™)
Tween20	Sigma-Aldrich
urea	Carl Roth
X-Gal	AppliChem
yeast extract	Carl Roth

2.1.2 Antibiotics

Tab. 3: Used antibiotics for *E. coli* and human cells and their working concentrations.

antibiotic	working concentration	company
for <i>E. coli</i>		
ampicillin	60-100 μg/mL	Sigma-Aldrich
kanamycin	50 μg/mL	AppliChem
for human cells		
Antibiotic-Antimycotic (100X)	penicillin 100 U/mL streptomycin 100 μg/mL amphotericin B 0.25 μg/mL	Thermo Fisher Scientific (GIBCO™)
blasticidin S	5 μg/mL	Carl Roth

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doxycycline	2 µg/mL	AppliChem
penicillin / streptomycin (PS)	penicillin 100 U/mL streptomycin 100 µg/mL	Thermo Fisher Scientific (GIBCO™)
puromycin	2-5 µg/mL	Carl Roth
Zeocin™ Selection Reagent	400 µg/mL	Thermo Fisher Scientific

2.1.3 Enzymes

Tab. 4: Restriction enzymes used for cloning and CoBRA (combined bisulfite restriction analysis) digestion (*Bst*UI). Enzymes were used together with the buffer recommended by the manufacturer.

restriction enzyme	company
<i>Bam</i> HI (10 U/µL)	Thermo Fisher Scientific
<i>Bbs</i> I (10 U/µL)	Thermo Fisher Scientific
<i>Bgl</i> II (10 U/µL)	Thermo Fisher Scientific
<i>Bst</i> UI (10 U/µL)	Thermo Fisher Scientific
<i>Eco</i> RI (10 U/µL)	Thermo Fisher Scientific
<i>Hind</i> III (10 U/µL)	Thermo Fisher Scientific
<i>Kpn</i> I (10 U/µL)	Thermo Fisher Scientific
<i>Sal</i> I (10 U/µL)	Thermo Fisher Scientific
<i>Xho</i> I (10 U/µL)	Thermo Fisher Scientific

Tab. 5: Additional enzymes used during cloning, DNA isolation, reverse transcription and PCR. Enzymes were used together with the buffer recommended by the manufacturer.

enzyme	company
DNaseI (1 U/µL)	Thermo Fisher Scientific
FastAP, thermosensitive (1 U/µL)	Thermo Fisher Scientific
M-MLV Reverse Transcriptase (200 U/µL)	Promega
Phusion High-Fidelity DNA-Polymerase (2 U/µL)	Thermo Fisher Scientific
Proteinase K (20 µg/µL)	Thermo Fisher Scientific
RNase A (10 mg/mL)	Thermo Fisher Scientific
<i>Taq</i> Polymerase	AG Dammann, homemade
T4 DNA Ligase (3 U/µL)	Thermo Fisher Scientific
T4 Polynucleotide Kinase (PNK) (10 U/mL)	Thermo Fisher Scientific

2.1.4 Molecular weight standards

Tab. 6: DNA molecular weight standards and loading dyes used for agarose gel electrophoresis and the protein molecular weight standard used for SDS-PAGE.

product	company
GeneRuler 100 bp DNA Ladder	Thermo Fisher Scientific
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific
6x DNA Loading dye	Thermo Fisher Scientific
PageRuler Prestained Protein Ladder	Thermo Fisher Scientific

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2.1.5 Transfection reagents

Tab. 7: Reagents used for the transfection of human cell lines

product	company
Lipofectamine™ RNAiMax transfection reagent	Thermo Fisher Scientific / Invitrogen
polyethylenimin, branched (PEI)	Sigma-Aldrich
TurboFect™ transfection reagent	Thermo Fisher Scientific

2.1.6 Inhibitors

Tab. 8: Inhibitors used in cell culture experiments

product	company
α-amanitin	AppliChem
5-aza-2'-deoxycytidine (5-aza-dC)	Sigma-Aldrich
MG-132	Thermo Fisher Scientific

2.1.7 Antibodies

Tab. 9: Primary antibodies used for Western blot (WB) and immunofluorescence (IF)

antibody	origin	dilution		company
		WB	IF	
FLAG (M2, F3165)	mouse	1:200 - 1:500	-	Sigma-Aldrich
FLAG (clone 118 ZooMAb®)	rabbit	-	1:1,000	Sigma
GAPDH (sc-47724)	mouse	1:500	-	Santa Cruz Biotechnology
GAPDH (FL-335)	rabbit	1:2,000	-	Santa Cruz Biotechnology
GAPDH (14C10)	rabbit	1:2,000	-	Cell Signaling
GFP	rabbit	1:1,000 - 1:2,000	-	AG Renkawitz, JLU Gießen, homemade
HA (3F10, high affinity)	rat	1:1,000	1:1,000	Roche
mCherry / dsRed (632496)	rabbit	1:1,000	-	Clontech / Takara
Myc (631206)	mouse	-	1:500	BD Biosciences
p53 (Do-1, sc-126)	mouse	1:1,000	-	Santa Cruz Biotechnology
SIAH3 (ARP69564_P050)	rabbit	1:500	-	Aviva Systems Biology (via BIOZOL)
β-Tubulin (E7)	mouse	1:1,000	-	DSHB
YAP (H-125, sc-15407)	rabbit	1:200	-	Santa Cruz Biotechnology

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Tab. 10: Secondary antibodies used for WB and IF

antibody	origin	dilution		company
		WB	IF	
m-IgGk BP-HRP (anti-mouse) (sc-516102)	recombinant	1:1,000 - 1:2,000	-	Santa Cruz Biotechnology
anti-rabbit IgG-HRP (sc-2357)	mouse	1:1,000 - 1:2,000	-	Santa Cruz Biotechnology
anti-rabbit HRP-linked (#7074)	goat	1:1,000 - 1:2,000	-	Cell Signaling
anti-rat HRP-linked (112-035-143)	goat	1:3,000	-	Jackson ImmunoResearch (via BIOZOL)
Alexa Fluor™ Plus 488 anti-mouse IgG (H+L) (A32723TR)	goat	-	1:500	Thermo Fisher Scientific
Alexa Fluor™ 568 anti-mouse IgG (H+L) (A11004)	goat	-	1:500	Thermo Fisher Scientific
Alexa Fluor™ 594 anti-rat IgG (H+L) (A-11007)	goat	-	1:500	Thermo Fisher Scientific
Alexa Fluor™ 568 anti-rabbit IgG (H+L) (A11011)	goat	-	1:500	Thermo Fisher Scientific

2.1.8 Plasmids

Tab. 11: Empty plasmids used for cloning and/or as controls in experiments

name / backbone	tag	resistance	producer
pCMV-Tag1 (EcoRI restriction site instead of stop-codon)	FLAG (N-term.) & Myc (C-term.)	kanamycin	Stratagene (mod. by A. Richter)
pcDNA3	-	ampicillin	Invitrogen
pEBG	GST (N-term.)	ampicillin	Gerd Pfeifer Lab
pEYFP-C1	EYFP (N-term.)	kanamycin	Clontech
pEYFP-C2	EYFP (N-term.)	kanamycin	Clontech
pEGFP-C2	EYFP (N-term.)	kanamycin	Clontech
pEGFP-N3	EGFP (C-term.)	kanamycin	Clontech
pcDNA3-mCherry	mCherry (N-term.)	ampicillin	A. Jimenez / V. Deutschmeyer
pcDNA4/TO/myc-His A/B	Myc-His(6x) (C-term.)	ampicillin	Invitrogen
pGL3	Firefly Luciferase (C-term.)	ampicillin	Promega

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pRL-null	<i>Renilla</i> Luciferase (C-term.)	ampicillin	Promega
pSpCas9 (BB)-2A-Puro V2.0 / pX459 (<i>Bg</i> /II inserted; Δ Cas9)	3xFLAG (N-term.)	ampicillin	Addgene (mod. by T. Zenz)
pGEM-T Easy	-	ampicillin	Promega
pJet1.2/blunt	-	ampicillin	Thermo Fisher Scientific

Tab. 12: Plasmids used for transfection of human cells. Corresponding plasmid maps and insert sequences are available in supplementary chapter 6.1, except for vectors obtained from collaboration partners or vectors that were already available in the laboratory prior to this study.

name	vector (backbone)	insert / Tag	resistance	producer/ cloned by
EYFP-SIAH3	pEYFP-C2 (<i>Eco</i> RI inserted)	SIAH3 Wt	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Δ S3UNS	pEYFP-C2	SIAH3 Δ S3UNS	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Δ SZF	pEYFP-C2	SIAH3 Δ SZF	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Δ SBS	pEYFP-C2	SIAH3 Δ SBS	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Δ DIMER	pEYFP-C2	SIAH3 Δ DIMER	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Δ Bind	pEYFP-C2	SIAH3 Δ DIMER+ Δ SBS	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Ubi1	pEYFP-C2	SIAH3 K23R	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Ubi2	pEYFP-C2	SIAH3 K25R	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Ubi3	pEYFP-C2	SIAH3 K45R	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Ubi4	pEYFP-C2	SIAH3 K173R	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Ubi5	pEYFP-C2	SIAH3 K213R	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Ubi1-2	pEYFP-C2	SIAH3 K23R, K25R	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Ubi1-3	pEYFP-C2	SIAH3 K23R, K25R, K45R	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Ubi1-4	pEYFP-C2	SIAH3 K23R, K25R, K45R, K173R	kanamycin	V. Deutschmeyer
EYFP-SIAH3 Ubi1-5	pEYFP-C2	SIAH3 K23R, K25R, K45R, K173R, K213R	kanamycin	V. Deutschmeyer
SIAH3-EGFP	pEGFP-N3	SIAH3 Wt	kanamycin	V. Deutschmeyer
FLAG-SIAH3	pCMV-Tag1	SIAH3 Wt	kanamycin	V. Deutschmeyer
GST-FLAG-SIAH3	pEBG	SIAH3 Wt	ampicillin	V. Deutschmeyer
GST-FLAG-SIAH3 Δ S3UNS	pEBG	SIAH3 Δ S3UNS	ampicillin	V. Deutschmeyer
GST-FLAG-SIAH3 Δ SZF	pEBG	SIAH3 Δ SZF	ampicillin	V. Deutschmeyer
GST-FLAG-SIAH3 Δ SBS	pEBG	SIAH3 Δ SBS	ampicillin	V. Deutschmeyer
GST-FLAG-SIAH3 Δ DIMER	pEBG	SIAH3 Δ DIMER	ampicillin	V. Deutschmeyer

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GST-FLAG-SIAH3 ΔBind	pEBG	SIAH3 ΔDIMER+ ΔSBS	ampicillin	V. Deutschmeyer
mCherry-SIAH3	pcDNA3	SIAH3 Wt	ampicillin	V. Deutschmeyer
EGFP-SIAH3	pEGFP-C2	SIAH3 Wt	kanamycin	S. Walesch / J. Moyer
SIAH3 (Tet-on)	pcDNA4/TO/myc- His B	SIAH3 Wt (not in frame with tags)	ampicillin	S. Walesch
SIAH3 ΔS3UNS (Tet-on)	pcDNA4/TO/myc- His B	SIAH3 Wt (not in frame with tags)	ampicillin	V. Deutschmeyer
SIAH3-Myc	pcDNA4/TO/myc- His A	SIAH3 Wt	ampicillin	V. Deutschmeyer
SIAH3 ΔS3UNS-Myc	pcDNA4/TO/myc- His A	SIAH3 ΔS3UNS	ampicillin	V. Deutschmeyer
SIAH3-promoter	pRL-null	SIAH3 promoter from HEK293T	ampicillin	V. Deutschmeyer
FLAG-SIAH1 WT	pCMV-Tag1	SIAH1 Wt	kanamycin	V. Deutschmeyer
FLAG-SIAH1 Rm	pCMV-Tag1	SIAH1 Rm C44S	kanamycin	V. Deutschmeyer
HA-SIAH1 Wt	pcDNA3.1	SIAH1 Wt	ampicillin	gift from AG Schmitz
HA-SIAH1 Rm	pcDNA3.1	SIAH1 Rm C44S	ampicillin	gift from AG Schmitz
mCherry-SIAH1 Wt	pcDNA3	SIAH1 Wt	ampicillin	V. Deutschmeyer
mCherry-SIAH1 Rm	pcDNA3	SIAH1 Rm C44S	ampicillin	V. Deutschmeyer
FLAG-SIAH2 WT	pCMV-Tag2B	SIAH2 Wt	kanamycin	gift from AG Schmitz
FLAG-SIAH2 Rm	pCMV-Tag2B	SIAH2 Rm H98A & C101A	kanamycin	gift from AG Schmitz
(HA)3-SIAH2 Rm	pcDNA3.1	SIAH2 Rm H98A & C101A	ampicillin	gift from AG Schmitz
mCherry-SIAH2 Wt	pcDNA3	SIAH2 Wt	ampicillin	V. Deutschmeyer
mCherry-SIAH2 Rm	pcDNA3	SIAH2 Rm H98A & C101A	ampicillin	V. Deutschmeyer
CHCHD4-EGFP	pEGFP-N3	CHCHD4	kanamycin	V. Deutschmeyer
FLAG-CHCHD4	pFLAG-CMV2	CHCHD4	ampicillin	V. Deutschmeyer
FLAG-LATS2	pcDNA3	LATS2	ampicillin	C. Seidel
mitoGFP	pLV	mitoch. targeting sequence of Cox8 + EGFP	ampicillin	gift from AG Schmitz
(His)6-HA-Ubi	pCMV	ubiquitin	ampicillin	gift from AG Schmitz
EGFP-STUB1	pEGFP-N3	STUB1	kanamycin	N. Schlaudraff
FLAG-STUB1	pCMV-Tag1	STUB1	kanamycin	N. Schlaudraff
TIMM50-EGFP	pEGFP-N3	TIMM50	kanamycin	V. Deutschmeyer

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Tab. 13: Plasmids used for epigenetic editing. Plasmid maps and sequences of the *SIAH3* guides are available in the supplementary chapter 6.1.2.8.

name	vector	insert	resistance	producer/ cloned by
p300	pcDNA-dCas9- p300 Core	dCas9-p300Core	ampicillin	Addgene (#61357)
VP160 (del U6)	pAC154-dual- dCas9VP- sgExpression	dCas9-VP160 (deletion of U6 promoter)	ampicillin	Addgene (#48240), (mod. by S. Walesch & F. Völker)
DME (demethylation enzyme)	Novel demethylation enzyme under non-disclosure agreement			
Ezh2	Ezh2[SET]-dCas9 (pcDNA3.3-TOPO)	Ezh2-dCas9	ampicillin	Addgene (#100087)
DNMT3A	DNMT3A-dCas9 (pcDNA3)	DNMT3A-dCas9	ampicillin	Addgene (#100090)
DNMT3L	pcDNA3/Myc- DNMT3L	Myc-DNMT3L	ampicillin	Addgene (#35523)
SIAH3-guide I	pX459-ΔCas9	SIAH3 sgRNA I	ampicillin	T. Zenz
SIAH3-guide II	pX459-ΔCas9	SIAH3 sgRNA II	ampicillin	T. Zenz
SIAH3-guide III	pX459-ΔCas9	SIAH3 sgRNA III	ampicillin	T. Zenz
SIAH3-guide IV	pX459-ΔCas9	SIAH3 sgRNA IV	ampicillin	F. Geyer
SIAH3-guide V	pX459-ΔCas9	SIAH3 sgRNA V	ampicillin	F. Geyer
SIAH3-guide VI	pX459-ΔCas9	SIAH3 sgRNA VI	ampicillin	F. Geyer
ctrl. guide	pX459-ΔCas9	none targeting sgRNA	ampicillin	A-K. Hinrichs

2.1.9 Primers

Tab. 14: Primers used for reverse transcription (RT) and quantitative PCR (qPCR)

name	sequence 5' - 3'	usage	gene	temp.	product
Hexamer	NNNNNN	RT	-	-	-
poly-dT	TTTTTTTTTTTTTTTT	RT	-	-	-
bACTRTFW	CCTTCCTTCCTGGGCATGGAGT C	qPCR	<i>ACTB</i>	60 °C	226 bp
bACTRTRV	CGGAGTACTTGCCTCAGGAG GA	qPCR	<i>ACTB</i>		
ANKRD1RTF1	AGCGCCCGAGATAAGTTGCT	qPCR	<i>ANKRD1</i>	60 °C	240 bp
ANKRD1RTR1	CACCAGATCCATCGGCGTCT	qPCR	<i>ANKRD1</i>		
CCN1RTF	AGCAGCCTGAAAAAGGGCA	qPCR	<i>CCN1</i>	60 °C	176 bp
CCN1RTR	CGGAACCGCATCTTCACAGT	qPCR	<i>CCN1</i>		
CTGFRTF1	CATCTTCGGTGGTACGGTGT	qPCR	<i>CTGF</i>	60 °C	295 bp
CTGFRTR1	GACCAGGCAGTTGGCTCTAA	qPCR	<i>CTGF</i>		
DUSP1RTF1	GTAAGCGTCCCTGACAGC	qPCR	<i>DUSP1</i>	60 °C	163 bp
DUSP1RTR1	ATCAAGGCAGTGATGCCCAA	qPCR	<i>DUSP1</i>		

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EAF2RTF1	CAGGCTGAGGTGGCAGATAG	qPCR	<i>EAF2</i>	60 °C	83 bp
EAF2RTR1	AATCCCGCTGCGCTATTCAT	qPCR	<i>EAF2</i>		
EGR1RTFW	GTCCAGCTCATCAAACCA	qPCR	<i>EGR1</i>	60 °C	294 bp
EGR1RTRV	TCATCGCTCCTGGCAAACCT	qPCR	<i>EGR1</i>		
FILIP1LRTF1	TCAGTGCCAAGCATGCGATA	qPCR	<i>FILIP1L</i>	60 °C	342 bp
FILIP1LRTR1	AGGGTGAGCGTGGTCAGTTA	qPCR	<i>FILIP1L</i>		
hGadd45aF	CTGTGAGTGAGTGCAGAAAGC	qPCR	<i>GADD45A</i>	60 °C	123 bp
hGadd45aR	CCAGCCGAGAATTCCTCCAAA	qPCR	<i>GADD45A</i>		
UGAP389	TGGAGAAGGCTGGGGCTCAT	qPCR	<i>GAPDH</i>	60 °C	176 bp
LGAP545	GACCTTGCCAGGGGTGCTA	qPCR	<i>GAPDH</i>		
KLF10RTF1	TCGGTGCCTCTCTCCAGCAG	qPCR	<i>KLF10</i>	60 °C	147 bp
KLF10RTR1	TCCAAGTGCAGCTCATTGACAT AAG	qPCR	<i>KLF10</i>		
MXD1RTF1	AAATCGACCAGCTTCAGCGA	qPCR	<i>MXD1</i>	60 °C	137 bp
MXD1RTR1	AACGTCGATTCCCTGTCGG	qPCR	<i>MXD1</i>		
PLK2RTF1	CCGCCTCCCGTATATAAGAC	qPCR	<i>PLK2</i>	60 °C	199 bp
PLK2RTR1	GCTGGTAGGTGATAGTCCGC	qPCR	<i>PLK2</i>		
SIAH1V1RTF1	GCTCTCCGCCACAGAAAT	qPCR	<i>SIAH1</i> , variant 1, endogenous	60 °C	232 bp
SIAH1V1RTR1	CAACATGTGAGCTTTGGGCG	qPCR	<i>SIAH1</i> , variant 1, endogenous		
FLAG-SIAH1RT F1	TGGATTACAAGGATGACGACGA	qPCR	<i>FLAG-SIAH1</i> , overexpression	60 °C	102 bp
FLAG-SIAH1RT R1	GGGATGGTGGACACTTCGAG	qPCR	<i>FLAG-SIAH1</i> , overexpression		
SIAH2RTF1	CTCAACTTCTGGCGGCATTG	qPCR	<i>SIAH2</i>	60 °C	98 bp
SIAH2RTR1	CTCTTCGAGTGTCGGGTCTG	qPCR	<i>SIAH2</i>		
SIAH3 RTF1	CGCTGCCGGGCAACTTGTCTG	qPCR	<i>SIAH3</i>	60 °C	147 bp
SIAH3 RTR1	TGGTGGCGGTGGTGGCAGTG	qPCR	<i>SIAH3</i>		
SIAH3 RTF2	ACAGAAACCATCGGCGCCTC	qPCR	<i>SIAH3</i>	60 °C	172 bp
SIAH3 RTR2	AGGACCTCTGTCGCGGTGAT	qPCR	<i>SIAH3</i>		
SQSTM1RTF1	CTCACCTTTCTGGCCGCTGA	qPCR	<i>SQSTM1</i>	60 °C	250 bp
SQSTM1RTR1	CTGGAAAAGGCAACCAAGTCC	qPCR	<i>SQSTM1</i>		
VEGFARTF2	CACCATGCAGATTATGCGGA	qPCR	<i>VEGF</i>	60 °C	172 bp
VEGFARTR2	CCGGGATTTCTTGCCTTTC	qPCR	<i>VEGF</i>		

Tab. 15: Primers used for CoBRA and pyrosequencing. BIO = biotinylated version for PCR for pyrosequencing, Pyro-seq. = pyrosequencing.

name	sequence 5' - 3'	usage	gene	temp.	product
SIAH3BSU1	AAGGATAATATTTTGTTCCTT	BS (PCR 2)	<i>SIAH3</i>	53 °C	156 bp
SIAH3BSL1 (BIO)	AAAAACTATAAATCCTTAAACC CTAAAAA				

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SIAH3BSU2	GGAAGGTATTTGTTATTGGTTG TTTTTGT	BS (PCR 1)	<i>SIAH3</i>	51 °C	236 bp
SIAH3BSL2	AAACATCACAACTTTTAAAAAA TTATTAATCC				
SIAH3BSeq1	GTTTTATTTTGATGATGAATATG AAG	Pyro- seq.	<i>SIAH3</i>	-	-

Tab. 16: Primers used for cloning and mutagenesis. CDS = coding sequence, ORF = open reading frame.

name	sequence 5' - 3'	usage	gene / purpose	temp.	product
SIAH3PBamU1	CGGGATCCTGTGCTTCCT CCACCCCTCA	cloning	amplifying of <i>SIAH3</i> promoter from HEK293T DNA	62 °C	738 bp
SIAH3PEcoL1	GGAATTCTTTTGGGGGGT TGTTGGTCCG				
SIAH3RTECOF1	GAATTCATGCTTTTCTTTAC CCAGTGCTTTGG	cloning	amplifying of <i>SIAH3</i> CDS from C918 cDNA (S. Walesch/J. Moyer)	61 °C	822 bp
SIAH3RTXHOR1	CTCGAGTCACATTTAGCT TCTGAGGGGAGG				
SIAH3InsU1	ATGGCTCGAGTTTTTCAGC AAGAATGCTTTTCTTTACC CAGTGC	mutage- nesis	correction of <i>SIAH3</i> CDS	60 °C	whole plasmid
SIAH3InsL1	CAAAGCACTGGGTAAAGA AAAGCATTCTTGCTGAAAA ACTCG				
Mut_pEYFP- SIAH3_EcoRI_F1	CGGACTCAGATCTCGAGTT TTTCAGAATTCATGCTTTTC TTTACCCAGTGCTTTG	mutage- nesis	insertion of an <i>EcoRI</i> restriction site in pEYFP vector	60 °C	whole plasmid
Mut_pEYFP- SIAH3_EcoRI_R1	CAAAGCACTGGGTAAAGA AAAGCATGAATTCTGAAA AACTCGAGATCTGAGTCC G				
mCh-SIAH3- delG_F1	CGGCCGCCAGTGTGCTGA ATTCATGCTTTTCTT	mutage- nesis	deletion 1 bp -> in frame with tag	60 °C	whole plasmid
mCh-SIAH3- delG_R1	AAGAAAAGCATGAATTCA GCACACTGGCGGCCG				
pcDNA4myc-S3- delStop_F1	CTCAGAAGCTGAAATGCTC GAGTCTAGAGGGC	mutage- nesis	deletion of stop-codon (ORF with C- term. Tag)	60 °C	whole plasmid
pcDNA4myc-S3- delStop_R1	GCCCTCTAGACTCGAGCAT TTCAGCTTCTGAG				
pcDNA4TO- S3dS3UNS- insATG_F1	GACACATACTTTAGGTTGT GCATGAATTCTGAAAACT CGAGC	mutage- nesis	insertion of ATG in cloned <i>SIAH3ΔS3UNS</i>	60 °C	whole plasmid

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pcDNA4TO-S3dS3UNS-insATG_R1	GCTCGAGTTTTTCAGAATT CATGCACAACCTAAAGTAT GTGTC		Tet-on construct		
pcDNA4_del4bp_F1	TCGAGTCTAGAGGGCCCT TCGAACAAAACTCATC	mutagenesis	B- to A-frame	60 °C	whole plasmid
pcDNA4_del4bp_R1	GATGAGTTTTTGTTCTGAAG GGCCCTCTAGACTCGA				
S3-EGFPdelStopF1	CCCTCAGAAGCTGAAATG CTCGACGGTACCG	mutagenesis	deletion of stop-codon (ORF with C-term. Tag)	60 °C	whole plasmid
S3-EGFPdelStopR1	CGGTACCGTCGAGCATTTTC AGCTTCTGAGGG				
FLAG-SIAH3dS3UNS_F1	TGGACACATACTTTAGGTT GTGGAATTCTAAGATCTTA TCGTCG	mutagenesis	deletion of S3UNS domain (FLAG construct)	60 °C	whole plasmid
FLAG-SIAH3dS3UNS_R1	CGACGATAAGATCTTAGA ATTCCACAACCTAAAGTAT GTGTCCA				
SIAH3delSZFU1	CTGGAGGATGTCAACCGT CACCGGGTTGGC	mutagenesis	deletion of SZF domain	60 °C	whole plasmid
SIAH3delSZFR1	GCCAACCCGGTGACGGTT GACATCCTCCAG				
SIAH3delSBSU1	AGGAGTGCATGATGATCC TATGGATCTGCCGC	mutagenesis	deletion of SBS domain	60 °C	whole plasmid
SIAH3delSBSL1	GCGGCAGATCCATAGGAT CATCATGCACTCCT				
SIAH3delDIMERU1	CCGTTGTCAGAGAAGGCC TGGGTGGGGG	mutagenesis	deletion of DIMER domain	60 °C	whole plasmid
SIAH3delDIMERL1	CCCCACCCAGGCCTTCTC TGACAACGG				
SIAH3mK23R_F1	CCCGTTTAGCCCTGTAGTG CTGAAACCGGAGA	mutagenesis	mutation of ubiquit. site 1 (K to L)	60 °C	whole plasmid
SIAH3mK23R_R1	TCTCCGGTTTCAGCACTAC AGGGCTAAACGGG				
SIAH3mK25R_F1	GGAGAAAACCCGTCTAGC CTTGTAGTGCTGAAACCG	mutagenesis	mutation of ubiquit. site 2 (K to L)	60 °C	whole plasmid
SIAH3mK25R_R1	CGGTTTCAGCACTACAAG GCTAGACGGGTTTCTCC				
SIAH3mK45R_F1	CCGACTGGACACATACCTT AGGTTGTGTGTGGG	mutagenesis	mutation of ubiquitinat. site 3 (K to L)	60 °C	whole plasmid
SIAH3mK45R_R1	CCCACACACAACCTAAGGT ATGTGTCCAGTCGG				
SIAH3mK173R_F1	CATGCCTCTCCTGTCTCCTC AGCACCAACAG	mutagenesis	mutation of ubiquit. site 4 (K to L)	60 °C	whole plasmid
SIAH3mK173R_R1	CTGTTGGTGCTGAGGAGA CAGGAGAGGCATG				

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SIAH3mK213R_F1	GTGGCCTCCACCTGAGG CGCCGAT	mutagenesis	mutation of ubiquit. site 5 (K to L)	60 °C	whole plasmid
SIAH3mK213R_R1	ATCGGCGCCTCAGGTGGG AGGCCAC				
S3Ubi12F1	GAGAAAACCCGTCTAGCC CTGTAGTGCTGAAACCGG AGATGA	mutagenesis	mutation of ubiquit. site 1+2 (K to L)	60 °C	whole plasmid
S3Ubi12R1	TCATCTCCGGTTTCAGCAC TACAGGGCTAGACGGGTT TTCTC				
CHCHD4UBAM1	GGATCCATGTCCTATTGCC GGCAGG	cloning	amplifying of <i>CHCHD4</i> CDS from HEK293T cDNA	61-63 °C	442 bp
CHCHD4LECO1	GAATTCATTAACCTTGATCC CTCCTCTTCTT				
CHCHD4-EGFPdelSTOPTAF1	CCGTCGACTGCAGAATTAC TTGATCCCTCCTCTT	mutagenesis	deletion of stop-codon -> in frame with C-term. EGFP-tag	60 °C	whole plasmid
CHCHD4-EGFPdelSTOPTAR1	AAGAGGAGGGATCAAGTA ATTCTGCAGTCGACGG				
Mut_HA-SIAH1_WT_a-t_F1	ATGGCCACTCTGACATTGA AGAATGGGCGGTAAC	mutagenesis	correction of <i>SIAH1</i> CDS back to Wt	60 °C	whole plasmid
Mut_HA-SIAH1_WT_a-t_R1	GTTACCGCCCATTCTTCAA TGTCAGAGTGGCCAT				
Mut_HA-SIAH1_Sall_F1	CTAGACTAGCTAGCTAGG TAGTCGACCGCTCAACACA TGGAAATA	mutagenesis	insertion of <i>Sall</i> restriction site downstream of <i>SIAH1</i> CDS	60 °C	whole plasmid
Mut_HA-SIAH1_Sall_R1	TATTTCCATGTGTTGAGCG GTCGACTACCTAGCTAGCT AGTCTAG				
mCh-SIAH1Wt-delT-ATG_F1	GTCTGACGGCTCATTCCCT TGTACAGCTCGTCCAT	mutagenesis	bringing in frame with N-term. mCherry-tag, correction CCG to ATG	60 °C	whole plasmid
mCh-SIAH1Wt-delT-ATG_R1	ATGGACGAGCTGTACAAG GGAATGAGCCGTCAGAC				
SIAH1-WTzuRM_F1	GGTAACACATAGTCAAAG CTGACTGGACACTCAAAA AGA	mutagenesis	mutagenesis of Wt to Rm (C44S / T130A)	60 °C	whole plasmid
SIAH1-WTzuRM_R1	TCTTTTGTAGTGTCC AGTCAGCTTTGACTATGTG TTACC				

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STUB1RTECO1	GAATTCATGAAGGGCAAG GAGGAGAAGGAG	mutagenesis	amplifying of <i>STUB1</i> CDS from HEK293T cDNA (N. Schaudraff)	70 °C	924 bp
STUB1RTXHO1	CTCGAGTCAGTAGTCCTCC ACCCAGCCATT				
mCh-STUB1-delG_F1	GGCCGCCAGTGTGCTGAA TTCATGAAGGGC	mutagenesis	bringing in frame with mCherry-tag	60 °C	whole plasmid
mCh-STUB1-delG_R1	GCCCTTCATGAATTCAGCA CACTGGCGGCC				
STUB1delSTOP_F1	GGTGGAGGACTACCTCGA CGGTACCG	mutagenesis	deletion of stop-codon (ORF with C-term. Tag)	60 °C	whole plasmid
STUB1delSTOP_R1	CGGTACCGTCGAGGTAGT CCTCCACC				
TIMM50BGLF1	AGATCTATGGCGGCCTCG GCAGCGGT	mutagenesis	amplifying of <i>TIMM50</i> CDS from HEK293T cDNA	70-72 °C	1071 bp
TIMM50SALR1	GTCGACGGGCTGTTTGA GCGAGGCC				

2.1.10 sgRNAs for epigenetic editing

Tab. 17: Oligonucleotide sequences for cloning sgRNAs in pX459-ΔCas vector. The 20-21 nt target sequences (sense orientation) are shown in bold. sgRNAs recruit the Sp(d)Cas9 coupled with a modifier to the *SIAH3* locus. fwd = forward, rev = reverse.

name		sequence 5' - 3'	former name / description
SIAH3 guide I	fwd	CACCGCTTTTGGGGGTTGTTGGTC	guide 5
	rev	AAAC GACCAACAACCCCCAAAGC	
SIAH3 guide II	fwd	AAACGCTTAGCGCGCCTTCATATTC	guide 1
	rev	CACCGA AATATGAAGGCGCGCTAAGC	
SIAH3 guide III	fwd	CACCGCAGTGAGGCGGCAATCAAA	guide 4
	rev	AAACT TTTGATTGGCCGCCTCACTGC	
SIAH3 guide IV	fwd	CACCGCCCTGATGAATTGAGCCTGG	guide D
	rev	AAAC CCAGGCTCAATTCATCAGGGC	
SIAH3 guide V	fwd	CACCGT CCATCTGCCTACTTTGGAG	guide C
	rev	AAACCTCCAAAGTAGGCAGATGGAC	
SIAH3 guide VI	fwd	CACCGGAGAGTAAGATTTATCAAG	guide B
	rev	AAACCTTGATAAATCTTACTCTCC	
ctrl. guide	fwd	CACCG CAAAATTGCAAGCGTCAAAG	non-targeting sequence (in human)
	rev	AAACCTTTGACGCTTGCAATTTTG	

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2.1.11 Organisms

Cloning and amplifying of plasmids was done using chemically competent *Escherichia coli* (*E. coli*) (Tab. 18). The further experiments were conducted using human cell lines (Tab. 19). All cell lines were authenticated using short tandem repeat (STR) profiling within the last 3 years, HUVEC were obtained from PromoCell. T-REx-293 were obtained from Invitrogen / Thermo Fisher Scientific. All experiments were performed with mycoplasma-free cells.

Tab. 18: *E. coli* strains used for cloning and amplifying of plasmids

strain	producer
DH5α	homemade AG Dammann
XL10-Gold	homemade AG Dammann
XL10-Gold Ultracompetent	Agilent Technologies

Tab. 19: Cell lines used for analysis and experiments. Tet = tetracycline

entity	cell line	RRID	description	medium
kidney	HEK293T	CVCL_0063	human, embryonic kidney	DMEM
	T-REx-293	CVCL-D585	human, embryonic kidney, stably expressing tetracycline-regulated repressor	DMEM (Tet free)
	T-REx SIAH3 Wt (clone 6+8+15)	derived from T-REx-293	T-REx-293 cells stably expressing SIAH3 Wt upon tetracycline/ doxycycline induction	DMEM (Tet free)
	T-REx SIAH3 ΔS3UNS (clone 1+3+7)	derived from T-REx-293	T-REx-293 cells stably expressing SIAH3 ΔS3UNS upon tetracycline/ doxycycline induction	DMEM (Tet free)
melanoma	C918	CVCL_8471	human uveal melanoma	DMEM
	IGR-1	CVCL_1303	human cutaneous melanoma	DMEM
	MeWo	CVCL_0445	human cutaneous melanoma	DMEM
	SK-MEL-13	CVCL_6022	human melanoma	DMEM
	SK-MEL-19	CVCL_6025	human cutaneous melanoma	DMEM
	SK-MEL-28	CVCL_0526	human cutaneous melanoma	DMEM
lung	A-549	CVCL_0023	human lung adenocarcinoma	DMEM
breast	MDA-MB-231	CVCL_0062	human breast adenocarcinoma	RPMI
umbilical cord	HUVEC	-	human umbilical vein endothelial cells	Endothelial cell growth medium (PromoCell)

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2.1.12 Tissue samples

Methylation analysis was performed on bisulfite-treated (BS) DNA from tissue samples described in Tab. 20. Analyses were performed in accordance with the Declaration of Helsinki. All patients signed informed consent at initial clinical investigation. The study was approved by local ethic committees (University of Heidelberg, Heidelberg, Germany or University of Halle, Halle an der Saale, Germany).

Tab. 20: Tissue samples used for methylation analyses

entity	normal / tumour	n	obtained from
skin	nevus cell nevi (normal)	59	Dermatology (University Hospitals Heidelberg & Halle (Saale)), Neuropathology (University Hospital Heidelberg)
	primary tumours (malignant melanoma)	42	
	metastases (skin / brain / lymphocytes)	47	
lung	normal tissues	50	Lungbiobank Heidelberg
	adenocarcinoma	50	
head and neck	normal tissues (cheek mucosa)	14	Department of Otolaryngology (University of Halle-Wittenberg)
	squamous cell carcinoma	14	

2.1.13 Media, solutions & buffers

Tab. 21: Media for the cultivation of human cell lines

name	ingredients
DMEM (Dulbecco's modified Eagle medium)	500 mL DMEM high glucose / no glucose 50 mL FCS 5 mL PS or Antibiotic-Antimycotic
RPMI (Roswell Park Memorial Institute medium)	500 mL RPMI 1640 50 mL FCS 5 mL PS or Antibiotic-Antimycotic

Tab. 22: Media for bacteria culture. The media have been autoclaved and appropriate antibiotics (see Tab. 11-13) were added before use. For blue-white screening, 50 µL X-Gal (20 mg/mL) and 20 µL IPTG (0.5 M) were mixed in 100 µL SOC medium (per plate) and distributed on the LB agar.

name	ingredients
LB medium (Lysogeny broth)	10 g tryptone 5 g yeast extract 10 g NaCl 1 g glucose (for solid medium: + 15 g Agar-Agar) ad 1 L ddH ₂ O adjusted to pH 7.5

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SOC medium (super optimal broth with catabolite repression)	20 g tryptone 5 g yeast extract 0.5 g NaCl 10 mL 250 mM KCl 5 mL 2 M MgCl ₂ (sterile-filtered, added after autoclaving) 10 mL 2 M glucose (sterile-filtered, added after autoclaving) ad 1 L ddH ₂ O
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Tab. 23: Buffers for protein analyses by SDS-PAGE, WB and mass spectrometry (MS) cell preparation

name	ingredients
Blocking buffer	3 % milk-powder in 1x TBS-T
Blotting buffer	20 mL 1 M NaH ₂ PO ₄ 14 mL 0,5 M Na ₂ HPO ₄ ad 1 L ddH ₂ O
Coomassie stain	45 % methanol 10 % acetic acid 0.25 % Coomassie Brilliant Blue G250
Coomassie destain	30 % methanol 10 % acetic acid
FLAG lysis buffer	50 mM tris/HCl, pH 7.4 150 mM NaCl 1 mM EDTA 0.5 % TritonX100 1x tablet cOmplete™ Protease Inhibitor / 50 mL adjusted to pH 7.4 with HCl
Laemmli buffer (4x)	200 mM tris/HCl, pH 6.8 32 % glycerol 8 % SDS 12 % β-mercaptoethanol (added before use) bromophenol blue (until dark blue)
MS lysis buffer	4 % SDS 100 mM tris/HCl, pH 7.6 1x tablet cOmplete™ Protease Inhibitor (EDTA-free) / 50 mL
RIPA buffer	50 mM tris/HCl, pH 7.4 150 mM NaCl 1 mM EDTA 1 % TritonX100 0.5 % sodium deoxycholate 0.1 % SDS 1x tablet cOmplete™ Protease Inhibitor / 50 mL
SDS-PAGE running buffer (10x)	250 mM tris 1.92 M glycine 0.1 % SDS
Separation gel buffer	1.5 M tris/HCl, pH 8.8
Stacking gel buffer	0.5 M tris/HCl, pH 6.8

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Stripping buffer - recipe I	25 mM glycine 1 % SDS adjusted to pH 2 with HCl
Stripping buffer - recipe II (Thermo Fisher Scientific)	15 g glycine 1 g SDS 10 mL Tween20 ad 1,000 mL ddH ₂ O adjusted to pH 2.2 with HCl
TBS (10x) (tris-buffered saline)	250 mM tris 1.5 M NaCl 27 mM KCl adjusted to pH 7.4 with HCl
TBS-T (1x)	TBS (1x) 0.05 % Tween20

Tab. 24: Buffers for Ni-NTA purification (ubiquitination assay)

name	ingredients	
Buffer A	6 M Gu-HCl 10 mM tris 100 mM NaH ₂ PO ₄ adjusted with NaOH to pH 8.0	+ 10 mM imidazole + 5 mM β-mercaptoethanol added prior to use
Buffer B	8 M urea 10 mM tris 100 mM NaH ₂ PO ₄ adjusted with NaOH to pH 8.0	
Buffer C	8 M urea 10 mM tris/HCl 100 mM NaH ₂ PO ₄ 0.2 % TritonX100 adjusted with NaOH to pH 6.3	
Buffer D	8 M urea 10 mM tris/HCl 100 mM NaH ₂ PO ₄ 0.1 % TritonX100 adjusted with NaOH to pH 6.3	
Elution buffer	125 mM tris/HCl, pH 6.8 20 % glycerol 5 % SDS bromophenol blue (until dark blue)	+ 200 mM imidazole + 7.5 % β-mercaptoethanol added prior to use
SDS sample buffer (1x)	4x Laemmli buffer diluted with ddH ₂ O (1 part buffer + 3 parts ddH ₂ O)	

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Tab. 25: Buffers and solutions used for the preparation of microscopy slides

name	ingredients
Blocking solution	3 % BSA 0.3 % TritonX100 in PBS
Dilution buffer for 1 st & 2 nd antibody	1 % BSA 0.3 % TritonX100 in PBS
Embedding reagent	6 g glycerol 2.4 g MOWIOL™ 4-88 reagent 6 mL ddH ₂ O 12 mL 0,2 M tris (pH 8.5) 0.1 % DABCO
Fixing solution	3.7 % formaldehyde in PBS
Washing solution I	PBS
Washing solution II	PBS-T (0.3 % TritonX100)
Solution for permeabilisation	0.3 % TritonX100 in ddH ₂ O

Tab. 26: Buffers and solutions for DNA isolation, agarose gel electrophoresis, PCR and further experiments

name	ingredients
100 bp / 1 kb gene marker	100 µL GeneRuler 100 bp / 1 kb Plus DNA Ladder (0.5 µg/mL) 83 µL 6x Loading Dye (Thermo Fisher Scientific) 25 µL 10x TBE ad 500 µL ddH ₂ O
dNTPs (2,5 mM each)	25 µL dATP (100 mM) 25 µL dCTP (100 mM) 25 µL dGTP (100 mM) 25 µL dTTP (100 mM) ad 1,000 µL ddH ₂ O
NH ₄ buffer	500 mM tris pH 8,8 HCl 160 mM (NH ₄) ₂ SO ₄ 0.1 % Tween20
PBS (10x) (phosphate-buffered saline)	140 mM NaCl 2.7 mM KCl 10 mM Na ₂ HPO ₄ 1.8 mM KH ₂ PO ₄ adjusted to pH 7.3
Proteinase K buffer	50 mM tris 25 mM EDTA 0.5 % NP-40 0.5 % SDS adjusted to pH 7.6

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TBE buffer (10x) (tris-borate-EDTA buffer)	1 M tris 10 mM EDTA 0.8 M boric acid adjusted to pH 8.5
TE buffer (tris-EDTA buffer)	10 mM tris/HCl 1 mM EDTA adjusted to pH 7.5

2.1.14 Kits and Microarray

Tab. 27: Kits for cloning, promoter assay, plasmid/PCR/BS-DNA purification, determination of protein concentration and GFP-immunoprecipitation (IP). Kits were used according to the manufacturers' protocols, unless otherwise stated in the Methods section.

name	company
Affimetrix Clariom S Assay, human	Thermo Fisher Scientific
CloneJET PCR Cloning Kit	Thermo Fisher Scientific
Dual-Luciferase® Reporter Assay System	Promega
GFP-Trap® A Kit	ChromoTek
NucleoBond® Xtra Midi	MACHEREY-NAGEL
NucleoSpin® Plasmid EasyPure	MACHEREY-NAGEL
NucleoSpin® Gel and PCR Clean-up Kit	MACHEREY-NAGEL
p-GEM®-T Easy Vector Systems	Promega
Pierce™ Rapid Gold BCA Protein Assay Kit	Thermo Fisher Scientific
QuikChange Lightning Site-Directed Mutagenesis Kit	Agilent Technologies
Seahorse XF Cell Mito Stress Test Kit, Seahorse XF Glycolysis Stress Test Kit, Seahorse XF Real-Time ATP Rate Assay Kit	Agilent Technologies

2.1.15 Equipment and instruments

Tab. 28: Amplifying and quantifying instruments as well as imaging systems. Standard laboratory instruments are not listed.

name	company
Axio Observer.Z1 microscope	Zeiss
Axiocam 506 mono digital camera	Zeiss
Axiophot fluorescence microscope	Zeiss
Aurox Unity imaging system	Aurox
BD FACS Canto II	BD Biosciences
Eppendorf Mastercycler	Eppendorf
FastGene FAS-DIGI PRO	NIPPON Genetics
Orion L Microplate Luminometer	Berthold Detection Systems
Mini-PROTEAN Tetra Cell	Bio-Rad
MOTIC AE21 inverse microscope	MOTIC
Moticam 3+ 3.0 MP	MOTIC

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PeqSTAR Thermocycler	PEQLAB
PyroMark Q24	Qiagen
Rotor-Gene3000	Corbett Research, Qiagen
Seahorse XF96 Analyzer	Agilent Technologies
Tecan Infinite M200 PRO	Tecan
Versadoc Imaging System 400 MP	Bio-Rad

2.1.16 Software and online tools

Tab. 29: Software and online tools

name	description / usage	company / source
Benchling	cloud-based platform for biotech research & development; design of sgRNAs and plasmid maps	Benchling, San Francisco, CA, USA (https://benchling.com/signin/welcome)
BD FACSDiva Software	FACS Canto II software	BD Biosciences
BioEdit 7.2.5	sequence alignment	by Tom Hall 1999
BioRender	creation of schematic figures	BioRender Inc., Toronto, Canada (https://www.biorender.com/)
DeepLoc 1.0	prediction of subcellular localisation	DTU Health Tech, Kongens Lyngby, Denmark; see Almagro Armenteros <i>et al.</i> , 2017 (https://services.healthtech.dtu.dk/services/DeepLoc-1.0/)
GEPIA	expression analysis of published data (TCGA & GTEx)	Zhang Lab, Peking University, Peking, China; see Tang <i>et al.</i> , 2017 http://gepia.cancer-pku.cn/
GraphPad QuickCalcs	t-test analyses	GraphPad Software, San Diego, CA, USA (https://www.graphpad.com/quickcalcs/)
Inkscape	vector graphics editor	Inkscape Project: (https://inkscape.org/)
Motic Images Plus 3.0	cell culture microscopy software	MOTIC Deutschland
NCBI	blasting of sequences, gene/protein information, paper search via PubMed	National Center for Biotechnology Information, U.S. National Library of Medicine (https://www.ncbi.nlm.nih.gov/)
NIPPON Genetics Camera Studio-Software	FAS-DIGI PRO software	NIPPON Genetics
QuantityOne 4.6.9	WB imaging	Bio-Rad
Quick Change Primer Design Program	Design of mutagenesis primer	Agilent Technologies (https://www.agilent.com/store/primerDesignProgram.jsp)

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R2: Genomics Analysis and Visualization Platform	expression and methylation analysis of published datasets, Kaplan-Meier survival curves	AMC, Amsterdam, Netherlands (http://r2.amc.nl)
R-Studio	R programming	Posit PBC, Boston, MA, USA
Rotor-Gene 6	qPCR software	Corbett Research, Qiagen
Seahorse Analytics	Analysis of Seahorse data	Agilent Technologies (https://seahorseanalytics.agilent.com/)
Seahorse XF Report Generators	Analysis of Seahorse data	Agilent Technologies
Simplicity 4.2	luminometer software	Berthold Detection Systems
SMART	analysis of the DNA methylation data of TCGA project	Yin Li, Di Ge & Chunlai, Lu Fudan University, Shanghai, China; see Li, Di Ge & Lu, 2019 (http://www.bioinfo-zs.com/smartapp/)
STRING	Protein-protein interaction networks & functional enrichment analysis	SIB/University of Zurich (Zurich, Switzerland), CPR (Copenhagen, Denmark), EMBL (Heidelberg, Germany); see Szklarczyk <i>et al.</i> , 2023 (https://string-db.org/)
Tecan i-control	Microplate reader software	Tecan
UCSC Genome Browser	information about gene locus, gene/mRNA sequences, in silico PCR	University of California, Santa Cruz, USA (https://genome.ucsc.edu/cgi-bin/hgGateway)
Wave Desktop and Controller 2.6 Software	Seahorse software	Agilent Technologies
Zen 3.2	software for fluorescence microscopy	Zeiss

2.2 Methods

This chapter describes the methods used in this thesis. These are techniques for DNA and cell manipulation, DNA, RNA and protein examination as well as for the analysis of cell metabolism.

2.2.1 Methods for DNA and plasmid manipulation and examination

2.2.1.1 Standard methods for DNA analysis

In the following, the basic protocols for PCR and agarose gel electrophoresis are described. Specific variations are described in the respective sections.

2.2.1.1.1 Polymerase chain reaction (PCR)

Standard PCR was used to amplify the CDS of *CHCHD4*, *SIAH3* (by S. Walesch / J. Moyer), *STUB1* (by N. Schlaudraff) and *TIMM50* from complementary DNA (cDNA), the promoter locus of *SIAH3* from

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genomic DNA as well as for the amplification of the fragments analysed by combined bisulfite restriction analysis (CoBRA) and pyrosequencing. Tab. 30 shows the basic PCR protocol. The annealing temperature (x) depends on the primers, the elongation time (y) on the length of the PCR product (homemade Taq polymerase: 10 s per 100 bp; Phusion: 15–30 s per 1 kb) (annealing temperature and product length are stated in Tab. 15 & 16). The number of cycles (z) is also variable.

Tab. 30: Basic PCR protocol. fwd = forward, rev = reverse.

basic PCR mixture			cycler programme		step
NH ₄ -Puffer (10x)	2.5	μL	95 °C	2 min	initial denaturation
dNTPs (2.5 μM each)	2	μL			
MgCl ₂ (50 mM)	1.5-1.75	μL	95 °C	30 s	denaturing
formamide	0-1	μL	x °C	30 s	annealing
betaine (5 M)	0-5	μL	72 °C	Y s	elongation
DMSO	0.75	μL			
primer 1 (fwd) (10 μM)	1	μL	72 °C	4 min	final elongation
primer 2 (rev) (10 μM)	1	μL			
Taq polymerase	0.5	μL			
template (e.g. DNA)	2	μL			
ddH ₂ O	ad 25	μL	4 °C	hold	

2.2.1.1.2 Agarose gel electrophoresis

DNA/RNA samples were mixed with 6x loading dye and separated on an agarose gel (1 or 2 % according to the expected fragment size, in 0.5x TBE buffer, plus 2.5 μL ethidium bromide or 3.5 μL Midori Green Xtra per 100 μL gel), which was run for 20-30 min at 200 V. Fluorescent DNA or RNA fragments were detected using UV light (ethidium bromide) or blue-green light (Midori Green).

2.2.1.2 Molecular Cloning

All recombinant plasmids used in this work already existed in our working group, were gifts from collaboration partners or were produced by myself, my Master's students or colleagues. Plasmid maps of vectors cloned by me, students of mine or other lab members during the course of this study as well as for the SIAH3 guides for epigenetic editing are shown in supplementary chapter 6.1. Maps were created using benchling.com with sequence information retrieved from sapgene.com or addgene.org.

CDS or the promoter locus to clone were amplified by PCR and inserted to pGEM-T Easy or pJet1.2 vector (according to the manufacturer's protocol) as basis for further cloning. New constructs were then generated either by restriction-based cloning or site-directed mutagenesis. Finished plasmids were test-digested and sequenced (Microsynth SeqLab) to verify correctness.

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2.2.1.2.1 Restriction-based cloning

Plasmids were cut with site-specific restriction enzymes to obtain the insert and open the target vector (same or compatible ends). The preparative digest was done in a total volume of 50 μ L. The mixtures are listed in Tab. 31. The digests were incubated for 1 h at 37 °C and then separated by agarose gel electrophoresis. The corresponding bands were cut out and spin-column cleaned-up using the *NucleoSpin Gel and PCR clean-up kit* after the manufacturer's protocol (elution of the insert in 15 μ L ddH₂O, backbone in 30 μ L ddH₂O). If both parts were cut with the same enzyme, the vector backbones were dephosphorylated to avoid recirculation through intra-molecular religation. Dephosphorylation of the 5'-ends was done with FastAP enzyme for 10 min at 37 °C and heat inactivated for 5 min at 75 °C (mixture described in Tab. 31). Afterwards the dephosphorylated plasmid was purified again using *NucleoSpin Gel and PCR clean-up kit* (elution in 30 μ L ddH₂O). The clean insert was then ligated into the linearised vector backbone using a T4 DNA ligase. The used ratio between backbone to insert ranged from 1:1 to 1:3. The ligation was done in a total volume of 10 μ L, containing 1 μ L backbone, 1-3 μ L insert, 1 μ L T4 DNA ligase enzyme and 1 μ L of the corresponding buffer. The mixture was incubated at room temperature (RT) from 1 h up to overnight. After being amplified by *E. coli* the final plasmid was test digested for successful insertion in a 10 μ L mixture.

Tab. 31: Reaction mixtures for restriction digestion and dephosphorylation of plasmids

preparative digest	test digest	dephosphorylation
10 μ g plasmid DNA	1,000 ng plasmid DNA	28 μ L vector backbone DNA
5 μ L buffer (10x)	1 μ L buffer (10x)	5 μ L FastAP buffer (10x)
2.5 μ L enzyme 1	0.5 μ L enzyme 1	5 μ L FastAP enzyme
2.5 μ L enzyme 2 (if applicable)	0.5 μ L enzyme 2 (if applicable)	-
ad 50 μ L ddH ₂ O	ad 10 μ L ddH ₂ O	ad 50 μ L ddH ₂ O

2.2.1.2.2 Mutagenesis

Site-directed mutagenesis (e.g. base-exchanges, deletion of domains, altering the frame) was done using the *QuikChange Lightning Site-Directed Mutagenesis kit* according to the manufacturer's protocol. The primers were designed with the corresponding online tool *Quik Change Primer Design Program*. The mutagenesis PCR was followed by a digest with methylation sensitive *DpnI* enzyme (20 min at 37 °C) to eliminate the parental plasmid (methylated) and only leave the mutated PCR-products (unmethylated). 2 μ L digested PCR-product was transformed to *E. coli* (see 2.2.1.2.3.) to amplify the mutated plasmid.

2.2.1.2.3 Transformation and culturing of *E. coli* bacteria

The transformation of chemically competent *E. coli* was done using the heat shock method. 50 μ L bacteria strain DH5 α or XL10Gold (thawed on ice) were mixed with 2 μ L PCR product (mutagenesis)

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or 5 µL ligation product, kept on ice for 30 min, heat-shocked in a 42 °C water bath for 45-60 s and cooled on ice for 1 min. For XL10Gold, 2 µL of XL10-Gold β-mercaptoethanol mix (*QuikChange Lightning Site-Directed Mutagenesis Kit*) was added 10 min before the addition of PCR/ligation product. 250 µL SOC medium was added after the short cooling step, bacteria were incubated shaking at 37 °C for 1 h and were finally plated on LB agar plates containing the appropriate antibiotics. The plates were incubated over night at 37 °C. If applicable, blue-white screening using IPTG and X-Gal within the LB agar (see Tab. 22) was performed.

2.2.1.2.4 Plasmid preparation

Grown colonies were transferred to a liquid culture (5 mL LB medium plus appropriate antibiotic) and incubated overnight at 37 °C with shaking. The plasmids were extracted using the *NucleoSpin Plasmid EasyPure Kit* after the manufacturer's protocol (exception: resuspension of plasmids in ddH₂O instead of TE buffer). The DNA concentration was determined using a spectrophotometer (*Tecan Infinite M200 PRO*). To gain larger amounts of plasmids, 100 mL liquid *E. coli* culture was used, and the plasmids were isolated using the *NucleoBond Xtra Midi Kit*.

2.2.2 Handling of human cell lines

2.2.2.1 Cultivation of human cell lines

This work is a cell line-based study. The experiments were conducted in human cancer lines (see Tab. 19). All used cell lines grow adherent. The cells were cultivated according to their needs in DMEM or RPMI (see Tab. 19) containing 10 % FCS (fetal calf serum) and 1 % PS or Antibiotic-Antimycotic, at 37 °C, 5 % CO₂ concentration and an air humidity of 95 %. Cells were splitted density-dependent every 2-4 days.

The cells were frozen in liquid nitrogen for later use. For this, detached cells were centrifuged at 300 x g for 3 min, resuspended in medium without antibiotics or FCS but containing 10 % DMSO (1 mL medium per 10 cm dish), were cooled down at -80 °C in a double-walled Styrofoam box and then stored in a liquid nitrogen container.

2.2.2.2 Transfection of human cell lines

This work is mainly based on the examination of the effect of the overexpression of a gene of interest. For this, cells were transiently transfected with recombinant plasmid(s) starting at a cell confluence of 60-90 % (C918 >95 %). Tab. 32 shows the reactions mixtures for the transfection of different cell lines. Two mixtures in different tubes were prepared: Mix 1 contains the plasmid/DNA in OptiMEM (vortexed), mix 2 the transfection reagent in OptiMEM (not vortexed, only inverted several times). Mix 2 was then added to mix 1 and mixed gently by inverting. After an incubation of 15 min at RT, the DNA/transfection reagent/OptiMEM mix was carefully added to the cells (full medium exchanged before with reduced serum medium (OptiMEM)). After 4-6 hours, OptiMEM

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was replaced with full, serum containing, medium (DMEM). Cells were harvested 24 to 72 h (or up to 144 h for epigenetic editing) after transfections, depending on the experimental setup.

2.2.2.3 Knockdown experiments by siRNA

Knockdown of a certain gene was done using small interfering RNA (siRNA). Cells were transfected equivalently to the procedure done for overexpression (see 2.2.2.2). Transfection setups, including the reagents, are listed in Tab. 32. Cells were incubated for 72 h before harvesting. For *SIAH2* knockdown, the ON-TARGETplus Human SIAH2 siRNA SMARTpool was used, whereas the ON-TARGETplus Human SIAH3 siRNA SMARTpool was applied for *SIAH3* knockdown. The ON-TARGETplus Non-targeting Pool served as a control (all reagents from Dharmacon).

Tab. 32: Setups for the transfection of different human cell lines.

	overexpression			knockdown	
cell line	HEK293T, T-REx-293		C918	T-REx-293, C918	
Size of the dish	6-well	10 cm	6-well	6-well	10 cm
plasmid/siRNA	plasmid			siRNA	
	4 µg	10 µg	4 µg	5 µL (10 µM)	12.5 µL (10 µM)
transfection reagent	PEI			Lipofectamine RNAiMAX	
	8 µL	20 µL	6 µL	5 µL	12.5 µL
OptiMEM (for each mix)	100 µL	250 µL	250 µL	100 µL	250 µL
OptiMEM (added to cells)	500 µL	3 mL	200 µL	600 µL	3 mL

2.2.2.4 Generation of a stable cell line expressing inducible *SIAH3*

T-REx-293 cells (stably expressing the tetracycline (Tet) repressor protein) were transfected with pcDNA4/TO/myc-His-SIAH3 Wt or Δ S3UNS plasmid for stable insertion of doxycycline-inducible *SIAH3*. Cells were cultured in Tet-free medium and selected for the genomic integration using Zeocin (500 µg/mL) and blasticidin S (5 µg/mL). Colonies were transferred to 96-well plates, cultured, and subsequently transferred to larger wells (24- and 6-wells). Single clones were screened for induction levels of *SIAH3* on RNA and protein level. Expression of *SIAH3* was induced by adding doxycycline (Dox; 2 µg/mL) for (at least) 24 h.

Either a single clone (scratch assay: clones #8 and #15) or a pool of three individual clones (Wt: #6 + #8 + #15, Δ S3UNS: #1 + #3 + #7) were used (all other experiments). T-REx-293 cells stably expressing only the Tet repressor served as control cell line (T-REx ctrl.).

Sara Walesch primarily generated the inducible cell line for *SIAH3* Wt. She built the pcDNA4/TO/myc-His B plasmid containing the *SIAH3* CDS and transfected the cells. I continued with selecting and screening the clones and created the *SIAH3* Δ S3UNS cell line.

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2.2.3 Analysis of DNA

Genomic DNA from (transfected) cell lines was isolated, bisulfite-converted and methylation was analysed by CoBRA and pyrosequencing.

2.2.3.1 DNA isolation

Genomic DNA from cell lines was isolated using phenol-chloroform purification.

After being pelletised and washed with PBS, cells were digested with Proteinase K (500 μ L Proteinase K buffer and 5 μ L Proteinase K enzyme) overnight at 55 °C (shaking). Then, 500 μ L phenol/chloroform/isoamyl alcohol (25:24:1) was added, shaken vigorously for 30 s and centrifuged for 3 min at 13,000 rpm. DNA, which was then located in the upper aqueous phase, was transferred to a new reaction tube. The separation with phenol/chloroform/isoamyl alcohol was repeated once more. A third separation step was done only with chloroform. Afterwards, 3 M NaAc (1:10 Vol.) and isopropanol (1 vol.) were added to the collected phase and inverted until the DNA precipitated. DNA was pelletised at 13,000 rpm for 5 min, washed in 150 μ L ethanol (70 %), dried and resuspended in 30-50 μ L DEPC H₂O, according to the pellet's size. DNA concentration was determined using a spectrophotometer (Tecan Infinite M200 PRO).

To remove leftover RNA, 2 μ L RNase A (10 mg/mL) was added to the samples, followed by incubation for 10 min at 37 °C.

2.2.3.2 Bisulfite conversion of DNA

During bisulfite-conversion, unmethylated cytosines are converted to uracils whereas methylated cytosines (5-methylcytosines) remain unchanged.

In a reaction volume of 20 μ L, 2 μ g DNA and 2 μ L 3 M NaOH were incubated at 37 °C for 10-15 min. 12 μ L 0.1 M hydroquinone (freshly made) and 208 μ L 1.9 μ L sodium metabisulfite (pH 5.5, freshly made, do not vortex) were added, mixed by pipetting and incubated at 50 °C (water bath) for 15-17 h (sulfonation and deamination of unmethylated cytosines). DNA was purified using the *NucleoSpin Gel and PCR Clean-up Kit* (after manufacturer's protocol, except the elution in 50 μ L NE buffer). The formed uracil sulfonate was subsequently desulfonated to uracil by the addition of 5 μ L 3 M NaOH and incubation at 37 °C (water bath) for 10 min. To precipitate DNA, 1 μ L glycogen (2 μ g/ μ L), 55 μ L 7.5 M ammonium acetate and 275 μ L 100 % ethanol were added, followed by an incubation at -20 °C for 10 min. Samples were centrifuged at 13,000 rpm for 10 min and the obtained DNA pellet was washed with 100 μ L 70 % ethanol. After being centrifuged again at 13,000 rpm for 2 min, the pellet was dried and resuspended in 30 μ L TE-buffer (shaking at 37 °C).

2.2.3.3 Combined Bisulfite Restriction Analysis (CoBRA)

The unmethylated cytosines converted to uracil were replaced by thymines in a subsequent PCR. In the resulting sequence, the formerly unmethylated cytosines are thus changed to thymines,

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while methylated cytosines are still present as cytosines. Restriction enzymes like *TaqI* or *BstUI* are then able to cut the PCR product in case the cytosine within their recognition site has not been converted. For *SIAH3*, the restriction enzyme *BstUI* with the recognition site CG'CG that cuts within the annotated CpG probe cg26667946 was used (see Fig. 6A).

In a fully nested PCR, a 156 bp long fragment of the promoter region of *SIAH3* was amplified. PCR conditions are listed in Tab. 33.

The PCR was followed by a restriction digest using *BstUI* in a reaction mix of 10 µL in total (see Tab. 31). A mock digest without enzyme served as negative control. The fragments were separated on a 2 % agarose gel.

Tab. 33: PCR conditions for amplifying the promoter fragment for CoBRA and pyrosequencing. In preparation for pyrosequencing, during PCR 2 a biotinylated lower primer (SIAH3BSL1Bio) was used.

PCR reaction mix:	PCR 1	PCR 2	cycler programme		step
NH ₄ -Puffer (10x)	2.5 µL	2.5 µL	95 °C	2 min	initial denaturation
dNTPs (2.5 µM each)	2 µL	2 µL			
MgCl ₂ (50 mM)	1 µL	1 µL	95 °C	20 s	denaturing
formamide	-	0.5 µL	x °C	20 s	annealing
SIAH3BSU- (10 µM)	U2: 1 µL	U1: 1 µL	72 °C	y s	elongation
SIAH3BSL- (10 µM)	L2: 1 µL	L1: 1 µL			
<i>Taq</i> -Polymerase	0.5 µL	0.5 µL	72 °C	4 min	final elongation
Template: PCR 1: BS-DNA PCR 2: PCR 1 product	2 µL	2 µL	4 °C	hold	cool down
ddH ₂ O	ad 25 µL	ad 25 µL			

x	PCR 1: PCR 2:	51 °C 53 °C
y	PCR 1: PCR 2:	20 s 30 s
cycles	PCR 1: PCR 2:	20 x 30 x

2.2.3.4 Pyrosequencing

By pyrosequencing, the methylation status of five CpGs within the *SIAH3* promoter region was analysed quantitatively (see Fig. 6A) using the PyroMark Q24 with the corresponding materials and reagents by Qiagen.

PCR was conducted as described in Tab. 33, using a biotinylated lower primer (SIAH3BSL1-Bio) that is supposed to bind the streptavidin-coupled Sepharose beads during sequencing. For each sample, 40 µL Binding Buffer, 2 µL Sepharose and 28 µL ddH₂O mixed with 10 µL PCR product was put in the PCR plate and was shaken at full speed for at least 4 min (RT). Nucleotides, enzyme and substrate were filled in the cartridge (according to the pre-run information of the assay in the software). 25 µL

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of sequencing primer (see Tab. 15, 0.3 μ M in Annealing Buffer) was filled in each position used of the Q24 plate. The workstation was prepared by filling the tanks with 70 % ethanol (tank 1), Denaturation Solution (tank 2), Washing Buffer (tank 3) and ddH₂O (tanks 4 & 5). At the workstation, samples were aspirated (from the PCR plate) with the vacuum tool. The tool was then put in ethanol for 5 s, then in Denaturation Solution for 5 s, followed by 15 s in Washing Buffer. The vacuum tool was raised upright in between to drain liquid from the filter probes. Vacuum tool and vacuum pump were then switched off. The beads were released by shaking the filter probes on the Q24 plate for 1 min. The Q24 plate was then heated up on an 80 °C metal carrier for 2 min, followed by a cool down for 5 min on a metal carrier at RT. In the meanwhile, the filter probes were cleaned by aspiration of ddH₂O from tank 4 and 5. The Q24 plate and the filled cartridge were then put inside the pyrosequencing machine and the run was started. Results were analysed using the PyroMark Q24 software.

Assay information:

Assay name: SIAH3

Type: CpG

Sequence to analyse: (Y = CpG position 1-5)

GYGYGTAAAGTYGGGTTGGAGYGGAGTGTTTTAGAGGTTGYGTTTTTTTTAGGGTTTAAGGATTTA

Dispensation order: TGTCAGTCGCTATGTCGTGATGTCGAGTGTTAGAGTAGTCGTTA

2.2.4 Analysis of gene expression

The analysis of the expression level of a gene is done on messenger RNA (mRNA) level. RNA was isolated from cultured cells (dependent on the experiment non-transfected, transfected or induced), DNase digested to remove contaminating DNA and reversely transcribed to complementary DNA (cDNA). cDNA levels were determined quantitatively using qPCR.

2.2.4.1 RNA isolation

Cells were lysed with TRIzol reagent (1 mL per 6-well). 200 μ L chloroform was added and after shaking vigorously for 15 s and incubation at RT for 2 min samples were centrifuged at 12,000 rpm for 30 min at 4 °C. The RNA, located in the upper aqueous phase, was transferred to a new reaction tube. RNA was then precipitated by adding 500 μ L isopropanol and shaking vigorously for 15 s. After an incubation for 10 min at RT, samples were centrifuged at 12,000 rpm for 10 min at 4 °C. The formed pellet, containing the RNA, was washed in 500 μ L ethanol (70 % in DEPC H₂O), centrifuged at 7,500 rpm for 5 min at 4 °C, dried (shortly) and resuspended in 20-50 μ L DEPC H₂O (shaking at 37 °C). The RNA concentration was determined spectrophotometrically.

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2.2.4.2 DNaseI digestion and reverse transcription of RNA to cDNA

The isolated DNA was digested with the endonuclease DNaseI to remove contaminating DNA.

1 µg RNA was mixed with 0.5 µL RiboLock RNase Inhibitor, 1 µL DNaseI, 1 µL of the according buffer (10x) and filled up to 10 µL with DEPC H₂O. The mixture was incubated at 37 °C for 30 min and DNaseI was then inactivated at 65 °C for 10 min. Afterwards, RNA was transcribed to cDNA by an M-MLV reverse transcriptase, an RNA-dependent DNA-polymerase. The reaction mix is depicted in Tab. 34. Where appropriate, cDNA was diluted 1:4 with ddH₂O (no dilution for the analysis of endogenous *SIAH3*).

Tab. 34: Conditions for the reverse transcription of RNA into cDNA.

mixture		cycler programme		step
DNaseI digested RNA	10 µL	62 °C	10 min	reduction of 2nd structure
5x M-MLV buffer	5 µL	+ 1 µL RiboLock RNase Inhibitor & M-MLV reverse transcriptase (1:1, premixed)		
dNTPs (2.5 µM each)	8 µL	42 °C	60 min	transcription of cDNA strand
poly-dT primer (10 µM)	1 µL	95 °C	5 min	enzyme inactivation
hexamer primer (10 µM)	1 µL	4 °C	∞	hold
<i>total</i>	25 µL			

2.2.4.3 Quantitative polymerase chain reaction (qPCR)

The quantitative PCR, or real-time PCR, is a method for the quantification of nucleic acids. cDNA is amplified in a PCR and the amplification product is quantified using a fluorescent reporter, here SYBR® Green dye.

In a reaction mixture, totalling 10 µL, 2 µL cDNA, 3 µL SYBR® Select Master Mix and 1 µL primer-mix (forward and reverse, each 4 µM) were mixed. Each sample was analysed in triplicates or quadruplicates. The reactions took place in the real-time cycler *Rotor-Gene3000*. One cycle consisted of a denaturing step for 15 s at 95 °C, a primer annealing step for 30 s at 60 °C, followed by the elongation for 30 s at 72 °C. At the end of each elongation step, fluorescence was measured. The SYBR® Green fluorescence was detected in the FAM channel at a wavelength of 510 nm. For normalisation purpose, the reference dye ROX was detected at 610 nm. At the end of each run, a melting curve between 72 °C and 99 °C was created for each primer-pair (in 1 °C steps, waiting for 45 s before the first step, 5 s for each of the following ones). For the evaluation, the data normalised to ROX were used. Further analysis was done in *MS Excel*. The average of the triplicates/quadruplicates was calculated and the expression of the gene of interest was put in relation to the housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) or *ACTB* (β-actin) to compensate for differences in the initial cell quantity.

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2.2.4.4 Microarray-based transcriptome analysis

The differential gene expression in a genome wide setting was analysed by using a microarray. The effect of EGFP-SIAH3 overexpression on the transcriptome was examined in HEK293T cells. A Clariom™ S Assay (human) from Affymetrix was used, which contains around 20,000 well annotated genes. Dr. Antje Richter did the preparation of the samples. HEK293T cells were transfected with either EGFP empty vector (ctrl.) or EGFP-SIAH3 and RNA was isolated 30 h later. 250 ng RNA was used for the microarray analysis. Marking of the samples and hybridisation on the chip (using the *Affymetrix® GeneChip WT terminal labeling and controls reagent kit*) as well as the preparation of the data and the normalisation was carried out by the staff of AG Böttger, Max-Planck-Institute in Bad Nauheim (using the corresponding software and probe library of Affymetrix). Further analysis was done with *MS Excel*, *R2: Genomics Analysis and Visualization Platform* and the *STRING* online tool.

2.2.5 Analysis of protein levels

The effect of different (gene) settings was also examined on protein level. Proteins were isolated and analysed via immunoblotting after separation by SDS-PAGE or using mass spectrometry (MS).

2.2.5.1 Protein analysis via SDS-PAGE and Western blot

2.2.5.1.1 Protein isolation

Cells were transfected, induced or treated otherwise, depending on the experimental setting. Proteins were isolated using FLAG lysis buffer or RIPA buffer. Cells were washed with PBS and lysis buffer was added (150-160 µL for a 6-well, 1 mL for a 10 cm-dish) to the cells. The plates were shaken vigorously (shortly) to detach the cells. The cell-buffer-mix was then transferred to a reaction tube and incubated on ice for 30 min (pipetting up and down every 10 min). After centrifugation (pelleting the remaining cellular components) for 10 min at 10,000 rpm at 4 °C, the protein lysate was transferred to a new reaction tube. An aliquot of 10 µL was taken away for the subsequent determination of the protein concentration. The lysate was denatured by adding Laemmli buffer (4x or 2x concentrated) and boiling for 5 min at 95 °C (shaking). Samples were kept at -20 °C until further processing.

2.2.5.1.2 Determination of protein concentration by Bradford or BCA assay

To be able to load equal amounts of protein, protein concentration was measured using either Bradford or BCA assay.

Bradford assay:

Bradford reagent was diluted 1:5 with ddH₂O. 1 mL diluted Bradford reagent was mixed with 1 µL sample lysate in a cuvette. A calibration curve was created with BSA dilutions (in ddH₂O; 0/1/5/10/20/30 µg/µL). FLAG lysis buffer without protein served as blank. Absorbance was

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measured at 595 nm using *GeneQuant pro* ("protein 595 Assay" program). The measurement could also be done using a 96-well plate and the *Tecan Infinite M200 PRO*: 1/5 of the mixture that was used for a cuvette was used for one 96-well. Thus, 200 μ L diluted Bradford reagent was mixed with 2 μ L 1:10 diluted sample (in ddH₂O) or BSA in a dilution series (0/1/2/4/6/8/10/20 μ g/ μ L). The samples were measured in triplicates. The absorbance was determined at 595 nm at 4 measuring points per well and the mean value was formed. The protein concentration of the samples was calculated with *MS Excel* based on the calibration curve.

BCA assay:

The *Rapid Gold BCA Protein Assay* from Thermo Fisher Scientific was used according to the manufacturer's protocol. The only differences were the following: The protein lysates were diluted 1:10 with FLAG/RIPA lysis buffer. For the calibration curve, 5 different concentrations of BSA (diluted in lysis buffer) are used (0/0.1/0.25/0.5/1.0 μ g/ μ L). The absorbance was determined using the *Tecan Infinite M200 PRO* at 480 nm at 4 measuring points per well from which the mean value was formed. The protein concentration of the samples was calculated with *MS Excel* based on the calibration curve.

2.2.5.1.3 SDS-PAGE and Western blot (WB)

Proteins were separated according to their size by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous gel consisting of stacking gel (lower percentage of acrylamide) and separation gel (higher percentage of acrylamide). Gel components are depicted in Tab. 35.

Denatured protein samples were loaded onto the polymerised SDS gels: equal volumes for pulldowns (20-45 μ L), equal amount according to the Bradford/BCA-determined concentration (20-80 μ g) for the other experiments. For protein size estimation the PageRuler™ Prestained Protein Ladder (2.5-5 μ L) was used. Both SDS-PAGE and WB were conducted using a *Mini-PROTEAN Tetra Cell*. Separation took place within the chamber filled with SDS-PAGE running buffer (1x), first at 80 V (during stacking gel passage), then at 100-120 V. Gels were then equilibrated in Blotting buffer for at least ½ h. Proteins were then transferred from the gel to a PVDF membrane by wet electroblotting. Membranes were activated in 100 % ethanol for 15 s, then soaked in H₂O for 10 min before being equilibrated in Blotting buffer for 20 min. Membrane and gel were packed according to the following order (from black = negative contact to white = positive contact): black sponge large pored – blotting paper – gel – membrane – blotting paper – white sponge fine pored. This assembly was put into the chamber filled with cooled Blotting buffer (membrane at the side of the anode, the SDS gel at the side of the cathode). Transfer took place at 4 °C on a magnetic stirrer at 18 V (Volt constant, 300 mA max.) for 12-15 h. Membranes were then kept in TBS.

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Tab. 35: Ingredients of SDS gels. The radical initiator APS and the catalyst TEMED were added immediately before pouring the gel. The quantities listed are sufficient for 4 gels.

ingredients	separation gel (10 %)	stacking gel (4 %)
ddH ₂ O	8.2 mL	6.1 mL
separation gel buffer	5 mL	-
stacking gel buffer	-	2.5 mL
acrylamide/bis-acrylamide (37.5:1) (ROTIPHORESE® Gel 30)	6.6 mL	1.3 mL
SDS (10 %)	0.2 mL	0.1 mL
APS (10 %)	120 µL	70 µL
TEMED	12 µL	12 µL
<i>total amount</i>	<i>20 mL</i>	<i>10 mL</i>

2.2.5.1.4 Protein detection

Proteins were detected using suitable primary antibodies (ABs) combined with secondary ABs coupled with horseradish peroxidase (HRP). When the corresponding substrate (peroxide solution) is converted by HRP, luminol is oxidised and emits visible light (chemiluminescence) that can be detected.

Membranes were blocked with Blocking buffer (3 % in TBS-T) for 1 h at RT to occupy unspecific binding sites. After being washed with TBS-T for 5 min, membranes were incubated with primary AB (diluted according to Tab. 9, in 3 % milk in TBS-T (1 % BSA in PBS-T for SIAH3-AB) containing 0.02 % sodium azide) rotating over night at 4 °C or for 1-4 h at RT. After 3 washing steps with TBS-T for 5 min, membranes were incubated with the secondary AB (diluted according to Tab. 10, in 3 % milk (2 % milk for HA-AB) in TBS-T, mouse or rabbit according to the host species of the primary AB) for 1 h at RT. Afterwards, membranes were washed again 3 times for 5 min in TBS-T. Membranes were developed using the *Immobilon Western Chemiluminescent HRP substrate solution* and imaged with the *Versadoc Imaging System 400 MP*. Dilution of the substrate solution (until 1:4 diluted with TBS) as well as the exposure time (300-800 s) depended on the strength of the reaction (amount of detected protein). The house keeping genes GAPDH or β -Tubulin were used as loading controls.

2.2.5.1.5 Coomassie staining

After protein separation, SDS gels were washed in ddH₂O and stained agitating on a shaker with Coomassie staining solution for 30 min (or until the gel turned dark blue). Afterwards, gels were destained using Coomassie destain solution until the background was transparent (3-5 changes of the destain solution).

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2.2.5.1.6 *Pulldown assays for interaction analysis*

Interaction between proteins was analysed by pulldown assays, either via immunoprecipitation (IP) (FLAG- or GFP-tag binding ABs) or via high affinity binding (Glutathione Sepharose binding to the Glutathion-S-Transferase (GST) tag). Together with the pulled protein of interest, its binding partners precipitate. This allows interaction partners to be found or verified.

FLAG- and GST-pulldown assay:

Cells were transfected in 10 cm dishes with a construct for the intended FLAG or GST fusion protein either alone (endogenous partner) or with a construct for a potential interactor protein. Cells were harvested and lysed in 1 mL FLAG lysis buffer as described in chapter 2.2.5.1.1. A 100 μ L aliquot of the lysate was denatured with 100 μ L Laemmli (2x concentrated), serving as input. The remaining lysate was used for the pulldown. For each sample, 50 μ L beads (*Anti-FLAG M2 Affinity Gel* or *Glutathione Sepharose 4B*) were used. The Anti-FLAG beads were equilibrated by 3x washing with TBS and centrifuging for 5 s at 10,000 rpm, the Glutathione Sepharose beads were washed with PBS and centrifuged for 4 min at 3,000 rpm (beads for all samples together in one tube). Afterwards the beads were distributed to the lysates and incubated, rotating overhead, at 4 °C for 1-3 h (GST pulldown) or overnight (FLAG-IP). To clean the beads from the unbound proteins, they were washed 3x with either TBS or PBS as described above. At the end, the supernatant was discarded and 50 μ L Laemmli (2x concentrated) was added to the beads. The proteins were denatured and detached from the beads by boiling at 95 °C for 5 min. The supernatant was transferred to a new tube and stored at -20 °C until further use.

GFP-IP using the *GFP-Trap A Kit*:

Pulldown was performed as described in the manufacturer's protocol (12.5 μ L beads per sample are enough, elution in 50 μ L 2x Laemmli).

2.2.5.1.7 *Ni-NTA purification*

To investigate the ubiquitination level of SIAH3, His-HA-ubiquitin was coexpressed together with SIAH3 (EYFP-tagged) and SIAH2 Wt or Rm (FLAG-tagged). Cells were treated with 10 μ M MG-132 12 h prior to harvest. Subsequently, ubiquitinated proteins conjugated to His-HA-tagged ubiquitin were enriched using nickel–nitrilotriacetic acid (Ni-NTA) affinity purification.

Cells were scraped from the culture dishes in ice-cold PBS (1 mL per 10 cm dish) and pelleted by centrifugation at 3,000 rpm for 2 min at 4 °C. The dishes were rinsed again with PBS, and the additional 1 mL was added to the pellet. The suspension was divided into an input fraction (200 μ L) and a purification fraction (800 μ L), both were pelleted again. The input fraction was resuspended in 150 μ L 1x sample buffer and boiled at 95 °C for 2-3 min, followed by sonification (2x 20 s, 10 % amplitude). The purification fraction was resuspended in 800 μ L Buffer A, sonicated 2x for 20 s

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(10 % amplitude) and centrifuged at 15,000 x g for 10 min at 4 °C. The supernatant was transferred to a new tube and 50 µL prewashed Ni-NTA agarose beads (beads mixed with Buffer A, centrifuged at 3,000 rpm for 2 min and resuspended in Buffer A again) were added. Lysates were incubated with the beads for 3 h at RT under rotation. To remove unbound proteins, the beads were washed sequentially with Buffer A–D (1 mL each), with each wash consisting of a 5 min incubation followed by centrifugation at 4,000 x g for 1 min. The beads were finally eluted in 70 µL Elution buffer and boiled at 95 °C for 5 min. After transfer of the supernatant to a new tube, the samples were stored at -20 °C until further use.

2.2.5.2 Mass spectrometry (MS) analysis

Preparation of cells, protein lysis and IP was carried out in our laboratory, MS measurement and data procession was performed by the MS facility of Prof. Dr. J. Graumann at the MPI in Bad-Nauheim. Both MS experiments were performed in triplicates. The mass spectrometric raw data as well as MaxQuant results and supporting metadata have been deposited to the proteomeXchange consortium (Deutsch *et al.*, 2023) via the MassIVE partner repository with the data set identifiers PXD048508 (interactomics) and PXD048509 (expression proteomics) (Deutschemeyer *et al.*, 2025). Graphs showing the volcano plots were created using *R* and graphs depicting the overrepresentation analysis (ORA) were produced with *MS Excel*.

2.2.5.2.1 MS – IP

MS analysis of a GFP-IP using an EGFP-tagged SIAH3 was performed to identify new binding partners of SIAH3.

2.2.5.2.1.1 Preparation of protein lysates and pulldown (by A. Richter)

EGFP-SIAH3 or EGFP-empty (ctrl.) were overexpressed in HEK293T cells (10 cm dish) for 24 h and enrichment of the EGFP-tagged protein was done by IP using the *GFP-Trap A Kit* according to the manufacturer's protocol (but only two washing steps for the beads after pulldown). Beads were frozen (not denatured with Laemmli) and handed over to the MS facility.

2.2.5.2.1.2 Measurement and data procession (MPI, MS facility, Prof. Dr. Graumann)

Proteins bound to the beads underwent off bead digest and liquid chromatography/tandem mass spectrometry (LC/MS2) analysis as described by Deutschemeyer *et al.* (2019), but opting for label-free quantification instead of chemical labelling. Instrument parameters were extracted and summarised using *MARMoSET* (Kiweler *et al.*, 2019) and are included in the supplementary Tab. S1. The LC gradients are depicted in supplementary Tab. S2. Peptide/spectrum matching as well as label free quantitation was performed using the MaxQuant suite of algorithms (Cox & Mann, 2008; Cox *et al.*, 2011; Cox *et al.*, 2014) (v. 1.6.2.6, parameters included in the supplementary Tab. S3) against the UniProt database (canonical & isoforms, downloaded 2018/07/17). For downstream statistical

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analysis, the analysis pipeline *autonomics* (<https://doi.org/doi:10.18129/B9.bioc.autonomics>) was used. ORA, based on a Fisher Exact Test, was performed for each contrast against the categories from the Kyoto Encyclopedia of Genes and Genomes (KEGG, Kanehisa *et al.*, 2016) and Gene Ontology (Ashburner *et al.*, 2000).

(Text adapted from Deutschmeyer *et al.*, 2025.)

2.2.5.2.2 MS - whole proteome

Changes made by overexpression of SIAH3 were examined by MS analysis of the whole proteome. Here, the SIAH3-inducible cell system was used (mix of 3 clones of T-REx SIAH3 (Wt), induced and uninduced. T-REx-293 (induced) served as second control to estimate the effect of Dox. In this work, the comparison between T-REx SIAH3 induced and uninduced is presented.

2.2.5.2.2.1 Preparation of protein lysates

T-REx SIAH3 and T-REx ctrl. were induced with 2 µg/mL Dox for 24 h (10 cm dish for each condition). For harvesting, cells were washed twice with PBS, scraped from the dish and lysed in 1 mL MS lysis buffer. After transfer to a 2 mL reaction tube and incubation at 70 °C for 10 min, cells were sonicated with a Branson Digital Sonifier at an amplitude of 10 %, 8 pulses of 1 s. Samples were then centrifuged at 16,000 x g for 10 min and supernatant was transferred to a new tube. An aliquot of 100 µL was denatured with 100 µL Laemmli (2x concentrated) and boiled at 95 °C for 5 min for WB analysis. A 20 µL aliquot was separated for the determination of the protein concentration. Finally, the lysates were snap frozen in liquid nitrogen and stored at -80 °C until further processing at the MS facility in Bad-Nauheim.

2.2.5.2.2.2 Measurement and data procession (MPI, MS facility, Prof. Dr. Graumann)

Proteomic sample preparation including chemical labelling by reductive demethylation was performed as reported by Wu *et al.* (2020). LC/MS2 measurement and data analysis was done as reported in chapter 2.2.5.2.1.2, but using MaxQuant version 1.6.10.42 and the UniProt database was downloaded on 2020/02/05. Parameters for the instrument and data processing are shown in the supplementary Tab. S1 – S3. (Text reproduced from Deutschmeyer *et al.*, 2025.)

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2.2.6 (Immuno-)Fluorescence microscopy

The (co)localisation of proteins was examined by fluorescence microscopy. The cells were grown in 6-well plates on microscopy coverslips and transfected as explained in chapter 2.2.2.2.

Cells were washed with PBS (Washing solution I, within the 6-well plate), fixed with Fixing solution (3.7 % formaldehyde) for 15 min at RT and were washed 3x for 5 min each with PBS. Cells were permeabilised with 1 mL/well PBS + 0.3 % TritonX100 (Solution for permeabilisation) for 5 min at 4 °C and washed once with PBS. Non-specific binding sites were blocked by incubation with 1 mL/well Blocking solution for 1 h at RT. After being washed 3x with PBS, coverslips were bedded onto Parafilm lying on a damp cloth inside a 15-cm dish. The coverslips were coated with 150 µL diluted primary AB (see Tab. 9, in 1 % BSA + 0.3 % TritonX100) and incubated overnight at 4 °C. The next day, the coverslips were transferred back to a (new) 6-well plate and washed 3x for 5 min each with PBS-T (Washing solution II). Coverslips were placed again onto Parafilm, were layered with 150 µL diluted secondary AB (AlexaFluor, corresponding to the species of the primary AB, see Tab. 10, in 1 % BSA + 0.3 % TritonX100) and incubated in darkness for 1 h at RT. Afterwards, coverslips were put back in the 6-well plate, washed twice with PBS-T and once with PBS (5 min each time). To stain the nuclei, cells were incubated for 8 min (RT, darkness) in 1 mL/well DAPI solution. After being washed 3x with PBS (5 min each time), coverslips were mounted upside-down onto microscopy slides using 40 µL Embedding reagent (containing MOWIOL). The dry slides were stored in the refrigerator.

Slides were examined using an *Axio Observer.Z1 microscope* and representative images were acquired with an *Axiocam 506 mono digital camera* and the software *ZEN3.2*. Confocal images were obtained using the *Aurox Unity imaging system* and processed with *FIJI*. Colocalisation was estimated using Pearson's coefficient, calculated with the *Coloc2* plugin in *FIJI*.

2.2.7 Proliferation and migration assays

Both assays were conducted on 6-well plates using the SIAH3-inducible T-REx-293 cells.

Growth curves were created to examine cell proliferation. For SIAH3, a pool of 3 clones was used (Wt: #6 + #8 + #15 or Δ S3UNS: #1 + #3 + #7). 500,000 cells per well were seeded for the experiments comparing ctrl. vs. SIAH3 Wt within high (4.5 g/L), low (1 g/L) or no glucose containing medium, 250,000 cells were seeded for the comparison between SIAH3 Wt and Δ S3UNS. Cells were induced with 2 µg/mL Dox immediately before plating. Each day, the cells were counted using a Neubauer counting chamber and microscopy images of the cells were taken. The experiment was conducted in triplicates.

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Cell migration was assessed by scratch assay. For this, cells were seeded and after 24 h three scratches per well were introduced with a sterile pipette tip. After washing with PBS, new medium containing 2 µg/mL Dox (except for the controls) was added to the cells. Scratches were photographed under the microscope daily for 6 days at 3-4 sites and quantified using *ImageJ*. The experiment was performed with two different T-REx SIAH3 clones (#8 & #15).

2.2.8 Cell cycle analysis

Cell cycle distribution was analysed by flow cytometry. T-REx SIAH3 cells (pool of clone #6 + #8 + #15) and T-REx empty cells (ctrl.) were plated on 10 cm dishes with low density and induced with 2 µg/mL Dox at the same time. For long induction times, cells were splitted again after one day onto 15 cm dishes to ensure proliferation. At the intended time points, cells were fixed according to the following protocol:

To gather dead cells as well, medium was collected in a reaction tube and centrifuged (5 min at 1,500 rpm) and supernatant was discarded. Cells were trypsinised, resuspended in fresh medium, and transferred to the same reaction tube in which the corresponding dead cells had been collected. Cells were centrifuged for 5 min at 1,500 rpm and supernatant was discarded. Afterwards, cells were washed twice with PBS and were resuspended in PBS (1 mL PBS for 5×10^6 cells). Ice-cold 100 % ethanol (2.33 mL per 5×10^6 cells) was added dropwise while shaking on the vortex mixer (making 30 % PBS + 70 % ethanol). Samples were fixed at -20 °C until further processing (at least overnight). On the day of measurement, fixed cells were washed twice with 1 mL PBS, centrifuged for 5 min at 1,500 rpm and resuspended in PBS (1 million cells in 500 µL PBS). 500 µL cell suspension was then digested with 2.5 µL RNase A for 30 min at 37 °C. Flow cytometry analysis was done using a *BD FACS Canto II*. Within a 5 mL FACS tube, 500 µL cells in PBS were mixed with 25 µL propidium iodide. Propidium iodide intercalates into the DNA and fluoresces, thus the cells can be separated by DNA content. After short incubation (5 min), samples were vortexed and applied to the machine. Per sample, around 20,000 cells were measured. The analysis was done with the *BD FACSDiva software*. First, the cells were gated according to size and granularity using forward scatter (FSC-A) and sideward scatter (SSC-A). Then, doublets were excluded conferring to width and height of the forward scatter (FSC-W vs. FSC-H). Propidium iodide was detected in the PE channel. The PE signal was plotted against cell count and within this graph, cells were gated according to the cell cycle phases sub G1, G0/G1, S and G2M.

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2.2.9 Analysing the acidification of the cell culture medium

Cellular metabolism produces lactate through glycolysis. This leads to an acidification of the cell culture medium that could be assessed via the pH indicator included in the medium. The medium layered over the pre-treated cells was collected and centrifuged shortly to remove cell debris. 100 µL of clear medium was added per well of a 96-well plate (in triplicate per sample). An absorbance spectrum ranging from 350-750 nm (increments of 5 nm, 4 measurement spots per well) was created using a spectrophotometer (*Tecan Infinite M200 PRO*).

2.2.10 Analysis of cellular metabolism

Cellular metabolism was monitored by analysing mitochondrial respiration and glycolysis of T-REx-293 cells. The *Seahorse XFe96 Analyzer* measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), key indicators for mitochondrial respiration and glycolysis, respectively, of living cells.

Cellular metabolism of T-REx SIAH3 cells (Wt: pool of clone #6 + #8 + #15; ΔS3UNS: pool of #1 + #3 + #7) and T-REx-293 empty cells as well as the effect of a SIAH3 knockdown in C918 cells were analysed. For SIAH3 Wt three different experiments were conducted. First, a mitochondrial stress test (*Agilent Seahorse XF Cell Mito Stress Test Kit*) was performed, followed by a glycolysis stress test (*Agilent Seahorse XF Glycolysis Stress Test Kit*) and analysis of ATP production rates (*Agilent Seahorse XF Real-Time ATP Rate Assay Kit*). In advance, optimal cell number and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) concentration had to be determined. For this, seeding of 20,000, 30,000 or 40,000 cells and 0.25 µM, 0.5 µM, 0.75 µM and 1 µM FCCP were tested. Seeding 30,000 cells per wells turned out to be the best cell set and 0.75 and 1 µM FCCP worked well and were therefore used in the following experiments. For the comparison between SIAH3 Wt and ΔS3UNS as well as between Wt and knockdown of SIAH3 in C918, only the ATP rate assay was conducted. The experiment was done in cooperation with PD Dr. Wolfgang Meißner of the Core Facility for Cellular Metabolism (now Core Facility Genomics) and Prof. Dr. Verena Taudte of the Core Facility Metabolomics of the Philipps University in Marburg.

2.2.10.1 Cell preparation

The cells were analysed within an *Agilent Seahorse XFe96/ XFPro Cell Culture Microplate* (96-well). To allow the T-REx-293 cells to adhere better, the microplate was coated with poly-L-lysine. 40 µL poly-L-lysine solution was put into the wells and removed after a 5 min incubation. The plate was dried for 10 min and placed under UV-light of the laminar flow clean bench for 1 h. Cells were counted and the intended cell number (30,000 cell, unless otherwise stated in Fig. 7 & 8) was placed within a volume of 80 µL growth medium per well (induced with Dox immediately before seeding). Directly after seeding, the cells were brought to Marburg, kept at RT for 1 h to allow the cells to

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settle down evenly and then put into a 37 °C incubator. Measurement took place 24 h after induction.

Knockdown in C918 cells using siRNA was performed as described in chapter 2.2.2.3. 48 h later, cells were plated onto the microplate (without precoating) and cellular metabolism was assessed after an additional 24 h (72 h knockdown).

To verify SIAH3 induction and knockdown, RNA and protein levels were analysed (set of cells plated in parallel on 6-wells).

2.2.10.2 Measurement and analysis

Cartridge preparation as well as washing of cells and the measurement followed the manufacturer's protocol, considering the following specifications:

Assay medium consisted of Seahorse XF DMEM supplemented with 1 mM pyruvate and 2 mM glutamine (and 10 mM glucose for the ATP Rate Assay of T-REx SIAH3 Wt vs. Δ S3UNS and knockdown in C918). The ports of the cartridges were filled according to the schemes in Tab. 36 and Fig. 7 & 8. The three experiments comparing T-REx SIAH3 (Wt) to control cells were conducted using an acute injection of glucose instead of adding it directly to the Assay medium (named "Modified Assay" in the manual). The first experiment, determination of the optimal cell number and FCCP concentration for T-REx-293 cells, did not follow exactly the protocol of one of the kits, but combined the first steps of the glycolytic stress test with the mitochondrial stress test without the 2-deoxy-D-glucose (2-DG) or rotenone and antimycin A (Rot/AA) step.

Control of the machine and analysis was done using the *Wave Desktop and Controller Software*. The ports were injected in the order indicated by the letters A-D. Before the first and after each injection, three measurements were taken at three time points (except in Exp. 1, where 4 measurements were taken after glucose injection). During data analysis, outliers were excluded and the mean of the replicates was calculated. Further analysis was done using the Seahorse XF Report Generators (*Seahorse XF Cell Mito Stress Test Report Generator*; *Glycolysis Stress Test Report Generator* and *Real-Time ATP Rate Assay Report Generator*).

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Tab. 36: Loading conditions of the Seahorse XFe96/XF Pro sensor cartridge

Exp. 1: Determination of optimal cell number and FCCP concentration				
T-REx SIAH3 (Wt) vs. ctrl.				
port	compound	concentration (10x conc.)	amount	final concentration
A	glucose	100 mM	20 µL	10 mM
B	oligomycin	10 µM	22 µL	1 µM
C1	FCCP	2.5 µM	25 µL	0.25 µM
C2	FCCP	5.0 µM	25 µL	0.50 µM
D1	FCCP	5.0 µM	27 µL	0.75 µM
D2	FCCP	5.0 µM	27 µL	1.00 µM
Exp. 2: Cell mito stress test				
T-REx SIAH3 (Wt) vs. ctrl.				
port	compound	concentration (10x conc.)	amount	final concentration
A	glucose	100 mM	20 µL	10 mM
B	oligomycin	20 µM	22 µL	2 µM
C1	FCCP	7.5 µM	25 µL	0.75 µM
C2	FCCP	10.0 µM	25 µL	1.00 µM
D	Rot/AA	5.0 µM	27 µL	0.50 µM
Exp. 3: Glycolysis stress test (a) & ATP rate assay (b)				
T-Rex SIAH3 (Wt) vs. ctrl.				
port	compound	concentration (10x conc.)	amount	final concentration
A (a & b)	glucose	100 mM	20 µL	10 mM
B (a & b)	oligomycin	10 µM	22 µL	1 µM
C (a)	2-DG	500 mM	25 µL	50 mM
C (b)	Rot/AA	5.0 µM	25 µL	0,50 µM
Exp. 4: ATP rate assay				
T-REx SIAH3 Wt vs. ΔS3UNS				
port	compound	concentration (10x conc.)	amount	final concentration
A	oligomycin	15 µM	20 µL	1.5 µM
B	Rot/AA	5.0 µM	22 µL	0.5 µM
Exp. 5: ATP rate assay				
C918 Wt vs. SIAH3 knockdown				
port	compound	concentration (10x conc.)	amount	final concentration
A	oligomycin	15 µM	20 µL	1.5 µM
B	Rot/AA	5.0 µM	22 µL	0.5 µM

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Exp. 1: Determination of optimal cell number and FCCP concentration (T-Rex SIAH3 vs. ctrl.)															
		ctrl.						SIAH3							
		- Dox			+ Dox			- Dox			+ Dox				
		1	2	3	4	5	6	7	8	9	10	11	12		
Ports: A: glucose B: oligomycin C1: FCCP 2.5 μM C2: FCCP 5.0 μM D1: FCCP 5.0 μM D2: FCCP 5.0 μM	20,000 cells	A	A B C1 D1	A B C1 D1	A B C1 D1	A B C1 D1	A B C1 D1	A B C2 D2	A B C1 D1	A B C1 D1	A B C2 D2	A B C1 D1	A B C1 D1	A B C1 D1	A B C2 D2
		B	A B C2 D2	A B C1 D1	A B C1 D1	A B C1 D1	A B C1 D1	A B C2 D2	A B C1 D1	A B C1 D1	A B C2 D2	A B C1 D1	A B C1 D1	A B C2 D2	A B C2 D2
		C	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C1 D1	A B C2 D2	A B C2 D2	A B C1 D1	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2
30,000 cells	D	A B C1 D1	A B C1 D1	A B C2 D2	A B C1 D1	A B C1 D1	A B C1 D1	A B C1 D1	A B C1 D1	A B C1 D1	A B C1 D1	A B C2 D2	A B C1 D1	A B C1 D1	A B C1 D1
	E	A B C1 D1	A B C1 D1	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C1 D1	A B C1 D1
	F	A B C2 D2	A B C1 D1	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C1 D1	A B C2 D2
40,000 cells	G	A B C1 D1	A B C1 D1	A B C2 D2	A B C1 D1	A B C1 D1	A B C2 D2	A B C1 D1	A B C1 D1	A B C1 D1	A B C2 D2	A B C2 D2	A B C2 D2	A B C1 D1	A B C1 D1
	H	A B C1 D1	A B C2 D2	A B C2 D2	A B C1 D1	A B C1 D1	A B C2 D2	A B C1 D1	A B C1 D1	A B C2 D2	A B C2 D2	A B C2 D2	A B C2 D2	A B C1 D1	A B C1 D1

Exp. 2: Cell mito stress test (T-Rex SIAH3 vs. ctrl.)															
		ctrl.						SIAH3							
		- Dox			+ Dox			- Dox			+ Dox				
		1	2	3	4	5	6	7	8	9	10	11	12		
Ports: A: glucose B: oligomycin C1: FCCP 7.5 μM C2: FCCP 10.0 μM D: Rot/AA	0.75 μM FCCP	A	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D
		B	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D
		C	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D
D		A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	A B C1 D	
1.0 μM FCCP	E	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D
	F	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D
	G	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D
	H	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D	A B C2 D

Exp. 3: Glycolysis stress test (a) & ATP rate assay (b) (T-Rex SIAH3 vs. ctrl.)															
		ATP rate assay (b)						Glycolysis stress test (a)							
		ctrl.			SIAH3			ctrl.			SIAH3				
		1	2	3	4	5	6	7	8	9	10	11	12		
Ports: A: glucose B: oligomycin Ca: 2-DG Cb: Rot/AA	- Dox	A	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca
		B	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca
		C	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca
D		A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	
+ Dox	E	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca
	F	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca
	G	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca
	H	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Cb	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca	A B Ca

Fig. 7: Cell plating and cartridge filling schemes for the Seahorse XFe96/XFPro Cell Culture Microplate and sensor cartridge (T-Rex SIAH3 vs. T-Rex ctrl.).

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Exp. 4: ATP rate assay (T-REx SIAH3 Wt vs. Δ S3UNS)													
		ctrl.				SIAH3							
		empty				Wt				Δ S3UNS			
		1	2	3	4	5	6	7	8	9	10	11	12
Ports: A: oligomycin B: Rot/AA	- Dox	A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B
	+ Dox	A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B

Exp. 5: ATP rate assay (C918 SIAH3 knockdown)													
		cell number											
		10,000			13,300			16,600			20,000		
		1	2	3	4	5	6	7	8	9	10	11	12
Ports: A: oligomycin B: Rot/AA	si ctrl.	A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B
	si SIAH3	A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B
		A	B	A	B	A	B	A	B	A	B	A	B

Fig. 8: Cell plating and cartridge filling schemes for the Seahorse XFe96/XFPro Cell Culture Microplate and sensor cartridge (T-REx SIAH3 Wt vs. Δ S3UNS and C918 Wt vs. knockdown).

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2.2.11 Epigenetic editing

Silenced *SIAH3* was reactivated using epigenetic editing. Six different single guide RNAs (sgRNAs) were used to recruit an inactivated version of *Streptococcus pyogenes* Cas9 coupled with an epigenetic activator (modifier) to the promoter region of *SIAH3*.

2.2.11.1 Creation of guide RNAs

sgRNAs were designed using *Benchling*. Guide II-VI span a range of 600 bp upstream of the TSS, guide I lies downstream of the TSS near the translation start site (TSS). All guides are 20-21 nucleotides long. Guides were ordered as oligonucleotides (for sequences see Tab. 17), annealed to form double strand DNA and cloned into pX459 vector (without Cas9) inside the gRNA scaffold via the *BbsI* restriction site. Cloning was performed according to the following protocol:

pX459 vector was opened with *BbsI*, cleaned up by gel extraction and purified using the *NucleoSpin Gel and PCR Clean-up Kit*, followed by dephosphorylation (see chapter 2.2.1.2.1). Oligonucleotides were phosphorylated with a T4 Polynucleotide Kinase (PNK), conditions are listed in Tab. 37. Forward and reverse oligonucleotides were annealed by heating at 95 °C for 5 min and then slowly cooling down to RT (switching off the heating block and waiting until it reached RT). Annealed oligonucleotides were then ligated into the opened pX459 vector and transformed in and amplified by *E. coli*.

Guides I-III were cloned by T. Zenz and guides III-VI by my Master's student F. Geyer.

Tab. 37: Conditions for the phosphorylation of the oligonucleotides.

phosphorylation of oligonucleotides	
10 µL PNK buffer A	➤ incubation at 37 °C for 1 h
1 µL PNK enzyme	
1 µL ATP (10 mM)	
2 µL forward nucleotide (20 µM)	
2 µL reverse nucleotide (20 µM)	
ad 100 µL ddH ₂ O	

2.2.11.2 Experimental Setup

Cells were co-transfected with 1,000 ng guide plasmid (one guide or mix as indicated in figure legends) and 3,000 ng modifier-dCas9 plasmid (e.g. p300). For HEK293T, one day after transfection selection using puromycin (5-10 µg/mL) and co-treatment (under a non-disclosure agreement) started. Medium was changed each day to apply fresh puromycin and co-treatment. For C918, puromycin selection (5 µg/mL) and co-treatment was applied only once on day 3. After an incubation for 48-144 h (depending on the experiment), DNA and/or RNA was isolated. DNA was

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BS-converted and pyrosequencing was performed. RNA was DNase digested, reverse transcribed and *SIAH3* mRNA was quantified by qPCR.

Experiments were performed by my Master's student J. Bender.

2.2.11.3 Promoter Assay

SIAH3 promoter activity was examined using a dual-luciferase reporter system. Cells, grown in 6-well dishes, were co-transfected with 1,250 ng pRL-null vector (empty or containing the *SIAH3* promoter sequence), 750 ng pGL3, 1,000 ng modifier-dCas9 plasmid and 1,000 ng guide plasmid (mix of guide I-III). After 24-48 h, cells were analysed using the *Dual-Luciferase Reporter Assay System* according to the following procedure.

Medium was discarded and cells were lysed in 200-250 μ L cold PLB through shaking vigorously. After centrifugation at 13,000 rpm for 30 s, clear lysate was transferred to a new reaction tube. 10 μ L lysate per sample was put into a white 96-well plate, 33 μ L LARII was added and firefly luciferase luminescence was measured using *Orion L Microplate Luminometer*. 33 μ L Stop & Glo Reagent (substrate 1:50 diluted with Stop & Glo buffer) was added and luminescence created by *Renilla* luciferase was measured. Luminescence was measured in triplicates. *Renilla* luciferase luminescence signal was normalised to firefly luciferase luminescence.

2.2.12 Bioinformatics and data presentation

2.2.12.1 Statistics

The difference between two conditions or groups (in qPCR and pyrosequencing experiments, of WB quantifications, promoter and proliferation assays as well as measurement of cell metabolism) was statistically evaluated using Student's t-test (two-sample, unpaired, equal variances) calculated with R (command: `t.test(x, y, var.equal=TRUE)`, with x and y being numeric vectors of data values) or with the online t-test calculator from *GraphPad* (see Tab. 29). Significance, depicted by p-values, was categorised as shown in Tab. 38. The error bars within the graphs represent the standard deviation. Further statistics (e.g. for MS experiments) was done as described in the chapters corresponding to the experiment.

Tab. 38: Levels of significance.

levels of significance		
> 0.05	ns	not significant
0.01 - 0.05	*	significant
0.001 - 0.01	**	very significant
< 0.001	***	highly significant

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2.2.12.2 Analysis of published data

Analysis of large, published datasets (e.g. TCGA, GTEx, CCLE) was performed using *R2: Genomic Analysis and Visualisation Platform* (expression and methylation data, correlation between expression and methylation, heatmaps for the different CpGs and Kaplan-Meier survival curves), *GEPIA* (expression) and *SMART* (methylation). For more information, including the URLs, see Tab. 29.

2.2.12.3 Data assembly and presentation

Graphs (of own data) were created using *MS Office Excel*, graphs depicting results of large, published datasets were obtained from online tools, except the Kaplan-Meier Curves showing the methylation of SKCM, HNSC and LUAD which were created with *R* (based on data from *SMART*). Plasmid maps were created with *Benchling.com*. If necessary, layout was modified using *Inkscape*. Figures were assembled in *Inkscape*.

RESULTS

3 RESULTS

3.1 *SIAH3* is epigenetically downregulated in human cancer

Although there is no bona fide CpG island within the promoter region of *SIAH3*, a clustering of CpGs can be found, which also appears to be of regulatory relevance. 21 CpGs are annotated as belonging to *SIAH3* (R2 Genome Browser, see Fig. 10A). Of these, 10 are located upstream of the TSS. The annotated CpG probe cg26667946 lies 31 bp in front of the TSS and corresponds to the *Bst*UI restriction site used in CoBRA analyses. By pyrosequencing, five CpGs were examined, including the two CpGs of probe cg26667946 and three additional ones (see Fig. 6A). In the following, the methylation of these CpGs was analysed using large, published datasets and pyrosequencing of tissue samples from skin cutaneous melanoma (SKCM), head and neck squamous cell carcinoma (HNSC) and lung adenocarcinomas (LUAD). Furthermore, the expression of *SIAH3* was analysed in these tissues, as well as the survival probability of patients regarding the *SIAH3* status.

3.1.1 *SIAH3* is hypermethylated in cancer

A comparison between normal tissues and cancer revealed a decrease in *SIAH3* expression from normal tissue to tumours and further to cancer cell lines, whereas *SIAH3* methylation levels (cg26667946) significantly increase (Fig. 9A and B). To link these observations, *SIAH3* promoter methylation was correlated with its expression in human cancer cell lines, finding that hypermethylation is significantly associated with lower *SIAH3* mRNA levels (Fig. 9C), suggesting that its expression can be regulated by DNA methylation. This finding is further supported by

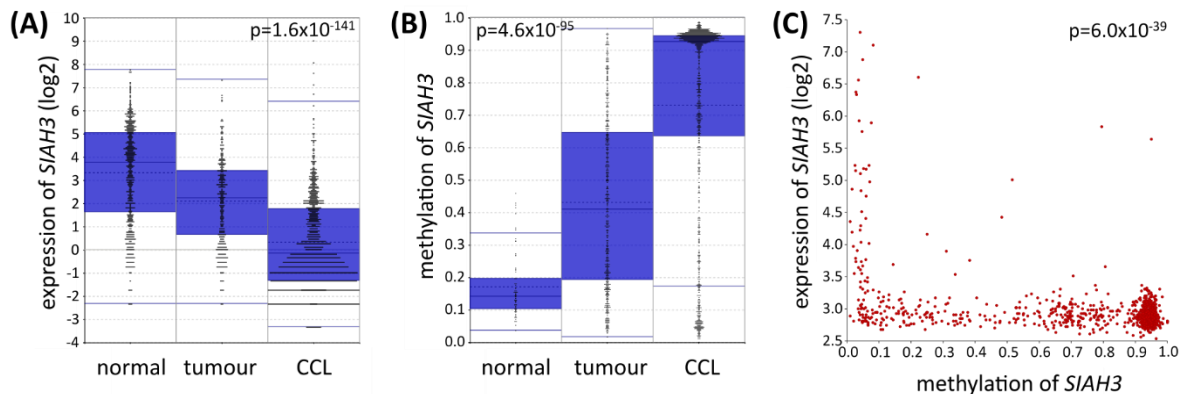


Fig. 9: *SIAH3* is hypermethylated in human cancer

(A) Cancer cell lines show significant lower *SIAH3* expression compared to primary tumours and normal tissues. Used datasets: Normal (GSE7307, Human body index, n = 504), tumour (GSE2109, Expression Project for Oncology, n = 296), CCL = cancer cell lines (GSE68378, Cancer Cell Line Encyclopedia, n = 917). **(B)** Methylation level (betavalue) of *SIAH3* at cg26667946 is significantly higher in cancer cell lines compared to tumours and normal tissue. Used datasets: Normal (GSE50192, normal tissues, n = 70), tumour (GSE76269, tumour types, n = 493), CCL = cancer cell lines (GSE36133, Cell Line Cancer Pharmacogenomic, n = 1028). **(C)** Correlation analysis of *SIAH3* methylation (cg26667946) and expression in cancer cell lines. Used datasets: Methylation (GSE36133, Cell Line Cancer Pharmacogenomic, n = 1028), expression (GSE68378, Cancer Cell Line Encyclopedia, n = 917).

All analyses were performed using the platform R2. Figure adapted from Deutschmeyer *et al.*, 2024.

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experimental evidence showing that 5-aza-dC treatment reduces methylation and increases *SIAH3* expression in melanoma cell lines (Fig. 6D and E). Closer examination of individual CpG sites revealed that 12 CpGs – spanning from 200 bp upstream of the TSS to the beginning of the first intron (cg25658983 to cg10775278) – are unmethylated in normal tissues but hypermethylated in cancer samples and cancer cell lines (Fig. 10).

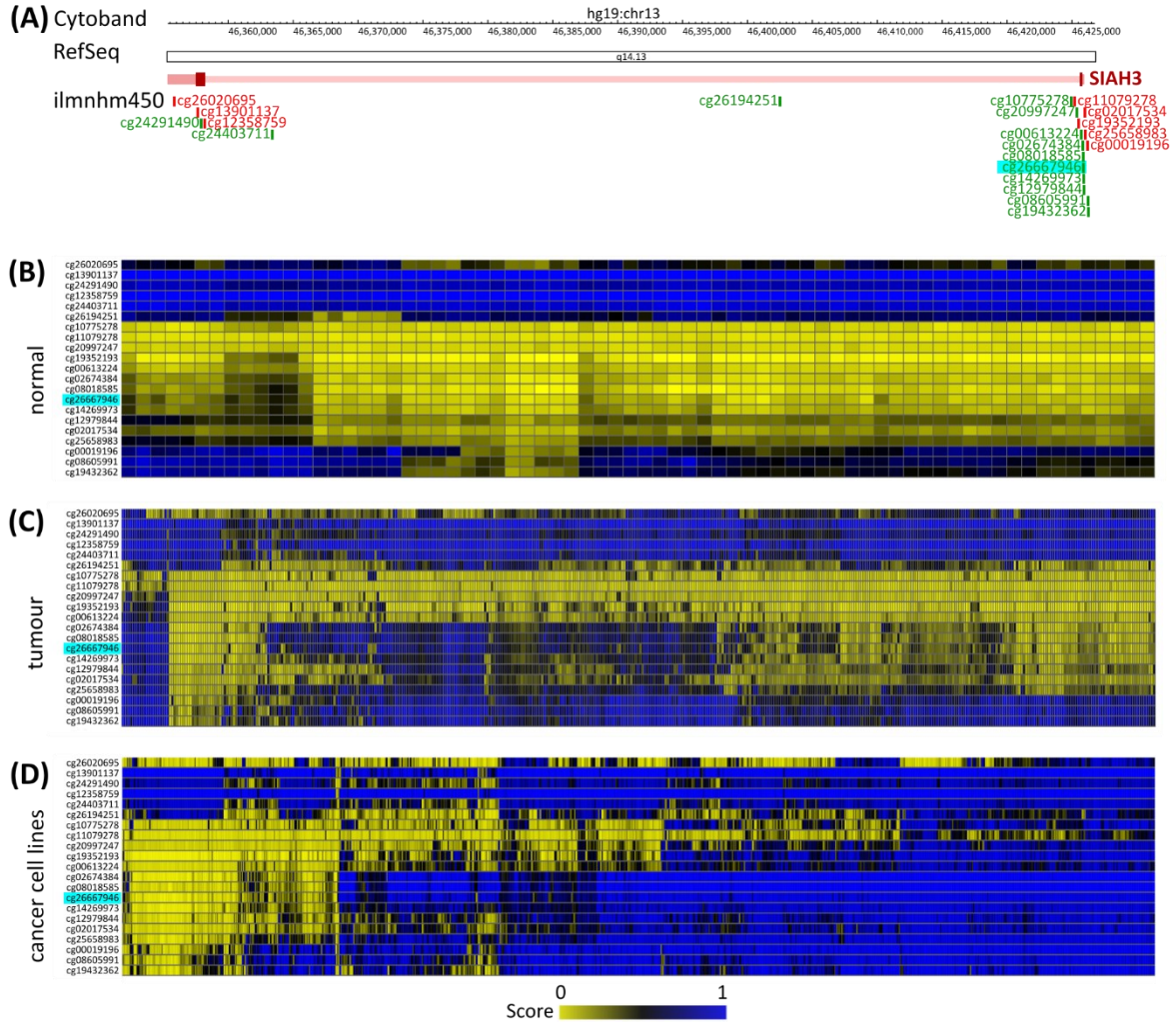


Fig. 10: Distribution of methylation across the *SIAH3* genomic region

(A) Localisation of *SIAH3* assigned CpG probes. Picture from the R2 Genome Browser. cg26667946 is marked. (B-D) Heatmaps showing all methylation probes for *SIAH3*. (B) Normal (GSE50192, normal tissues, n = 70), (C) tumour (GSE76269, tumour types, n = 493), (D) cancer cell lines (GSE36133, Cell Line Cancer Pharmacogenomic, n = 1028). Analyses from the platform R2.

Analysing DNA from primary tissue samples, *SIAH3* promoter methylation was found to be significantly higher in primary tumours and metastases from melanoma patients compared to normal skin (nevus cell nevi), as shown by pyrosequencing of five CpGs. Similarly, a significantly higher level of methylation was observed in head and neck and lung tumours (Fig. 11A-C). The expression pattern was reversed in these tumours (Fig. 11D-F), indicating that *SIAH3* is epigenetically silenced in these tumours.

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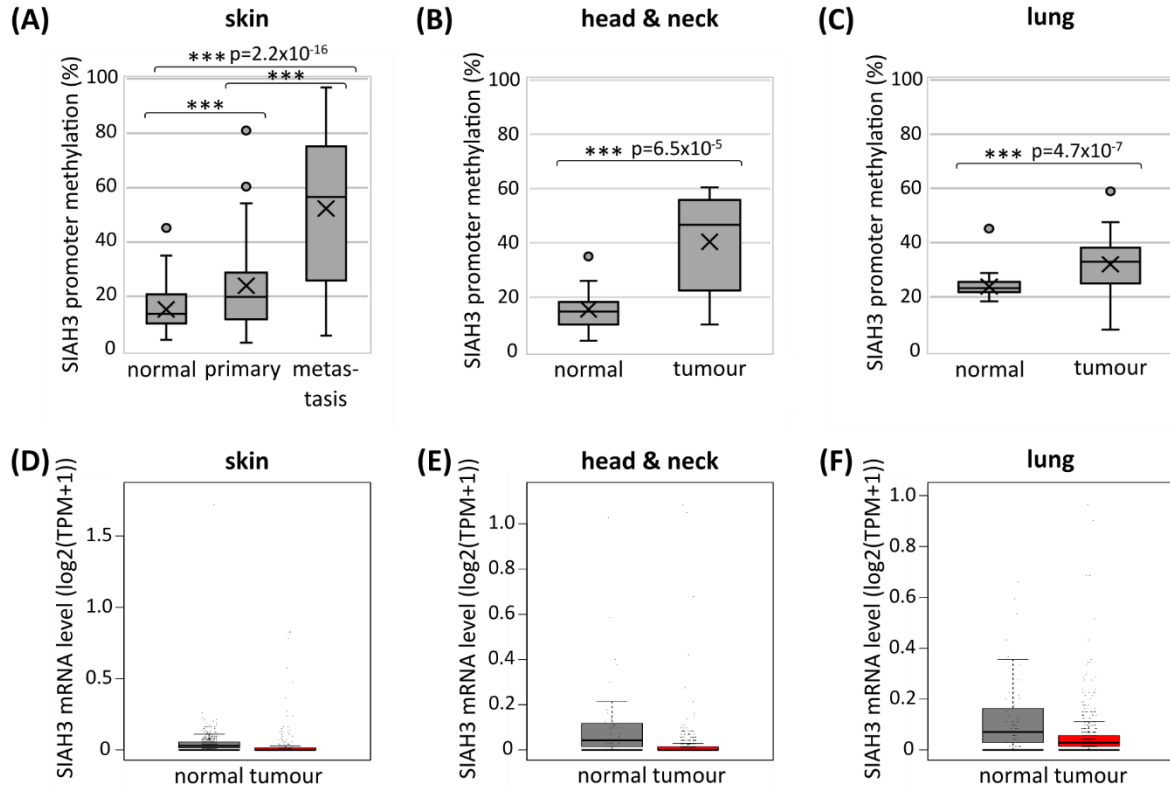


Fig. 11: *SIAH3* is epigenetically silenced by hypermethylation in melanoma, head and neck cancer and lung adenocarcinoma.

(A-C) Pyrosequencing of 5 CpGs in the promoter of *SIAH3*. Experiments were conducted by S. Walesch, J. Moyer and my Bachelor's student R. Schmalenberger. (A) *SIAH3* promoter hypermethylation in malignant melanoma of the skin. Normal n = 59, primary tumours n = 42, metastases n = 47. Student's t-test: Normal vs. primary tumours p = 0.0008, primary tumours vs. metastases p = 5.1x10⁻⁸, normal vs. metastases p = 2.2x10⁻¹⁶. (B) *SIAH3* hypermethylation in head and neck cancer. Normal n = 14, tumour n = 14, p = 6.5x10⁻⁵. (C) *SIAH3* hypermethylation in adenocarcinomas of the lung. Normal n = 50, tumour n = 50, p = 4.7x10⁻⁷. **(D-F)** Expression of *SIAH3* in (D) SKCM (TCGA + GTEx data; normal n = 558, tumour n = 461), (E) HNSC (TCGA + GETX data; normal n = 44, tumour n = 519) and (F) LUAD (TCGA data; normal n = 59, tumour n = 483). Expression analysis was performed with *GEPIA*. Panels A-C adapted from Deutschmeyer *et al.*, 2025.

Datamining also revealed significantly increased methylation of the *SIAH3* promoter, coupled with reduced expression, in kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), glioblastoma multiforme (GBM) and colon adenocarcinoma (COAD) (Fig. 12A and B), further broadening the observation that *SIAH3* is epigenetically silenced across a wide range of different cancer types.

On the other hand, there are also entities in which *SIAH3* is overexpressed in cancer, such as in the thymoma (THYM), uterine corpus endometrial carcinoma (UCEC) and pheochromocytoma and paraganglioma (PCPG). However, at least in THYM and UCES, the expression is not negatively correlated with methylation, indicating that here gene expression is not (only) determined by methylation (Fig. 12C and D).

RESULTS

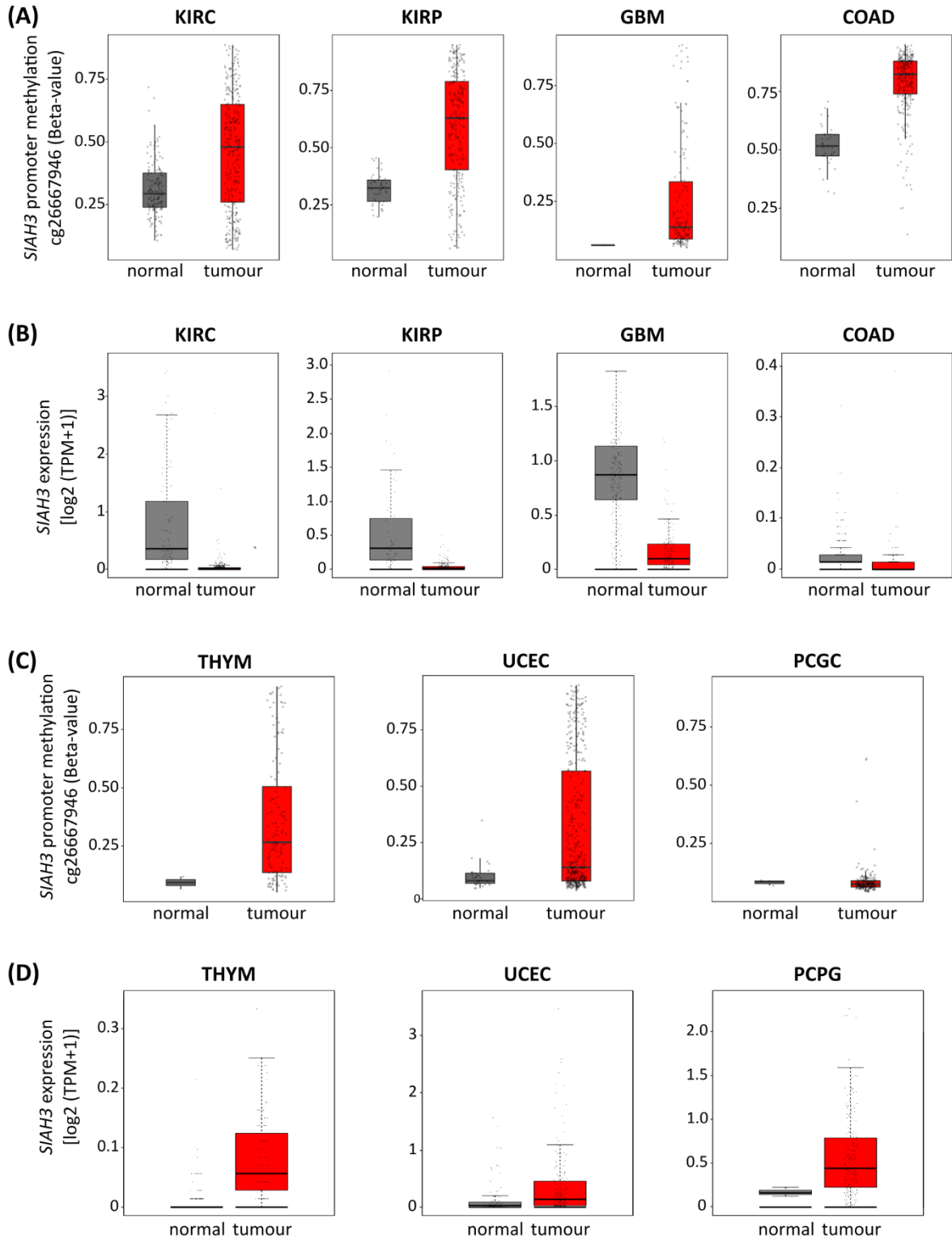


Fig. 12: *SIAH3* is epigenetically silenced in many different cancer types but upregulated in others.

(A) *SIAH3* is hypermethylated in KIRC (normal $n = 157$, tumour $n = 313$), KIRP (normal $n = 43$, tumour $n = 272$), GBM (normal $n = 1$, tumour $n = 159$) and COAD (normal $n = 34$, tumour $n = 288$). Analysis was performed with *SMART*. **(B)** *SIAH3* expression is reduced in KIRC (normal $n = 100$, tumour $n = 523$), KIRP (normal $n = 60$, tumour $n = 286$), GBM (normal $n = 207$, tumour $n = 163$) and COAD (normal $n = 349$, tumour $n = 275$). Analysis was performed with *GEPIA*. **(C)** *SIAH3* methylation in THYM (normal $n = 2$, tumour $n = 124$), UCEC (normal $n = 33$, tumour $n = 419$) and PCPG (normal $n = 3$, tumour $n = 183$). Analysis was performed with *SMART*. **(D)** *SIAH3* expression in THYM (normal $n = 339$, tumour $n = 118$), UCEC (normal $n = 91$, tumour $n = 174$) and PCPG (normal $n = 3$, tumour $n = 182$). Analysis was performed with *GEPIA*.

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3.1.2 Reduced *SIAH3* levels are associated with a poorer prognosis in patients across various cancer types

Furthermore, the observed epigenetic silencing of *SIAH3* was found to be associated with a poorer survival prognosis in several cohorts of cancer patients. As shown in Fig. 13, lower expression of *SIAH3* was related to a worse overall survival probability in SKCM, HNSC and LUAD. A corresponding higher methylation of *SIAH3* (cg26667946) was also associated with poor overall survival in SKCM and LUAD. And this finding extends to additional tissues such as the brain tumours, breast cancer (BRCA), and liver hepatocellular carcinoma (LIHC), where cancer patients exhibit a better survival probability with higher levels of *SIAH3* expression (Fig. 14).

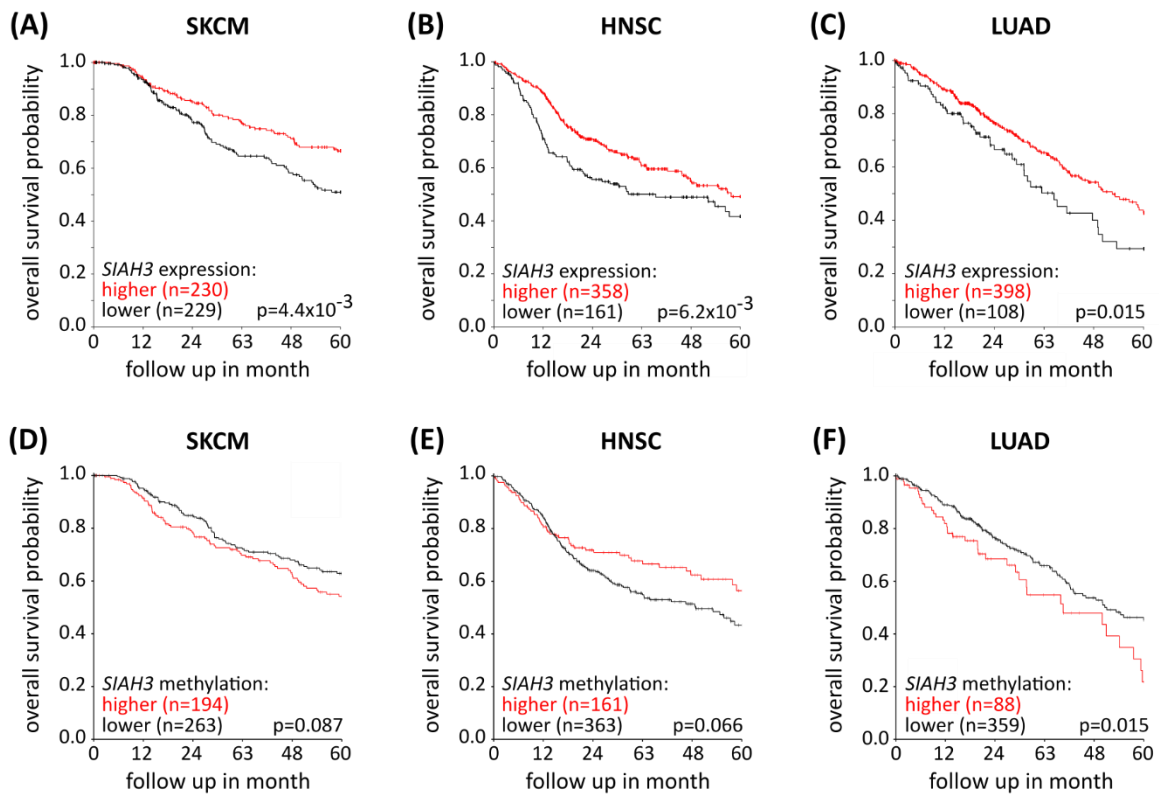


Fig. 13: Silencing of *SIAH3* is associated with poor prognosis in patients with SKCM, HNSC and LUAD.

(A-C) Kaplan-Meier survival curves of SKCM (A), HNSC (B) and LUAD (C) patients with higher (red) or lower (black) *SIAH3* expression. Used dataset: (A) TCGA-SKCM (n = 459), (B) TCGA-HNSC (n = 519), (C) TCGA-LUAD (n = 506). Analysis was performed using the platform R2. Expression cut-off: scan method. **(D-E)** Kaplan-Meier survival curves based on *SIAH3* methylation (cg26667946) of SKCM (A), HNSC (B) and LUAD (C) patients with higher (red) or lower (black) *SIAH3* methylation. Datasets were obtained via SMART. (D) TCGA-SKCM (n = 457), (E) TCGA-HNSC (n = 524), (F) TCGA-LUAD (n = 447). Graphs were created with R. Expression cut-off: maxstat method.

Panels A-C adapted from Deutschmeyer *et al.*, 2025.

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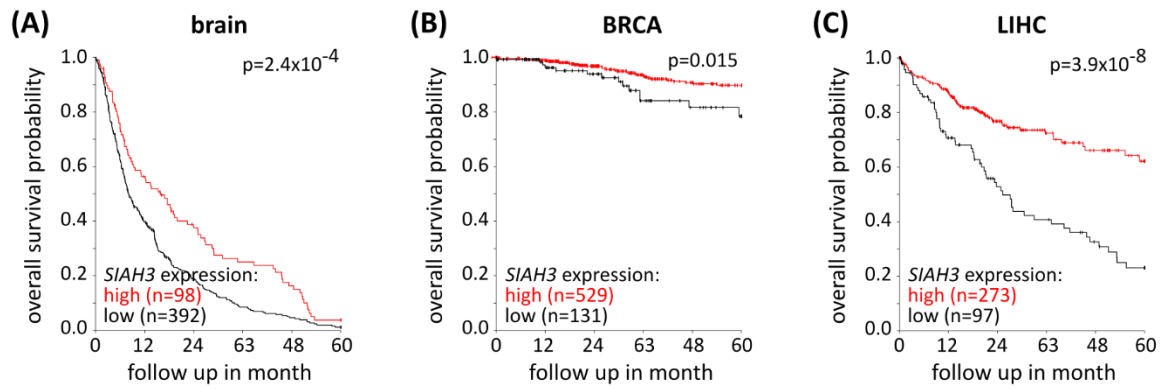


Fig. 14: Higher *SIAH3* expression is correlated with better survival in further cancer entities.

Kaplan-Meier survival curves regarding *SIAH3* expression of brain cancer (A), BRCA (B) and LIHC (C) patients with high (red) or low (black) *SIAH3* expression. Used dataset: (A) GSE108474, REMBRANDT study (n = 490), (B) TCGA-BRCA (2022-v32) (n = 660), (C) TCGA-LIHC (n = 370). Analysis was performed using the platform R2. Expression cut-off: scan method. Figure based on Deutschmeyer *et al.*, 2025.

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3.2 Additional mechanisms regulating the expression of *SIAH3*

Beyond regulation via DNA methylation, *SIAH3* expression was found to be modulated by additional mechanisms. Experiments with cells cultured at different densities revealed that *SIAH3* expression increases as cell density rises (Fig. 15A and supplementary Fig. S17A). Conversely, when densely grown cells were separated, prolonged isolation led to a gradual decline in *SIAH3* expression (Fig. 15B and Fig. S17B). Inhibition of RNA polymerase II with α -amanitin revealed an mRNA half-life of less than 3 h, indicating moderate stability of *SIAH3* mRNA. (Fig. 15C and Fig. S17C).

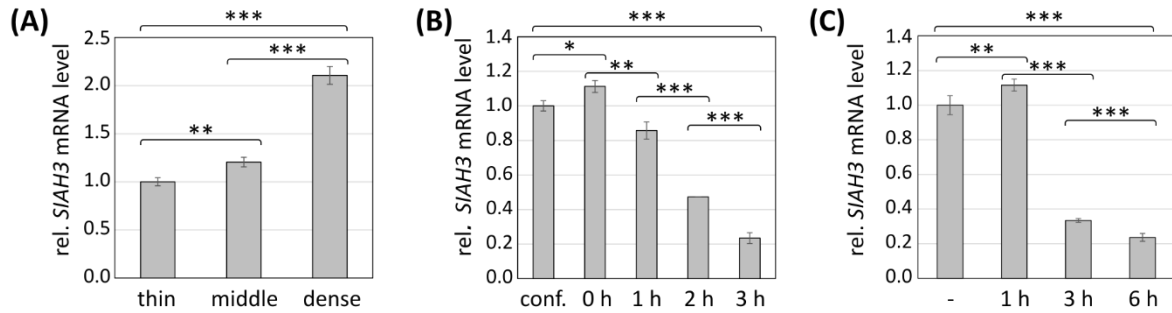


Fig. 15: *SIAH3* expression depends on cell density.

(A) Density dependence of *SIAH3* expression. Same numbers of C918 cells were cultured on dishes of 3 different sizes until the smallest was confluent. RNA was isolated and *SIAH3* mRNA level was determined by RT-qPCR. *SIAH3* expression was normalised to *ACTB*. **(B)** Separation of cells led to a decrease in *SIAH3* expression. C918 cells were cultivated until being confluent, then separated by trypsin and kept isolated by rotating for 1 to 3 h at 37 °C. RNA was isolated at the indicated time points and *SIAH3* expression was determined by RT-qPCR. *SIAH3* expression was normalised to *ACTB*. **(C)** Stability of *SIAH3* mRNA. C918 cells were treated with α -amanitin (10 μ M) 1 to 6 h before RNA was isolated. *SIAH3* expression was determined by qPCR and normalised to *28S* RNA. */**/** indicate the significance levels (Student's t-test).

Exposing C918 cells to low oxygen levels (1 % O₂) showed a decrease in *SIAH3* expression after 6 h and 24 h while the known hypoxia regulated gene *VEGF* (vascular endothelial growth factor) was upregulated. This effect was higher in the denser grown cells and more significant after 24 h than after 6 h. This finding suggests that *SIAH3* plays a role in hypoxia-regulated pathways (Fig. 16).

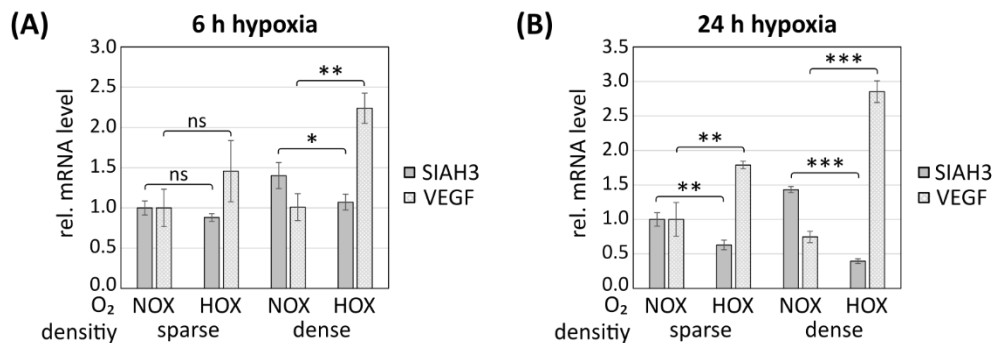


Fig. 16: *SIAH3* is downregulated under hypoxic conditions.

C918 cells were cultivated under normoxic (NOX) and hypoxic (HOX, 1 % O₂) conditions for 6 h **(A)** or 24 h **(B)**. RNA was isolated and mRNA level was determined by RT-qPCR. *SIAH3* and *VEGF* expression were normalised to *ACTB*. */**/** indicate the significance levels (Student's t-test).

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3.3 *SIAH3* overexpression inhibits cell proliferation

SIAH3's hypermethylation in tumours and its correlation with a poorer prognosis in cancer patients proposed that *SIAH3* may act as tumour suppressor. To support this hypothesis, the effect of *SIAH3* on cell proliferation and migration was analysed.

Cell migration was investigated by scratch assay using the T-REx *SIAH3* inducible cell line (Fig. 17). 6 days after scratching, the T-REx control cells closed the gap completely, whereas the scratch within the *SIAH3* expressing cells was still half-opened (both clones). The uninduced T-REx *SIAH3* cells filled the gap almost completely. Additionally, among the *SIAH3* expressing cells many dead ones were detected (Fig. 17C). These observations indicate that *SIAH3* hinders the cells from refilling the gap. Due to the observed dead cells, no conclusions can be drawn as to whether this effect is caused by decreased migration or by reduced proliferation and/or cell death. Therefore, cell proliferation was investigated in more detail by generating growth curves.

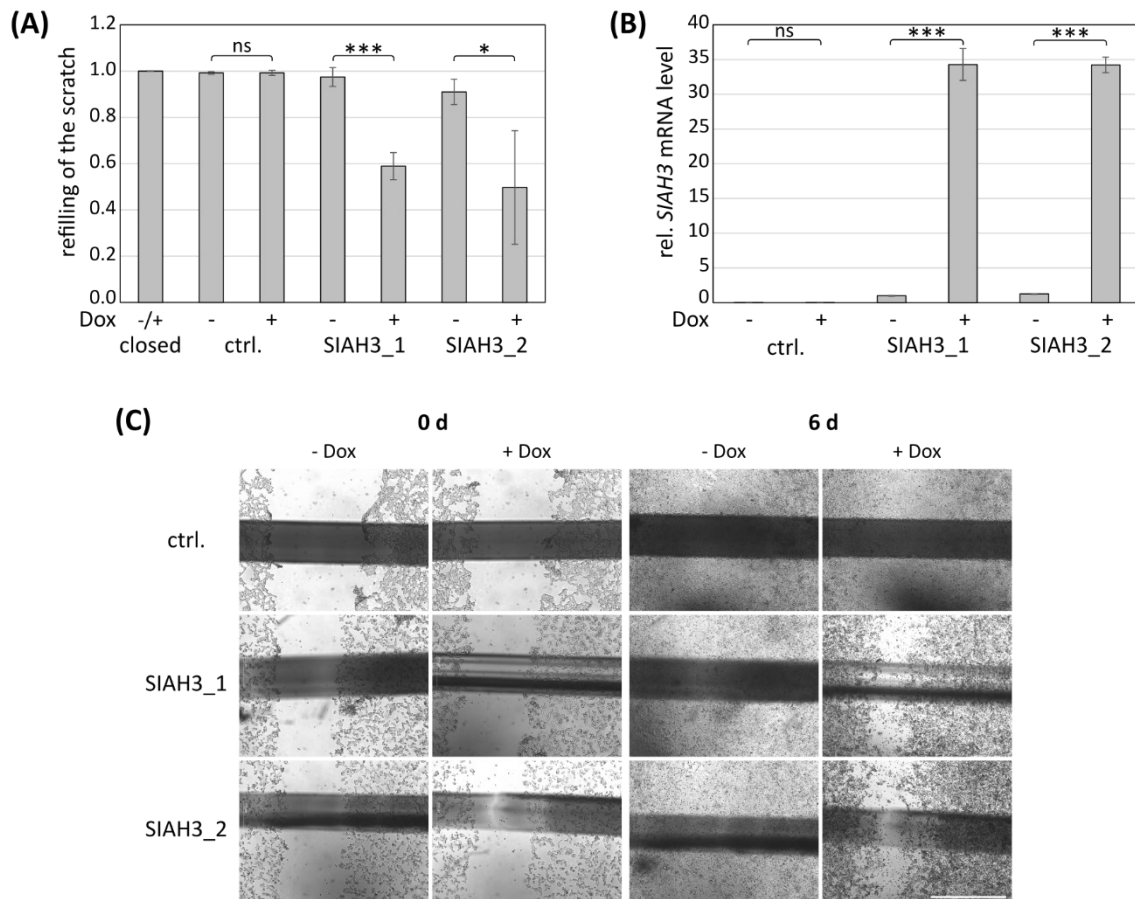


Fig. 17: Scratch assay of the T-REx cells.

(A) Scratch Assay of T-REx *SIAH3* cells (two different clones) and T-REx control cell ($\pm$ Dox). One day after plating, scratches were introduced, and the same regions were photographed daily for the following 6 days to measure scratch width. The extent of the refilling of the gap on day 6 is depicted. Complete gap closure was defined as 1. **(B)** *SIAH3* expression of the scratch assay experiment. RNA was isolated after day 6 and *SIAH3* mRNA level was determined by RT-qPCR (normalised to *ACTB*). **(C)** Corresponding microscopy photos of day 0 and day 6. Scale bar: 1,000 μ m. */**/** indicate the significance levels (Student's t-test). Panels A and C adapted from Deutschmeyer *et al.*, 2025.

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The growth curves showed that T-REx *SIAH3*-expressing cells achieved less than half of the cell number observed in control cells five days after induction (Fig. 18A). Uninduced T-REx *SIAH3* cells exhibited a slightly reduced cell growth, too, presumably due to the leaky expression of *SIAH3* in the uninduced state (see Fig. 18B). Additionally, the corresponding microscopy images showed that cells expressing *SIAH3* were not only growing slower but also started to die after four days of induction (Fig. 18D). Doxycycline alone did not influence cell proliferation.

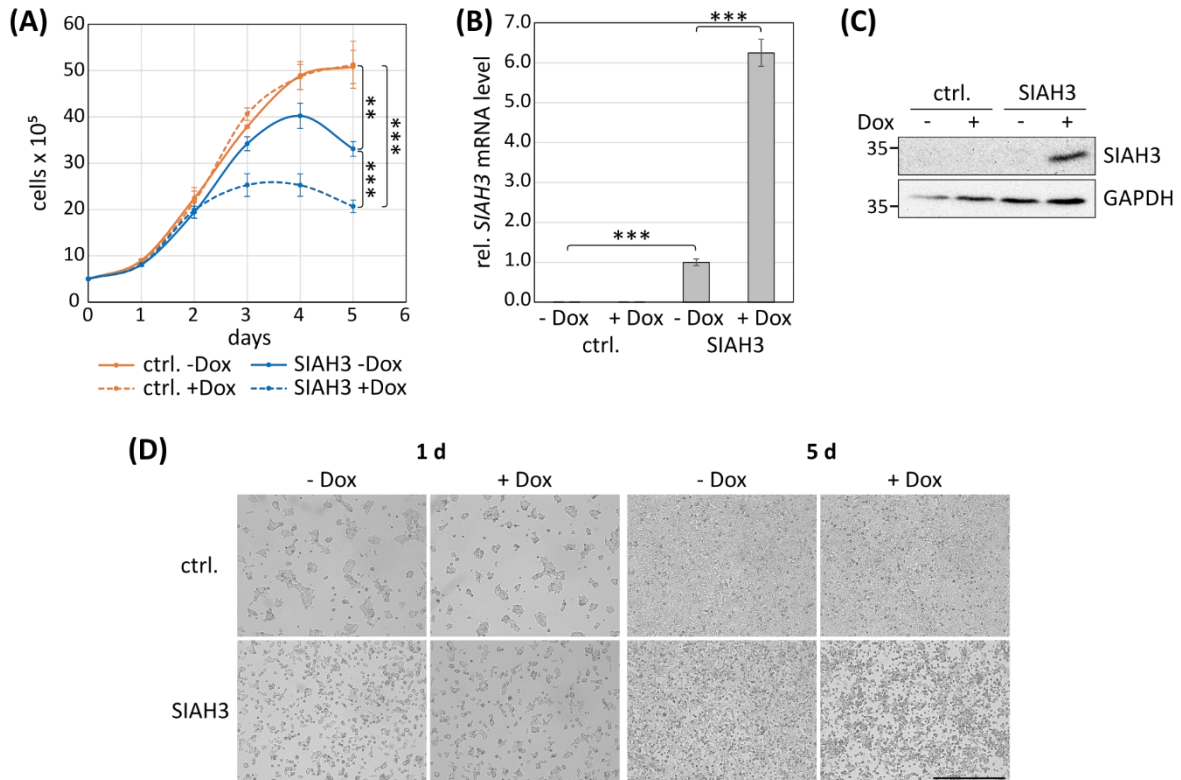


Fig. 18: *SIAH3* inhibits cell proliferation.

(A) Growth curve of T-REx control and *SIAH3* cells (pool of 3 clones). 500,000 cells were plated per well ($\pm$ Dox). Each day, cells were photographed under the microscope and counted. Experiment was conducted in triplicates. **(B)** *SIAH3* expression corresponding to the growth curve of A. RNA was isolated on day 5 and mRNA level of *SIAH3* was determined by RT-qPCR (normalised to *ACTB*). **(C)** Protein expression of *SIAH3* corresponding to the growth curve of A. Proteins were isolated on day 5, SDS-PAGE, followed by WB, was performed and *SIAH3* and GAPDH were detected by specific ABs. **(D)** Microscopy photos of day 1 and 5 corresponding to the growth curve of A. Scale bar: 500 μ m.

*/**/** indicate the significance levels based on Student's t-test.

Given that *SIAH3* is involved in mitophagy (Hasson *et al.*, 2013), I examined whether lowering the glucose concentration in the medium would enhance the observed effect, as cells would then be more reliant on mitochondrial metabolism. A test with different glucose concentrations revealed that without glucose and with 1 g/L glucose, *SIAH3* expressing cells exhibited a significantly reduced cell number five days after induction compared to the uninduced cells – comparable to that observed under standard glucose conditions (4.5 g/L) (Fig. 19A). Thus, additional growth curves were performed under low-glucose conditions. (Fig. 19B and C). However, without glucose, the cells were not healthy and grew badly even without *SIAH3*, questioning the reliability of the results

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(Fig. 19B). With 1 g/L glucose, cells grew better, but even though there was a significant growth reduction of T-REx SIAH3 cells compared to control cells, no significant difference between induced and uninduced SIAH3 could be observed (Fig. 19C). The findings let assume that the impact of SIAH3 on cell proliferation does not directly depend on the glucose levels provided.

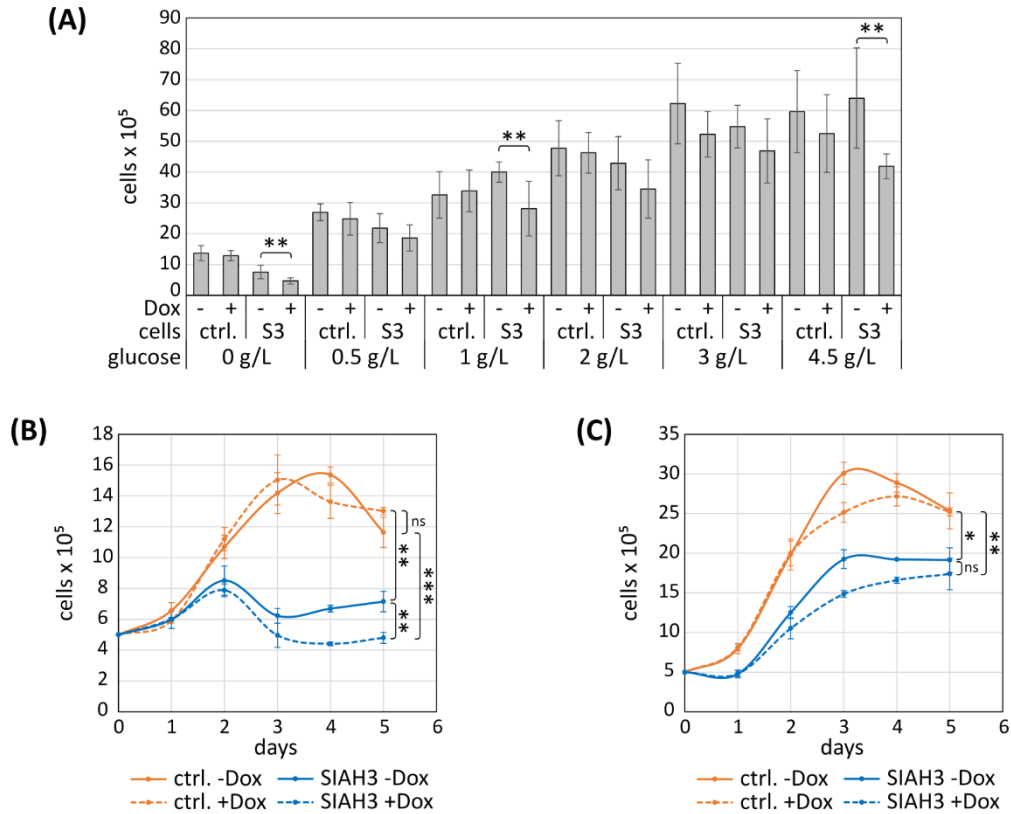


Fig. 19: Cell proliferation under different glucose conditions.

(A) Assessment of cell growth under different glucose concentrations. 500,000 cells were plated per well ($\pm$ Dox). Cells were counted after 5 days. S3 = SIAH3 **(B)** Growth curve of T-REx control and SIAH3 cells (pool of 3 clones) cultured in glucose-free medium. 500,000 cells were plated per well ($\pm$ Dox). Cells were counted each day. Experiment was conducted in triplicates. **(C)** Growth curve of T-REx control and SIAH3 cells (pool of 3 clones) cultured in medium containing 1 g/L glucose. 500,000 cells were plated per well ($\pm$ Dox). Cells were counted each day. Experiment was conducted in triplicates.

*/**/** indicate the significance levels based on Student's t-test.

In contrast to the other two family members SIAH1 and SIAH2, SIAH3 possess a SIAH3 unique sequence (S3UNS) near the N-terminus. To investigate whether this domain is relevant for growth inhibition, a subsequent growth curve experiment compared cells expressing wild-type SIAH3 with those expressing the deletion mutant. Interestingly, deletion of the S3UNS domain did not affect SIAH3's ability to inhibit cell proliferation: Six days after seeding, SIAH3 Wt as well as SIAH3 Δ S3UNS expressing cells count half the number of T-REx control and uninduced T-REx SIAH3 cells (Fig. 20A). However, looking at the dead cells revealed, that only among the induced SIAH3 Wt cells were significantly more dead cells compared to the controls (Fig. 20B). Microscopy images of the cells on day six showed less attached SIAH3 Wt cells than SIAH3 Δ S3UNS ones (Fig. 20E), leading to assume,

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that some cells may detach even if they are still alive. Overall, this experiment provides evidence that growth inhibition by SIAH3 is at least partly attributable to cell death. To explain the different effects of Wt SIAH3 and Δ S3UNS mutant, more experiments are needed.

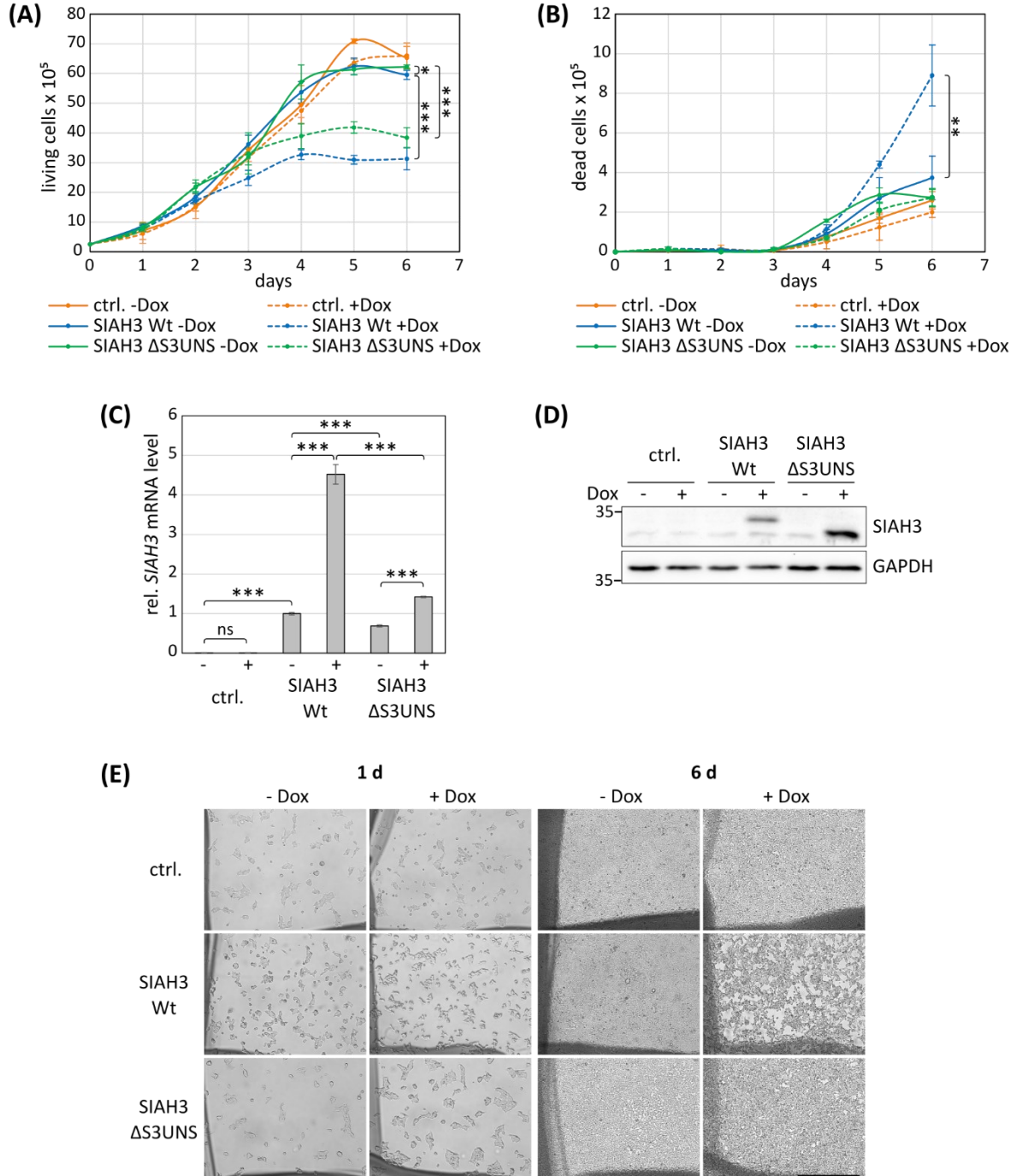


Fig. 20: SIAH3 inhibits cell proliferation partially by cell death.

Growth curve of T-REx control and SIAH3 Wt or Δ S3UNS expressing cells (pool of 3 clones). 250,000 cells per well were plated ($\pm$ Dox). Each day, cells were photographed under the microscope and counted. Experiment was conducted in triplicates. */**/** indicate the significance levels based on Student's t-test. **(A)** Numbers of living cells are depicted. **(B)** Numbers of dead cells are presented. **(C)** Corresponding *SIAH3* expression. RNA was isolated on day 6 and mRNA levels of *SIAH3* were determined by RT-qPCR (normalised to *ACTB*). **(D)** Corresponding protein level of SIAH3. Proteins were isolated on day 6 and WB analysis was performed. SIAH3 and GAPDH were detected by specific ABs. **(E)** Microscopy photos of day 1 and 6. Scale bar: 500 μ m. Figure modified from Deutschmeyer *et al.*, 2025; extended with SIAH3 Δ S3UNS.

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The effect of *SIAH3* of the cell cycle – and this as possible mechanism underlying the growth inhibition effect – was investigated by flow cytometry experiments. Analyses 1, 2 and 3 days after the induction of *SIAH3* expression in T-REx cells (Fig. 21) did not show a constant, significant influence of *SIAH3* on cell cycle distribution. The graphs showing the cell count per DNA-content are shown in supplementary Fig. S18. Even longer *SIAH3* induction over 4 or 5 days had no effect (supplementary Fig. S19).

Taken all these experiments together, *SIAH3* inhibits cell proliferation, in part by inducing cell death, but without affecting cell cycle distribution.

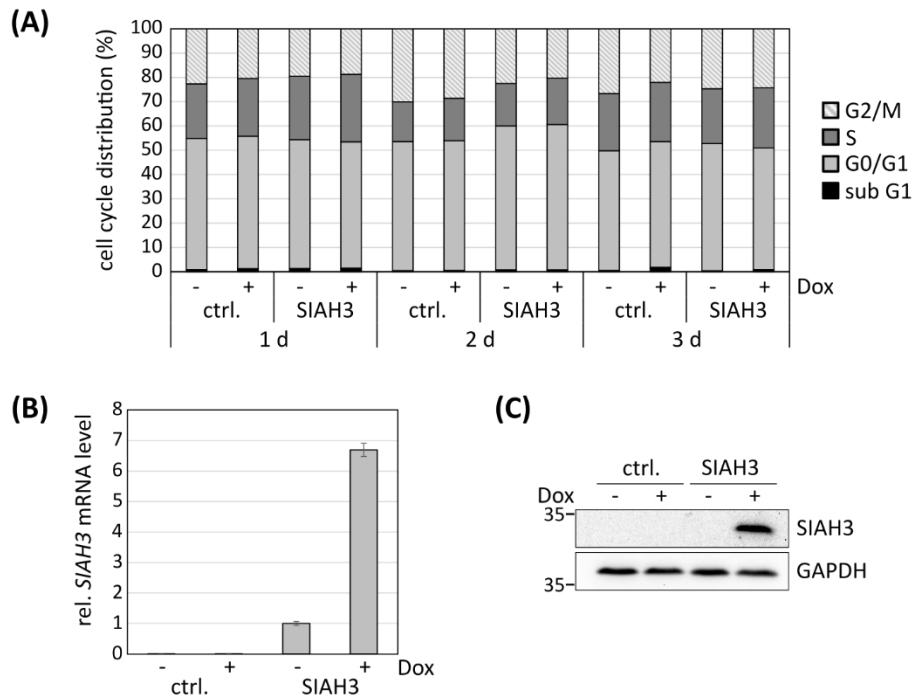


Fig. 21: *SIAH3* has no effect on cell cycle progression.

(A) Cell cycle distribution of T-REx *SIAH3* cells (pool of 3 clones), compared to T-REx control cells, was analysed at 3 different time points by flow cytometry and propidium iodide staining. **(B)** *SIAH3* expression corresponding to the flow cytometry experiment shown in A. RNA was isolated on day 3 and mRNA level of *SIAH3* was determined by RT-qPCR. *SIAH3* expression was normalised to *ACTB*. */**/** indicate the significance levels based on Student's t-test. **(C)** Protein expression of *SIAH3* corresponding to the flow cytometry experiment shown in A. Proteins were isolated on day 3, SDS-PAGE, followed by WB, was performed and *SIAH3* and GAPDH were detected by specific ABs.

RESULTS

3.4 Transcriptional effects of SIAH3

3.4.1 Microarray analysis

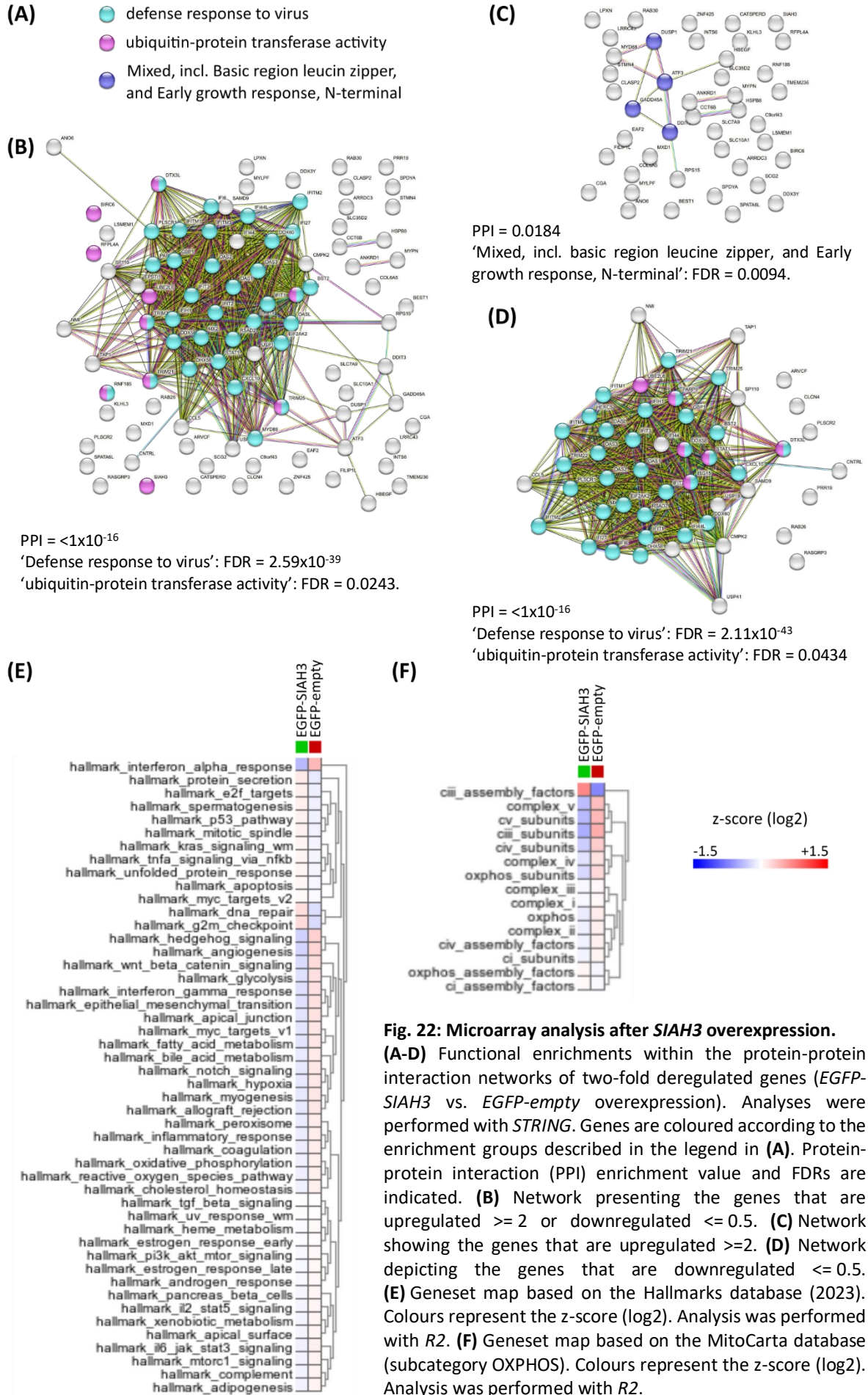
To investigate the mechanism by which SIAH3 controls cell growth, the expression profile of HEK293T cells after *EGFP-SIAH3* overexpression was examined by microarray analysis. Among the 20,893 tested genes, 45 genes were more than two-fold upregulated, and 50 genes were downregulated to at least half the original level. A summary of the number of deregulated genes is shown in Tab. 39.

Tab. 39: Overview of the number of deregulated genes in the microarray analysis

category	gene count
total tested genes	20,893
upregulated (> 1)	8,140
upregulated (>= 2)	45
downregulated (< 1)	7,941
downregulated (<= 0.5)	50
not deregulated (= 1)	4,812

Two-fold up- and downregulated genes were investigated using the protein-protein interaction networks generated by the *STRING* database. Functional enrichment analysis showed significantly more interactions than statistically expected. Fig. 22B shows the network presenting the genes that are upregulated or downregulated twice. Within the upregulated genes, only the small cluster named “Mixed, incl. basic region leucine zipper, and Early growth response, N-terminal” (from the *STRING* local network cluster) was found (Fig. 22C), but among the downregulated genes (Fig. 22D) a large interaction network of genes involved in the defense response to virus (from GO term “Biological Process”) occurred. The gene group “ubiquitin-protein transferase activity” (from GO term “Molecular function”) was significantly enriched, too. However, these two functional enrichments were also discovered in the analysis of another gene, that was analysed in the same microarray as SIAH3 (same sample preparation, same control) (supplementary Fig. S20). This suggests that the found enrichments were not SIAH3 specific. Looking at the geneset map presenting the subgroups of the “Hallmarks database” also showed that upon *SIAH3* overexpression more gene groups were downregulated than upregulated, but except the group “interferon alpha response” – matching to the above described geneset “Defense response to virus” – no other groups were strongly deregulated (Fig. 22E). However, the geneset map of the MitoCarta database (subset OXPHOS) revealed a downregulation of the subunits of the complexes III, IV and V of the respiratory chain whereas the assembly factors of complex III were upregulated (Fig. 22F). This finding suggests that SIAH3 may play a role in mitochondrial metabolism, what is analysed later.

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Sorting the deregulated genes, 10 interesting ones were picked for further analysis (Tab. 40). However, the deregulation of most candidate genes could not be validated by qPCR (Fig. 23). Only *GADD45A* (Fig. 23) and *ANKRD1* (Fig. 24) were significantly upregulated upon *SIAH3* overexpression. *EGR1* was significantly upregulated after 48 h, but only slightly after 24 h. *KLF10* was upregulated after 48 h but downregulated after 24 h. *SQSTM1* was downregulated whereas it was upregulated in the microarray analysis. And *MXD1* was only upregulated after 48 h of *SIAH3* overexpression. The other genes were not significantly deregulated at all.

Tab. 40: Candidate genes from the microarray analysis

gene	complete name	ratio SIAH3 / EGFP
<i>FILIP1L</i>	filamin A interacting protein 1-like	8.96
<i>ANKRD1</i>	ankyrin repeat domain 1 (cardiac muscle)	7.19
<i>DUSP1</i>	dual specificity phosphatase 1	2.53
<i>MXD1</i>	MAX dimerisation protein 1	2.16
<i>EAF2</i>	ELL associated factor 2	2.14
<i>GADD45A</i>	growth arrest and DNA-damage-inducible, alpha	2.12
<i>EGR1</i>	early growth response 1	1.97
<i>PLK2</i>	polo-like kinase 2	1.80
<i>KLF10</i>	Kruppel-like factor 10	1.76
<i>SQSTM1</i>	sequestosome 1	1.70

The second most upregulated gene found in the microarray analysis was *ANKRD1* (ankyrin repeat domain 1). Upregulation could be validated by qPCR in different datasets as shown in Fig. 24. *EGFP/EYFP-SIAH3* overexpression led to an increase of *ANKRD1* expression by more than double in HEK293T cells (Fig. 24A and B). The GST-SIAH3 construct increased *ANKRD1* expression slightly, but the FLAG-construct failed to upregulate *ANKRD1*. Upregulation of *ANKRD1* expression was diminished or did not occur at all upon overexpression of *SIAH3* domain deletion mutants. Specifically, deletion of the DIMER domain caused the complete loss of the upregulation (Fig. 24B and supplementary Fig. S21A and B). *SIAH3* did not increase *ANKRD1* expression in HeLa cells (supplementary Fig. S21C and D), but induction of *SIAH3* in T-TEEx-293 cells led to a remarkably upregulation of *ANKRD1*. After 6 days, an increase in *ANKRD1* expression by more than 15 times was observed (Fig. 24C).

These results suggest that the upregulation of *ANKRD1* is cell line and tag specific. The necessity of the binding domains led us to assume that *SIAH3* needs to interact with binding partners (or to form dimers) to induce this transcriptional effect.

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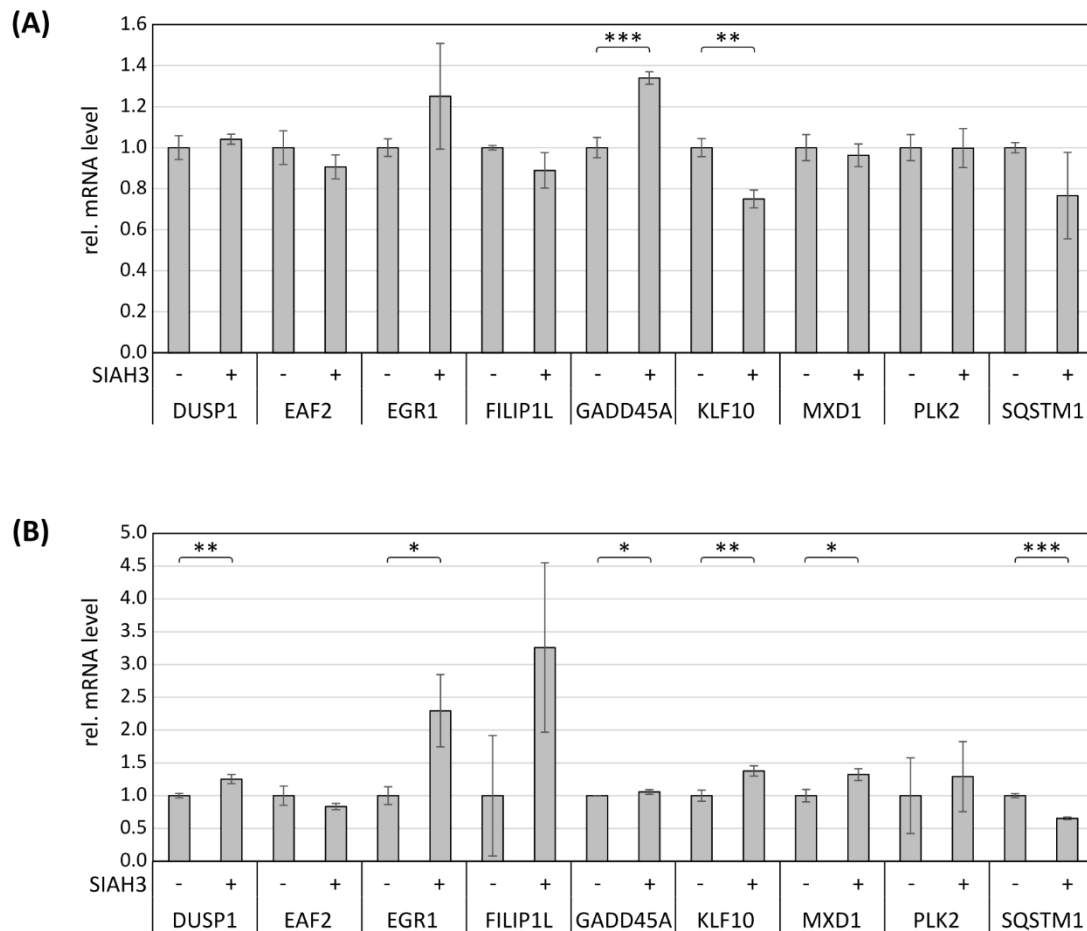


Fig. 23: Validation of candidate genes from the microarray analysis

EGFP-SIAH3 or *EGFP-empty* was overexpressed in HEK293T cells for 24 h **(A)** or 48 h **(B)**. RNA was isolated and the mRNA level of 9 candidate genes was determined by RT-qPCR. Expression was normalised to *ACTB*. */**/** indicate the significance levels based on Student's t-test. – *EGFP-empty*, + *EGFP-SIAH3* overexpression.

RESULTS

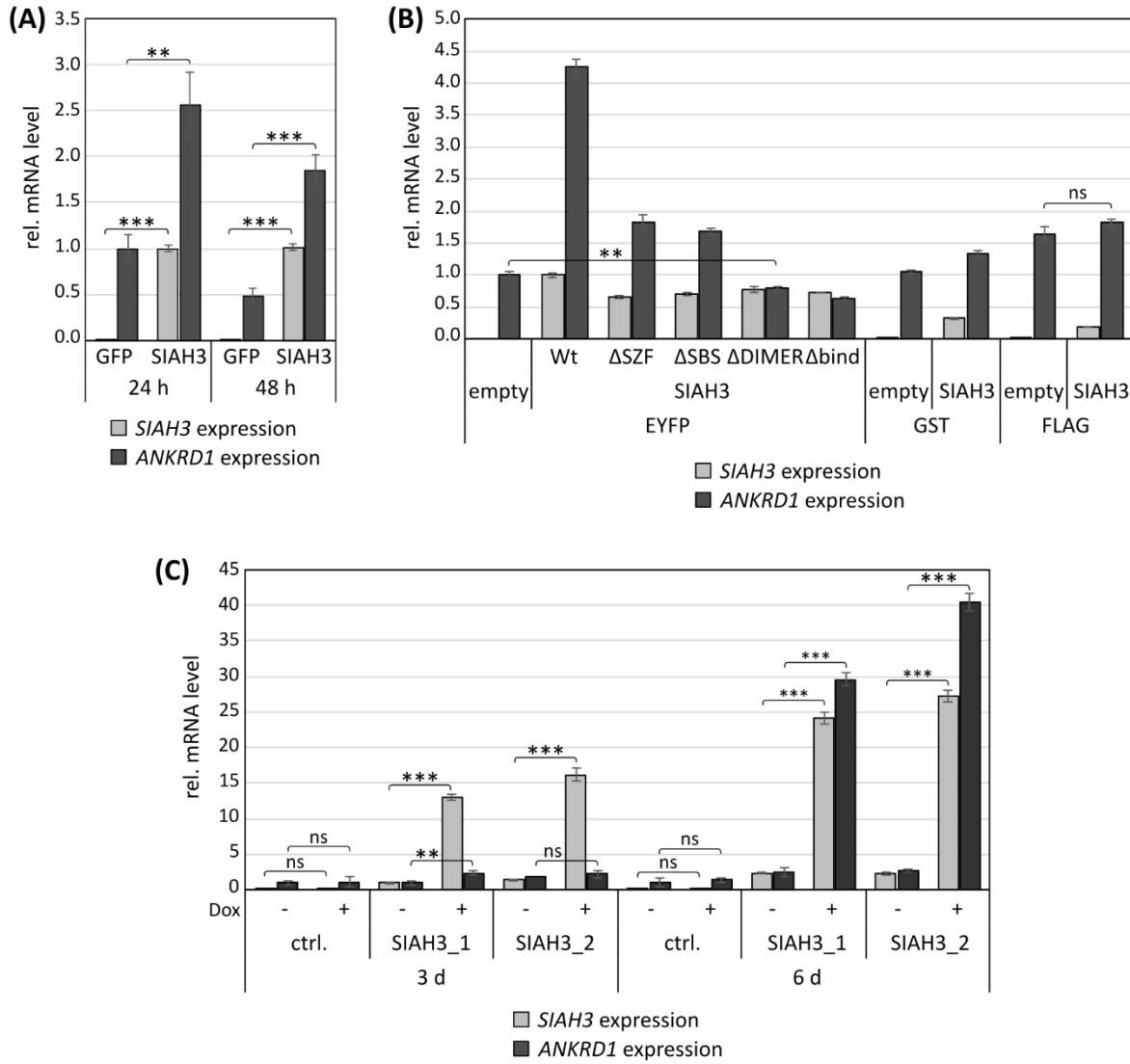


Fig. 24: *ANKRD1* is upregulated by *SIAH3*.

(A) *EGFP-SIAH3* or *EGFP-empty* were overexpressed in HEK293T cells for 24 or 48 h. RNA was isolated and *SIAH3* and *ANKRD1* mRNA level were determined by RT-qPCR. *SIAH3/ANKRD1* expression was normalised to *ACTB*. *ANKRD1* level for 24 h GFP overexpression as well as *SIAH3* level for 24 h *SIAH3* overexpression were set to 1. **(B)** *EYFP-SIAH3* (Wt/domain deletion mutants), *GST-SIAH3*, *FLAG-SIAH3* or the corresponding empty controls were overexpressed in HEK293T cells for 48 h. RNA was isolated and *SIAH3* and *ANKRD1* mRNA level were determined by RT-qPCR. *SIAH3/ANKRD1* expression was normalised to *ACTB*. *SIAH3* primer pair 2 was used for all constructs except for *ΔDIMER* and *Δbind*, where primer pair 1 was used (for comparison, Wt was assessed with both primers and amplification was set to 1). For *ANKRD1*, the level after *EYFP* overexpression was set to 1. All differences between the empty vector and *SIAH3* overexpression are highly significant (**), except where otherwise indicated (Student's t-test). *Δbind* = *ΔSBS*+*ΔDIMER*. **(C)** T-Rex control cells or T-Rex *SIAH3* cells (2 different clones) were induced with Dox for 3 or 6 days. RNA was isolated and *SIAH3* and *ANKRD1* mRNA level were determined by RT-qPCR. *SIAH3/ANKRD1* expression was normalised to *ACTB*. *SIAH3* level of uninduced *SIAH3*_1 cells as well as *ANKRD1* level of uninduced control cells was set to 1.

*/**/** indicate the significance levels based on Student's t-test.

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3.5 Involvement of SIAH3 in Hippo signalling

The effect of SIAH3 on *ANKRD1* expression, a known target gene of YAP (Yes-associated protein) and RASSF1A (Jiménez *et al.*, 2017), hints on an involvement of SIAH3 in Hippo signalling. Since it is known that SIAH2 degrades LATS2 (large tumour suppressor kinase 2) (Ma *et al.*, 2015), the participation of SIAH3 in this pathway was investigated. In one experiment, SIAH3 was able to reduce the degradative effect of SIAH2 on LATS2 (Fig. 25A), but this result was not reproducible (Fig. 25B). However, a protein-protein interaction between SIAH3 and LATS2 was found in pulldown experiments (Fig. 25C and D), suggesting that SIAH3 influences LATS2 by binding and maybe inhibiting its phosphorylation rather than preventing SIAH2-mediated degradation. Deletion of the interaction domains of SIAH3 (SZF, SBS or DIMER) abrogated the binding ability almost completely (Fig. 25D). The effector protein of the Hippo pathway is YAP, which is prevented to shuttle inside the nucleus when phosphorylated at the end of a phosphorylation cascade, involving LATS2 phosphorylation (Hippo on) (Boopathy & Hong, 2019). As expected, LATS2 overexpression led to a decrease in YAP protein level and shifting upwards of the YAP signal. Even though there was no effect on LATS2 protein level of SIAH3, a slight increase and a downshift of the YAP was detected in the T-REx SIAH3 cells compared to the signal in the control cells (Fig. 25E). This shift may represent a beginning dephosphorylation of YAP. Despite there was no increase in YAP levels in HEK293T cells upon SIAH3 overexpression, the slight downshift of the YAP band could also be observed in this experimental setting (Fig. 25F). Induction of *SIAH3* for 3-6 days in T-REx-293 cells yielded the slight downshift of YAP, too (Fig. 25G). Moreover, after 6 days a small increase in YAP signal occurred in the T-REx SIAH3 cells compared to T-REx control cells.

Even if these results need more support and further examination, these observations give hints that SIAH3 may activate YAP by inhibiting the phosphorylation cascade through the interaction with LATS2.

RESULTS

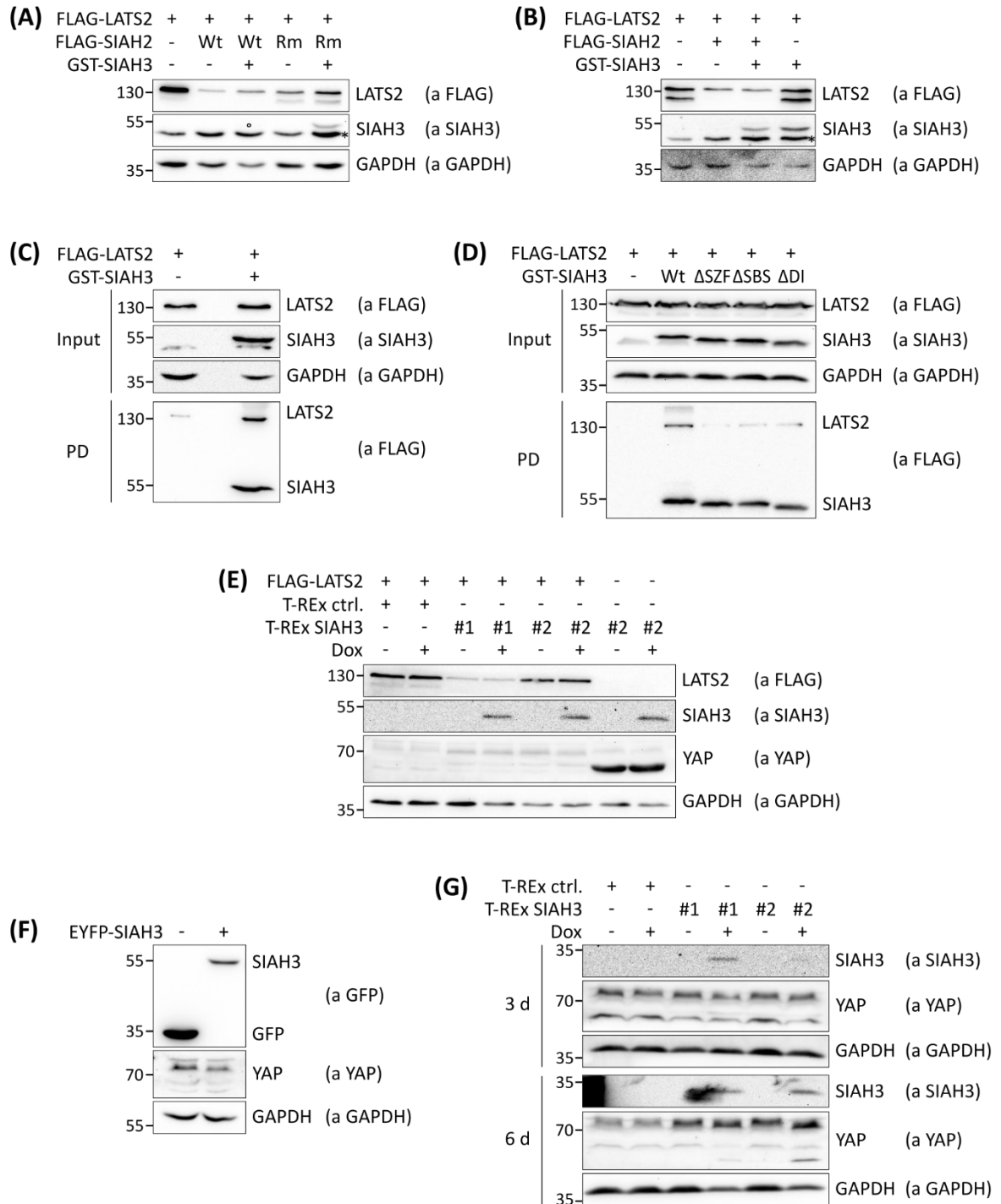


Fig. 25: SIAH3 is involved in Hippo signalling.

(A) SIAH3 reduced the degradation effect of SIAH2 on LATS2. WB analysis of FLAG-LATS2, FLAG-SIAH2 (Wt/Rm) and GST-SIAH3 (37 h) overexpression in HEK293T cells). **(B)** No Effect of SIAH3 on the degradation of LATS2 by SIAH2 was detected. WB analysis of FLAG-LATS2, FLAG-SIAH2 (Wt) and GST-SIAH3 (48 h overexpression in HEK293T cells). **(C)** SIAH3 interacts with LATS2. GST-pulldown (PD) of GST-SIAH3, coexpressed with FLAG-LATS2 in HEK293T cells for 30 h. **(D)** SIAH3's SZF, SBS and DIMER domains are necessary for the interaction with LATS2. GST-pulldown of GST-SIAH3 (Wt, Δ SZF, Δ SBS, Δ DIMER). SIAH3 was coexpressed with FLAG-LATS2 in HEK293T cells for 24 h. **(E)** LATS2 and (leaky) SIAH3 affect YAP. WB analysis of FLAG-LATS2, overexpressed in T-Rex SIAH3 cells (2 different clones) or control cells. SIAH3 was induced by Dox for 3 days. **(F)** SIAH3 leads to a small downshift of the YAP signal. WB analysis of EYFP-SIAH3, overexpressed in HEK293T cells for 24 h. **(G)** SIAH3 leads to a downshift of the YAP signal. WB analysis of T-Rex SIAH3 cells (2 different clones) or T-Rex control cells, induced with Dox for 3 or 6 days.

* unspecific bands. ° GST-SIAH3 was under detection limit. Used ABs are indicated in parentheses. a = anti.

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In addition to the described increase in *ANKRD1* expression, induction of SIAH3 in T-REx cells led to a remarkably upregulation of the Hippo target genes *CTGF* (connective tissue growth factor, *CCN2*), *GADD45A* and *CCN1* (cellular communication network factor 1) (Fig. 26), which validated the involvement of SIAH3 in Hippo signalling. However, through upregulation of both, tumour suppressive genes (*GADD45A*) and tumour progressive or ambivalent ones (*CCN1/2* and *ANKRD1*), it remains unclear if SIAH3 acts more as an oncogene or as a tumour suppressor.

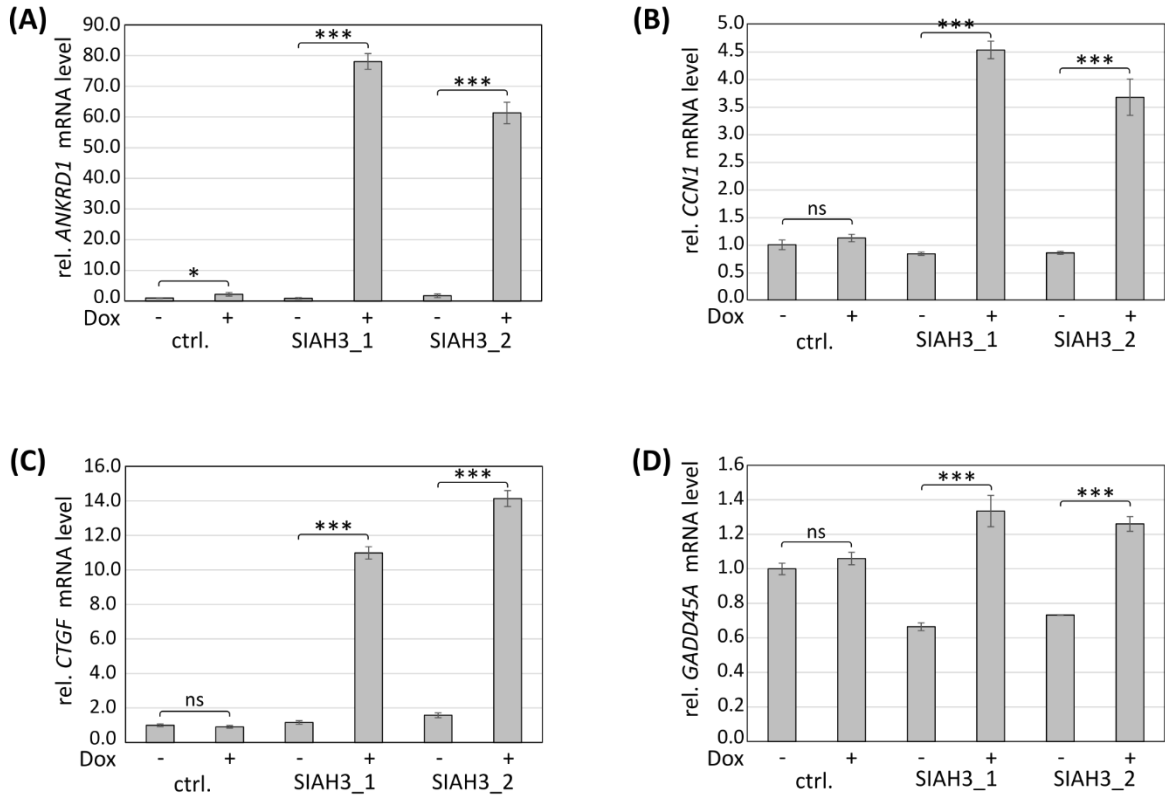


Fig. 26: SIAH3 upregulates Hippo target genes.

SIAH3 expression was induced by Dox for 6 days in T-REx *SIAH3* cells (2 different clones) or in T-REx control cells. RNA was isolated and *ANKRD1* (A), *CCN1* (B), *CTGF* (C) and *GADD45A* (D) mRNA levels were analysed by RT-qPCR. Expression was normalised to *ACTB*. */**/** indicate the significance levels based on Student's t-test.

RESULTS

3.6 The effect of SIAH3 on p53

In addition to the members of the Hippo pathway, the effect of SIAH3 on p53, one of the best-known proteins involved in tumorigenesis, was investigated. However, these experiments revealed an ambiguous effect of SIAH3 (Fig. 27). In the T-REx cells, SIAH3 led to an increase in p53 protein level (Fig. 27A and B), but overexpression in HEK293T cells resulted in a reduction of p53 level (deletion of either the SZF, SBS or DIMER domain abolished this effect) (Fig. 27C and D). Thus, the effect on p53 and the underlying conditions remain unclear and need further examination.

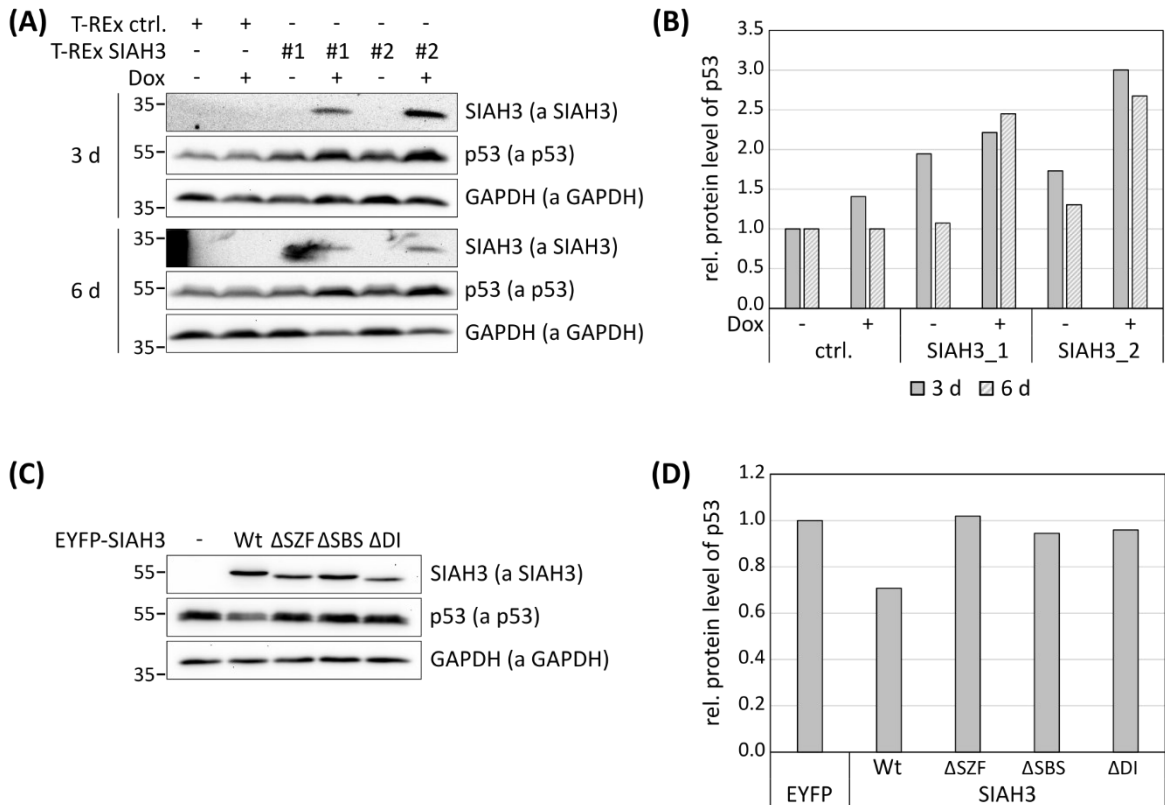


Fig. 27: SIAH3 affects p53 protein level.

(A) SIAH3 increased p53 level in T-REx cells. T-REx SIAH3 cells (2 different clones) or T-REx control cells were induced with Dox for 3 or 6 days and WB analysis was performed. For 6 days, it is the same experiment as shown in Fig. 25G, therefore lanes for GAPDH and SIAH3 are equivalent. **(B)** Quantification corresponding to the WB shown in A. p53 protein levels were normalised to GAPDH. **(C)** SIAH3 decreased p53 level in HEK293T cells. EYFP-SIAH3 (Wt or deletion mutants) were overexpressed in HEK293T cells for 24 h and WB was performed. **(D)** Quantification corresponding to the WB shown in C. p53 protein levels were normalised to GAPDH. ΔDI = ΔDIMER. All proteins were detected using protein specific ABs (as indicated in parentheses).

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3.7 SIAH3 interacts with several ubiquitin-protein ligases

3.7.1 SIAH3 interacts with SIAH1 and SIAH2

To identify further mechanisms underlying the inhibitory effect of SIAH3 on cell proliferation, MS-based proteomics was performed. In the first experiment, the interactome of SIAH3 was analysed. EGFP-tagged SIAH3 and EGFP controls were overexpressed in HEK293T cells, followed by GFP-IP. The analysis of three independent biological samples revealed the specific association of SIAH3 with 369 proteins (≥ 1.5 -fold, $p \leq 0.5$). The corresponding WB demonstrating successful SIAH3 overexpression is shown in supplementary Fig. 22A. Overrepresentation analysis (ORA) of the SIAH3 interactome for the GO subontology “molecular function” showed significant enrichment for the terms “RNA binding”, “ubiquitin conjugating enzyme binding” and “ubiquitin-protein ligase activity” (Fig. 28A). And proteins associated with the term “ubiquitin-protein ligase activity” were predominantly identified among SIAH3’s potential interaction partners, as shown in the volcano plot in Fig. 28B. Moreover, among these ubiquitin ligases, the other two family members SIAH1 and SIAH2 were found binding to SIAH3 (Fig. 28C).

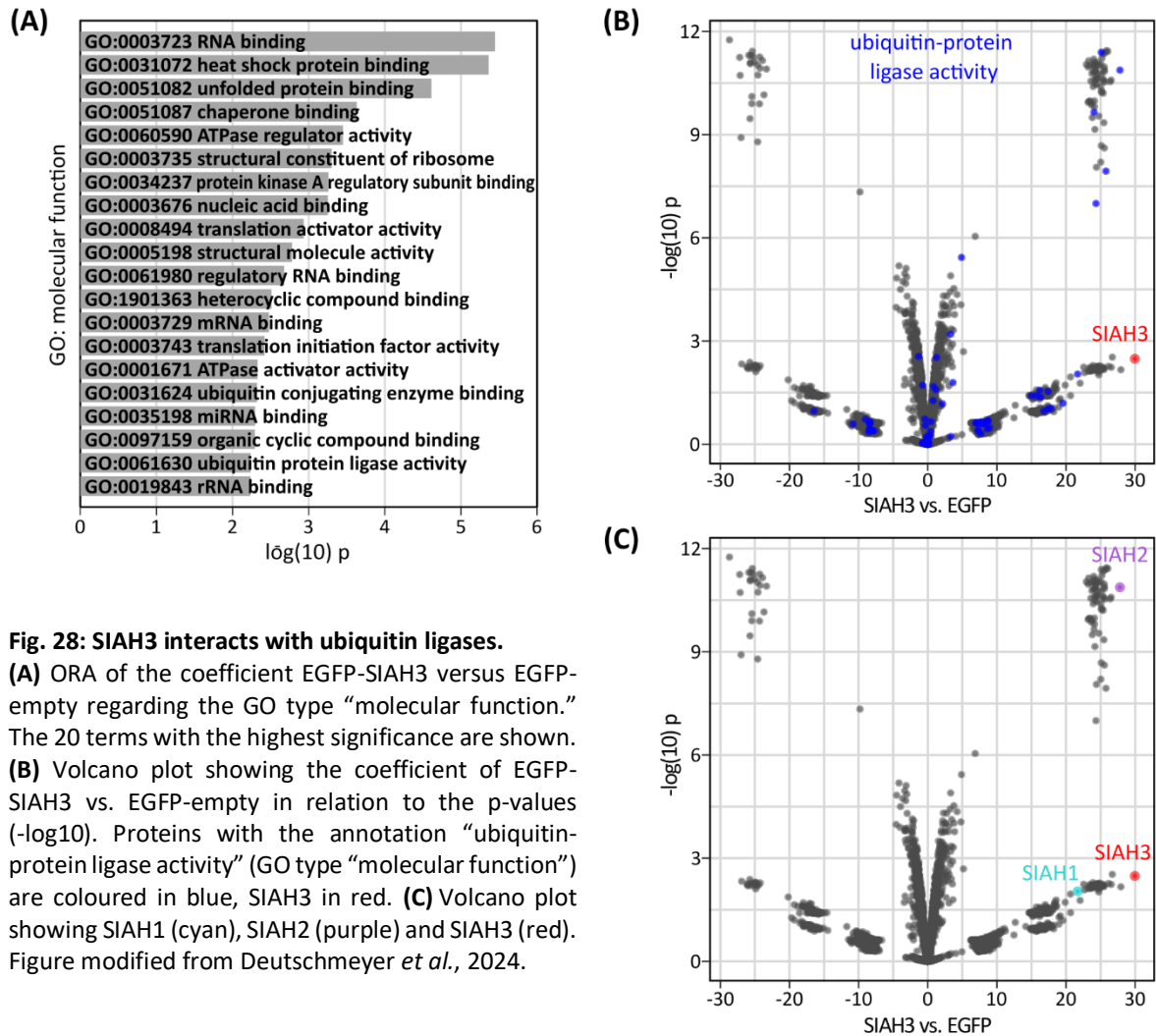


Fig. 28: SIAH3 interacts with ubiquitin ligases.
(A) ORA of the coefficient EGFP-SIAH3 versus EGFP-empty regarding the GO type “molecular function.” The 20 terms with the highest significance are shown.
(B) Volcano plot showing the coefficient of EGFP-SIAH3 vs. EGFP-empty in relation to the p-values ($-\log_{10}$). Proteins with the annotation “ubiquitin-protein ligase activity” (GO type “molecular function”) are coloured in blue, SIAH3 in red. **(C)** Volcano plot showing SIAH1 (cyan), SIAH2 (purple) and SIAH3 (red).
 Figure modified from Deutschmeyer *et al.*, 2024.

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The binding of SIAH3 to both SIAH1 Rm (Ring-domain mutant) and SIAH2 Rm was verified by GST-pulldown experiments (Fig. 29A). The Rm mutants were used as the wild-type molecules (Wt) undergo autoubiquitination and accordingly often yield a diffuse signal in WB.

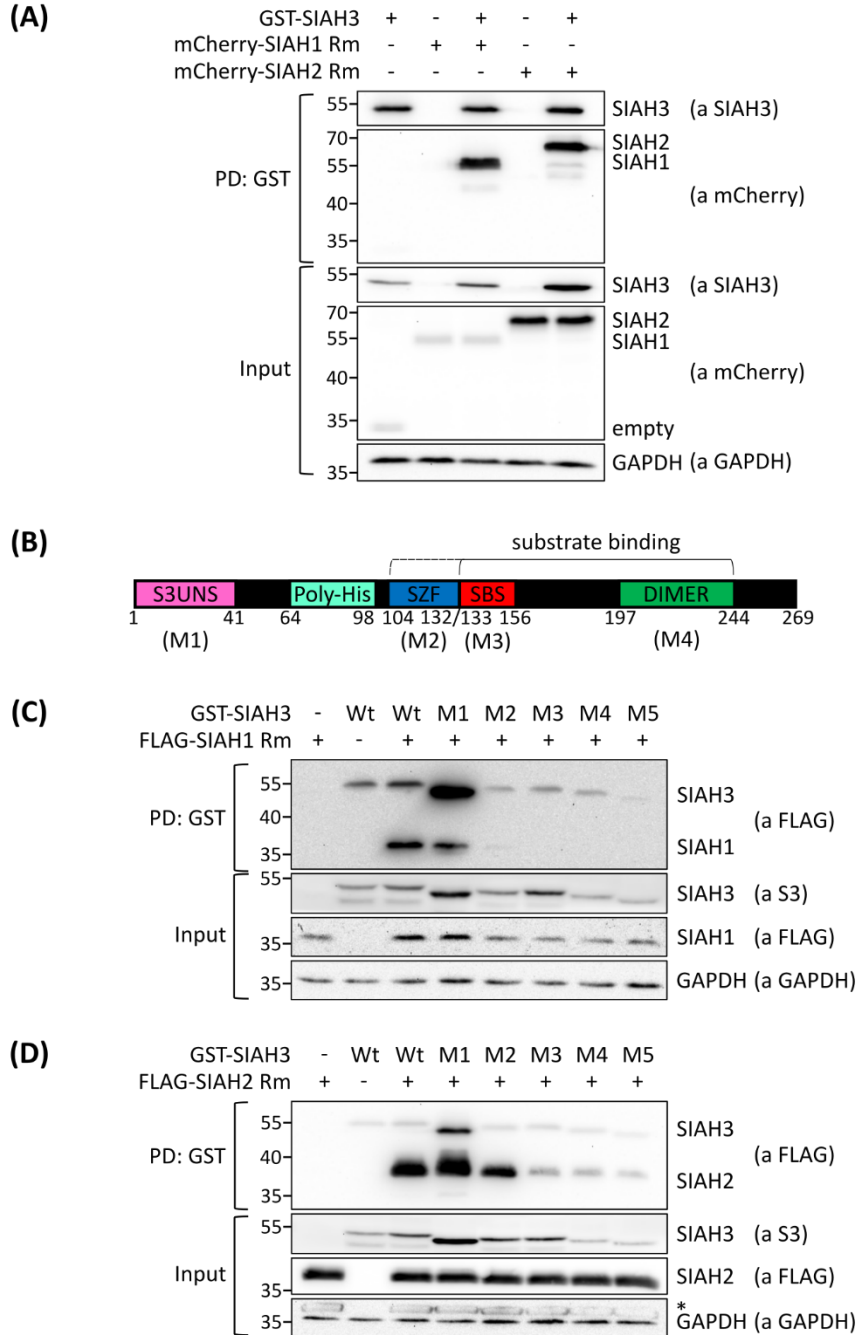


Fig. 29: SIAH3 interacts with the ubiquitin ligases SIAH1 and SIAH2.

(A) Interaction between SIAH3 and SIAH1 or SIAH2 RING-mutants (Rm). GST-pulldown (PD) of GST-SIAH3, coexpressed with either mCherry-SIAH1 Rm, mCherry-SIAH2 Rm or mCherry-empty in HEK293T cells for 24 h. **(B)** Domain structure of SIAH3. Domain deletion mutants: M1 = Δ S3UNS, M2 = Δ SZF, M3 = Δ SBS, M4 = Δ DIMER, M5 = Δ SBS Δ DIMER. Domains after Pepper *et al.* (2017) and Zhang *et al.* (2017). **(C)** and **(D)** Interaction of SIAH3 domain mutants with SIAH1 Rm (C) or SIAH2 Rm (D). GST-pulldown of GST-SIAH3 (Wt or mutants), coexpressed with FLAG-SIAH1 Rm or SIAH2 Rm (empty vector) in HEK293T cells. * remains from initial SIAH2 detection. ABs used in WB are indicated in parentheses.

Figure adapted from Deutschmeyer *et al.*, 2025.

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Furthermore, the binding of the SIAH3 deletion constructs (Fig. 29B) to SIAH1 Rm and SIAH2 Rm (Fig. 29C and D) was evaluated. For SIAH1 it was found that deletion of SZF diminished the binding and deletion of SBS and/or DIMER abolished the binding of SIAH3 to SIAH1. Regarding SIAH2, Δ SZF had no effect on binding, however, Δ SBS and Δ DIMER reduced the binding. These results indicate that the C-terminal substrate binding domain is involved in the interaction between SIAH3 and SIAH1/2. The observed interaction was further confirmed by colocalisation experiments of SIAH3-Myc with HA-SIAH1 Rm and SIAH2 Rm using fluorescence microscopy (Fig. 30). SIAH3 partially colocalised with SIAH1 and recruited it to its own loci. Coexpression of SIAH3 with SIAH2 led to the formation of large accumulations of SIAH3 in the cytoplasm, diminishing SIAH3's speckled localisation. (Passage reproduced from Deutschmeyer *et al.*, 2025.)

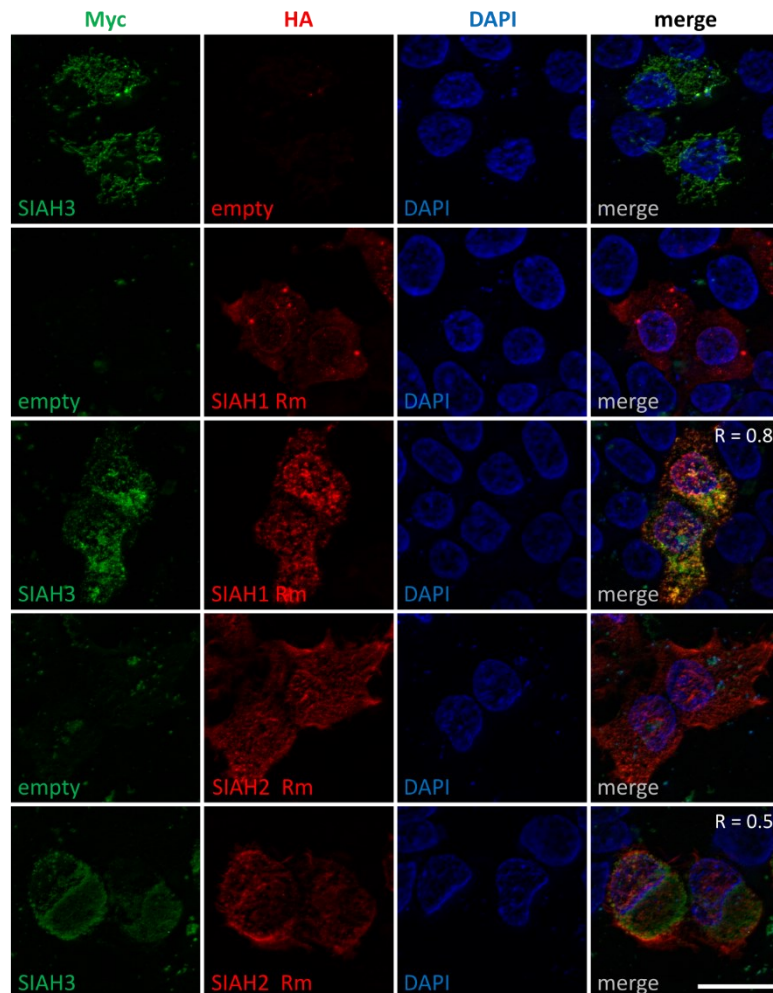


Fig. 30: Colocalisation of SIAH3 with SIAH1 or SIAH2.

SIAH3-Myc (or Myc-empty) was coexpressed with either HA-SIAH1 Rm or HA-SIAH2 Rm (or empty vector) in HEK293T cells. After 24 h, cells were fixed and immunostained using Myc- or HA-AB, respectively. Nuclei are marked with DAPI. Pearson's correlation coefficient (R) as a statistic for quantifying colocalisation is indicated. Scale bar: 20 μ m. Figure adapted from Deutschmeyer *et al.*, 2025.

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3.7.2 SIAH3 forms homodimers

Next to the shown heterodimerisation, SIAH3 was found to form homodimers. Pulldown experiments of GST-SIAH3, coexpressed with mCherry-tagged SIAH3 revealed an interaction between these two SIAH3 constructs (Fig. 31A). Likewise, an interaction of GST/FLAG-tagged SIAH3 with the SIAH3 expressed in the T-REx SIAH3 cells (without tag) was observed and this interaction was attenuated if the DIMER domain was deleted (Fig. 31B). This suggests that, equally to the interaction between SIAH3 and SIAH1/2, the DIMER domain is involved in the formation of homodimers between SIAH3 molecules, too.

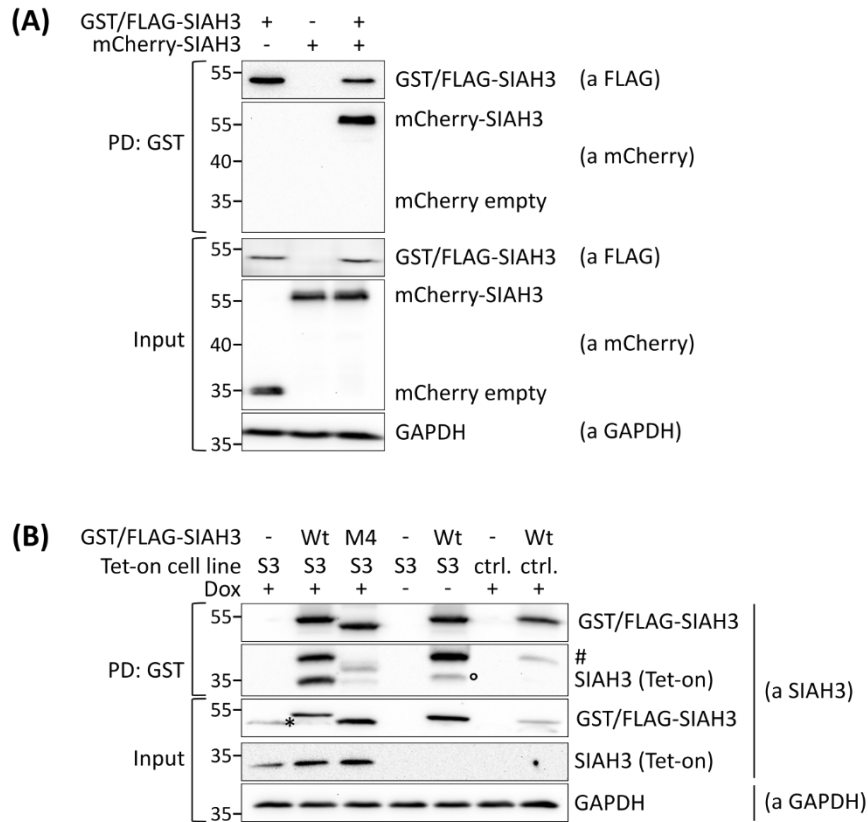


Fig. 31: Homodimerisation of SIAH3

(A) Homodimerisation in HEK2913T. GST-pulldown analysis of GST/FLAG-SIAH3, coexpressed with mCherry-SIAH3 (or corresponding controls) in HEK293T cells. **(B)** DIMER domain is involved in homodimerisation. GST/FLAG-SIAH3 (Wt or Δ DIMER mutant) was overexpressed in T-REx cells (pool of 2 SIAH3 clones or control cells). After 12 h, cells were induced with Dox for 24 h, followed by GST-pulldown, SDS-PAGE and WB. Corresponding ABs are indicated in parentheses.

presumably degradation product of GST/FLAG-SIAH3, * unspecific band, ° leaky SIAH3 expression.

Panel A adapted from Deutschmeyer *et al.*, 2025.

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3.7.3 SIAH3 interacts with STUB1

As shown in Fig. 28B, the volcano plot indicates that SIAH3 may interact with many more proteins with ubiquitin-protein ligase activity beyond SIAH1 and 2. One of these candidates is STUB1 (STIP1 homology and U-box containing protein 1; CHIP) (Fig. 32A). GST-pulldown verified this interaction (Fig. 32B). An immunofluorescence (IF) analysis revealed that STUB1 is localised in both cytoplasm and nucleus, while SIAH3 shows a punctate cytoplasmic staining pattern (Fig. 32C). In contrast to the effect on SIAH1 and SIAH2, SIAH3 did not alter the localisation of STUB1. Although this result did not demonstrate clear colocalisation, it does not exclude the possibility that both proteins can interact, as both are present in the cytoplasm.

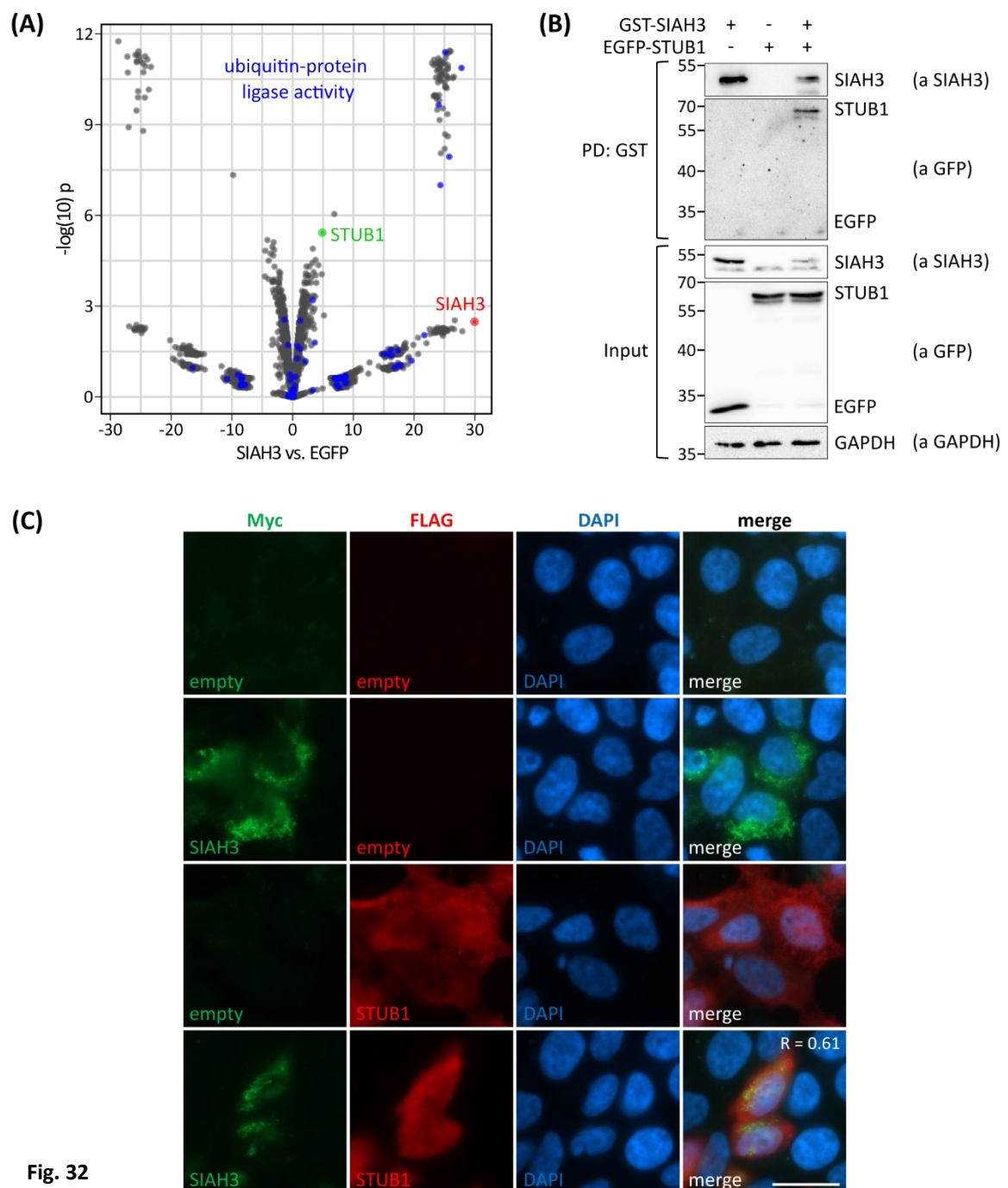


Fig. 32

RESULTS

Fig. 32: Interaction of SIAH3 with STUB1

(A) Volcano plot of the MS interactome data showing the coefficient of EGFP-SIAH3 vs. EGFP-empty in relation to the p-values ($-\log_{10}$). SIAH3 is coloured in red and STUB1 in green. **(B)** Interaction between SIAH3 and STUB1. GST-pulldown analysis of GST-SIAH3, coexpressed with EGFP-STUB1 (or the corresponding controls) in HEK293T cells for 24 h. The corresponding ABs are indicated in parentheses. **(C)** Localisation of SIAH3 and STUB1. SIAH3-Myc (or Myc-empty) was coexpressed with FLAG-STUB1 (or empty vector) in HEK293T cells. After 24 h, cells were fixed and immunostained using Myc- and FLAG-AB. Nuclei were marked with DAPI. Pearson's correlation coefficient (R) as a statistic for quantifying colocalisation is indicated. Scale bar: 20 μm . Panel A modified from Deutschmeyer *et al.*, 2025.

3.8 SIAH3 levels are restricted by SIAH1/2-mediated decay

The ubiquitin ligases SIAH1 and SIAH2 are known for their ability to mark their substrates for degradation by ubiquitination. In order to investigate whether they mediate SIAH3's degradation, too, experiments were performed to evaluate the SIAH3 protein and SIAH3 ubiquitination levels in the presence or absence of SIAH1 or SIAH2.

3.8.1 Both - SIAH1 and SIAH2 - mediate the degradation of SIAH3 protein

Titration experiments showed that SIAH3 was degraded by SIAH1 and SIAH2 in a dose dependent manner. This was observed in the transient overexpression setting in HEK293T cells (Fig. 33A and B) as well as in the SIAH3 inducible T-REx system (Fig. 33D and E). While the protein levels of SIAH3 were reduced proportional to the amount of SIAH2, mRNA levels did not change in a concentration-dependent fashion to the transfected quantity of SIAH2 (Fig. 33C and F). With regard to SIAH1, a SIAH3 reduction was observed when low amounts of SIAH1 were added, whereas higher quantities of SIAH1 appeared to be not as potent in reducing SIAH3 protein levels. And comparable to SIAH2, upon SIAH1 addition RNA levels of SIAH3 did not change in the same manner as the protein levels changed (Fig. 33C and F).

RESULTS

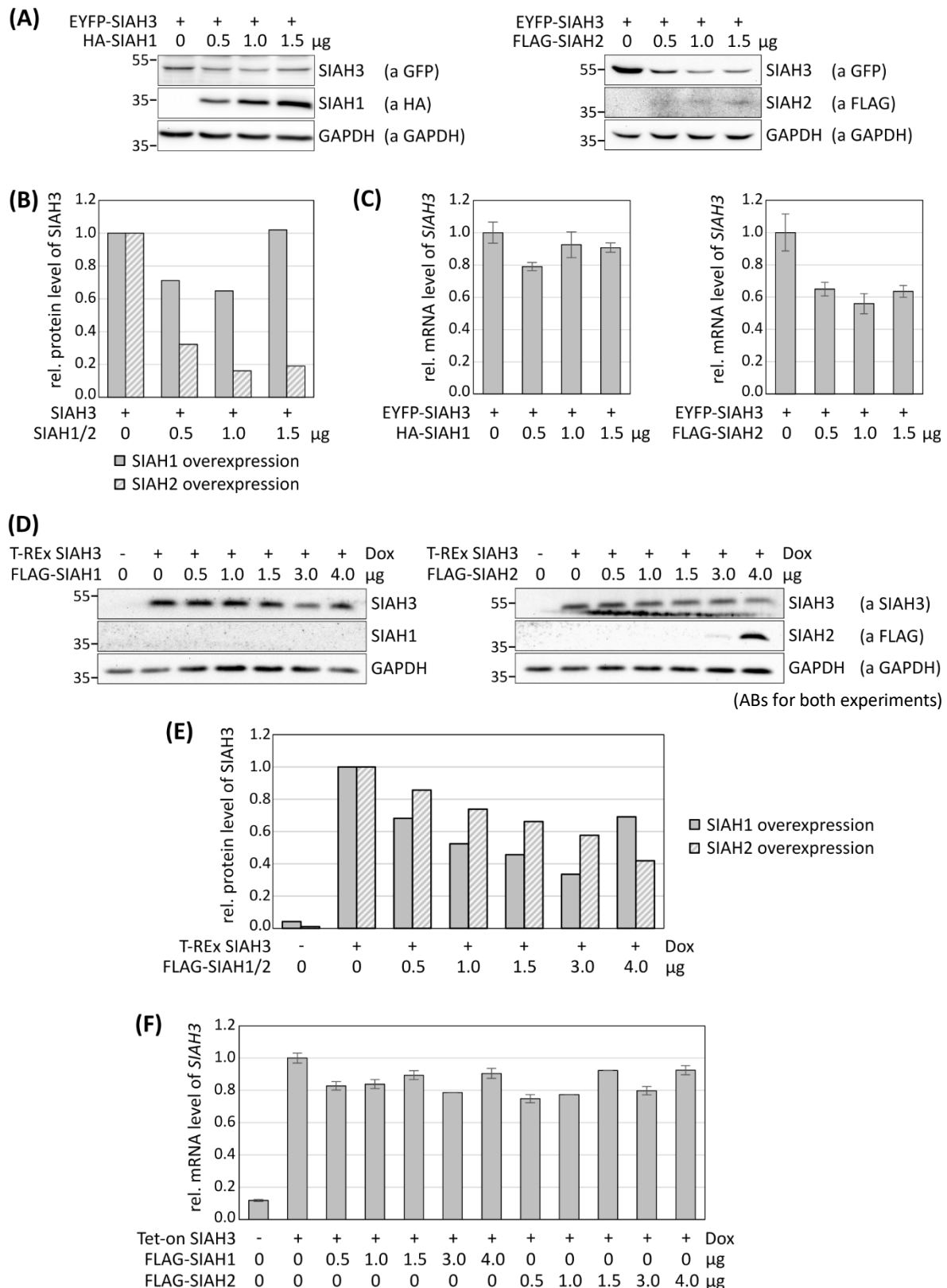


Fig. 33: SIAH1 and SIAH2 degrade SIAH3 in a dose dependent manner.

(A) SIAH1 and SIAH2 degrade SIAH3 in a dose dependent manner. WB analysis of EYFP-SIAH3, overexpressed together with increasing quantities of either HA-SIAH1 or FLAG-SIAH2 in HEK293T cells for 24 h. **(B)** Quantification of the SIAH3 protein levels (normalised to GAPDH protein level) corresponding to the experiment shown in A. **(C)** mRNA expression level of *SIAH3* (determined by RT-qPCR, normalised to *ACTB*) corresponding to the experiment shown in A. **(D)** SIAH3 is degraded by SIAH1 and SIAH2 in T-REx cells. Increasing quantities of FLAG-SIAH1 or FLAG-SIAH2 were overexpressed in T-REx SIAH3 cells for 30 h. 6 h after transfection cells were induced with Dox for 24 h, followed by WB analysis. SIAH1 was not detectable.

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(E) Quantification of the SIAH3 protein levels (normalised to GAPDH protein level) corresponding to the experiments shown in C. (F) mRNA expression level of *SIAH3* (determined by RT-qPCR, normalised to *ACTB*) corresponding to the experiments shown in C. ABs used in WB are indicated in parentheses. */**/** indicate the significance levels based on Student's t-test.

Consistent with the degradation of SIAH3 by SIAH2 overexpression, knocking down endogenous SIAH2 resulted in increased SIAH3 protein levels in T-REx SIAH3 cells whereas the mRNA levels were not upregulated significantly (considering three independent experiments) (Fig. 34).

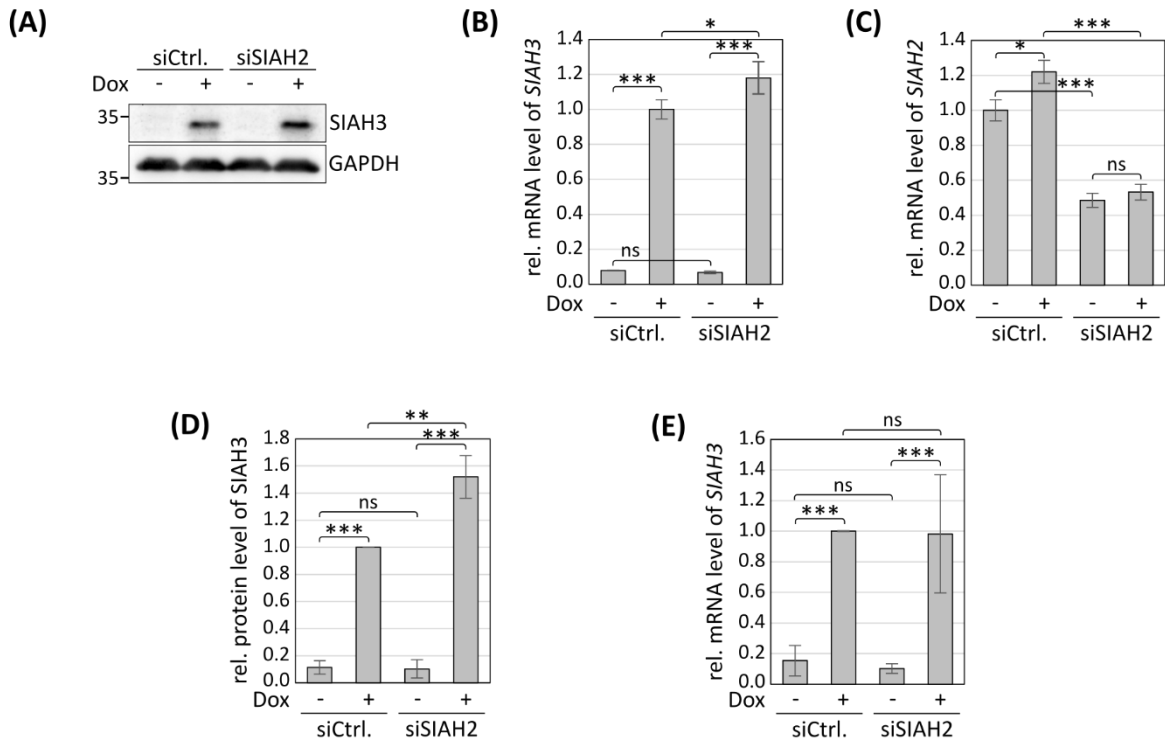


Fig. 34: SIAH2 knockdown increases SIAH3 protein levels.

(A) Knockdown of SIAH2 by siRNA in T-REx SIAH3 cells (pool of 3 clones) for 3 days. 24 h before harvesting, cells were induced with Dox. Proteins were isolated, followed by SDS-PAGE and WB. SIAH3 and GAPDH were detected using protein specific ABs. (B) mRNA levels of *SIAH3* corresponding to the experiment shown in A, analysed by RT-qPCR. Expression was normalised to *GAPDH*. (C) mRNA levels of *SIAH2* corresponding to the experiment shown in A, determined by RT-qPCR. Expression was normalised to *GAPDH*. (D) Quantification of SIAH3 protein levels after knockdown of SIAH2 (as described in A) of three independent experiments quantified by *ImageJ* (normalised to GAPDH). (E) Corresponding *SIAH3* mRNA levels of the 3 independent experiments quantified by qPCR (normalised to *GAPDH*).

*/**/** indicate the significance levels based on Student's t-test. Panels A and D modified from Deutschmeyer *et al.*, 2025.

While the SIAH3 Wt was strongly degraded by SIAH1 and SIAH2, the protein levels of the domain deletion constructs were not remarkably decreased. This degradation effect was even inverted for the DIMER deletion mutant. The same effect was observed for both, SIAH1 and SIAH2 (Fig. 35). This result suggests that all four domains are involved in the degradation mechanism mediated by SIAH1/2 and it seems that dimerisation of the SIAHs is needed to yield this reduction.

RESULTS

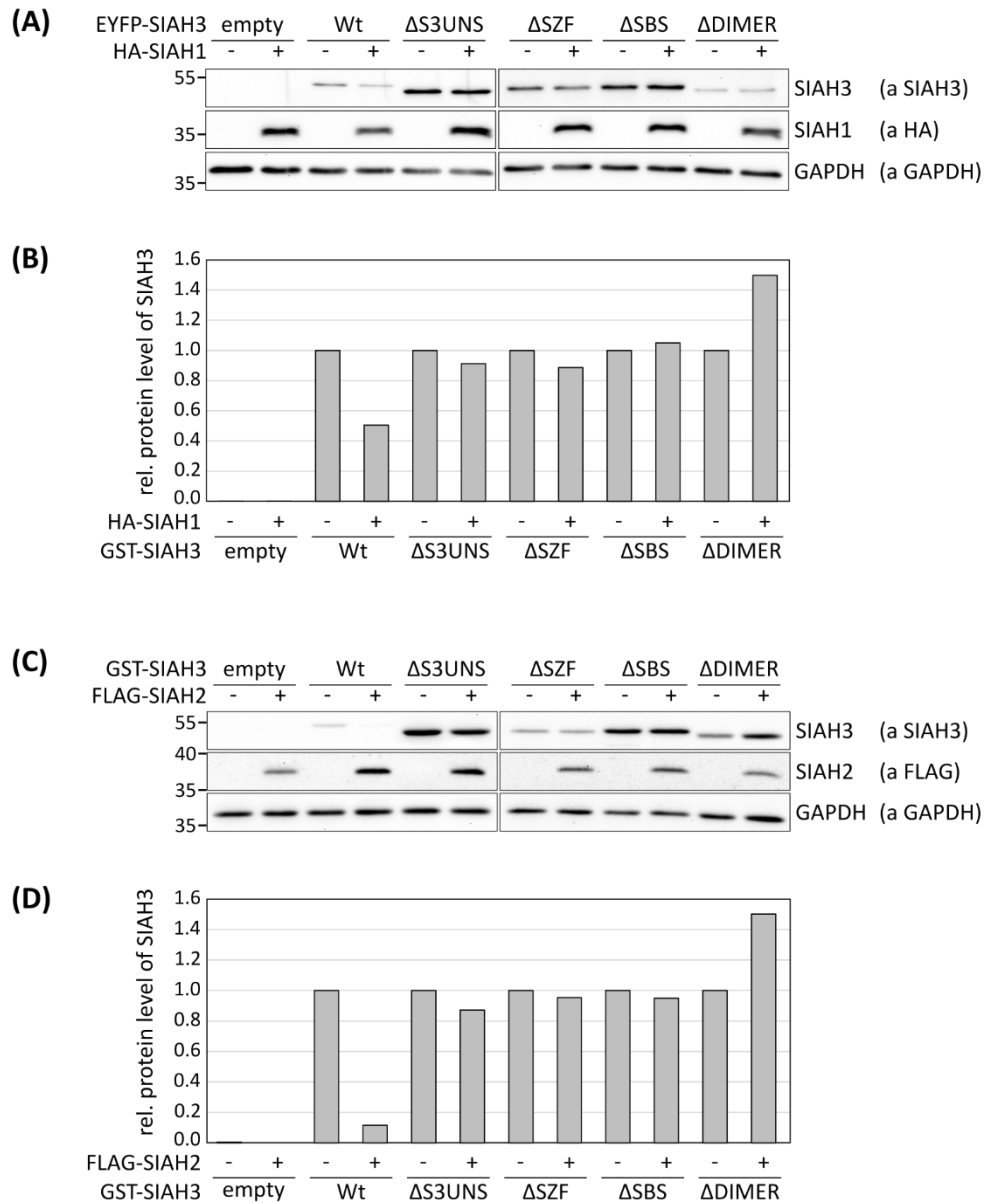


Fig. 35: Deletion of any of the SIAH3 domains abolished SIAH3 degradation by SIAH1 and 2.

(A) WB analysis of EYFP-SIAH3 (Wt or the domain deletion constructs) and HA-SIAH1 (or the corresponding controls), overexpressed in HEK293T cells for 24 h. ABs are indicated in parentheses. **(B)** Corresponding quantification of SIAH3 protein levels (normalised to GAPDH). **(C)** WB analysis of GST-SIAH3 (Wt or the domain deletion constructs) and FLAG-SIAH2 (or the corresponding controls), overexpressed in HEK293T cells for 24 h. ABs are indicated in parentheses. **(D)** Corresponding quantification of SIAH3 protein levels (normalised to GAPDH).

3.8.2 Degradation of SIAH3 depends on the ubiquitin-proteasome system (UPS)

Given that SIAH1 and SIAH2 are known E3 ubiquitin ligases, the necessity of their catalytic domain, the RING domain, for SIAH3 degradation was evaluated. The experiments showed that in contrast to the Wt SIAH1/2, the RING mutants did not decrease but even increase the SIAH3 protein level (Fig. 36A and B), demonstrating that this process depends on a functional RING domain. Thus, the regulation of SIAH3 by SIAH1/2 likely depends on their ability to mediated ubiquitination. Treatment of cells with the proteasome inhibitor MG-132 decreased SIAH3 reduction upon

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coexpression with either SIAH1 or SIAH2 (Fig. 36C-F), indicating that the degradation occurs via the proteasome.

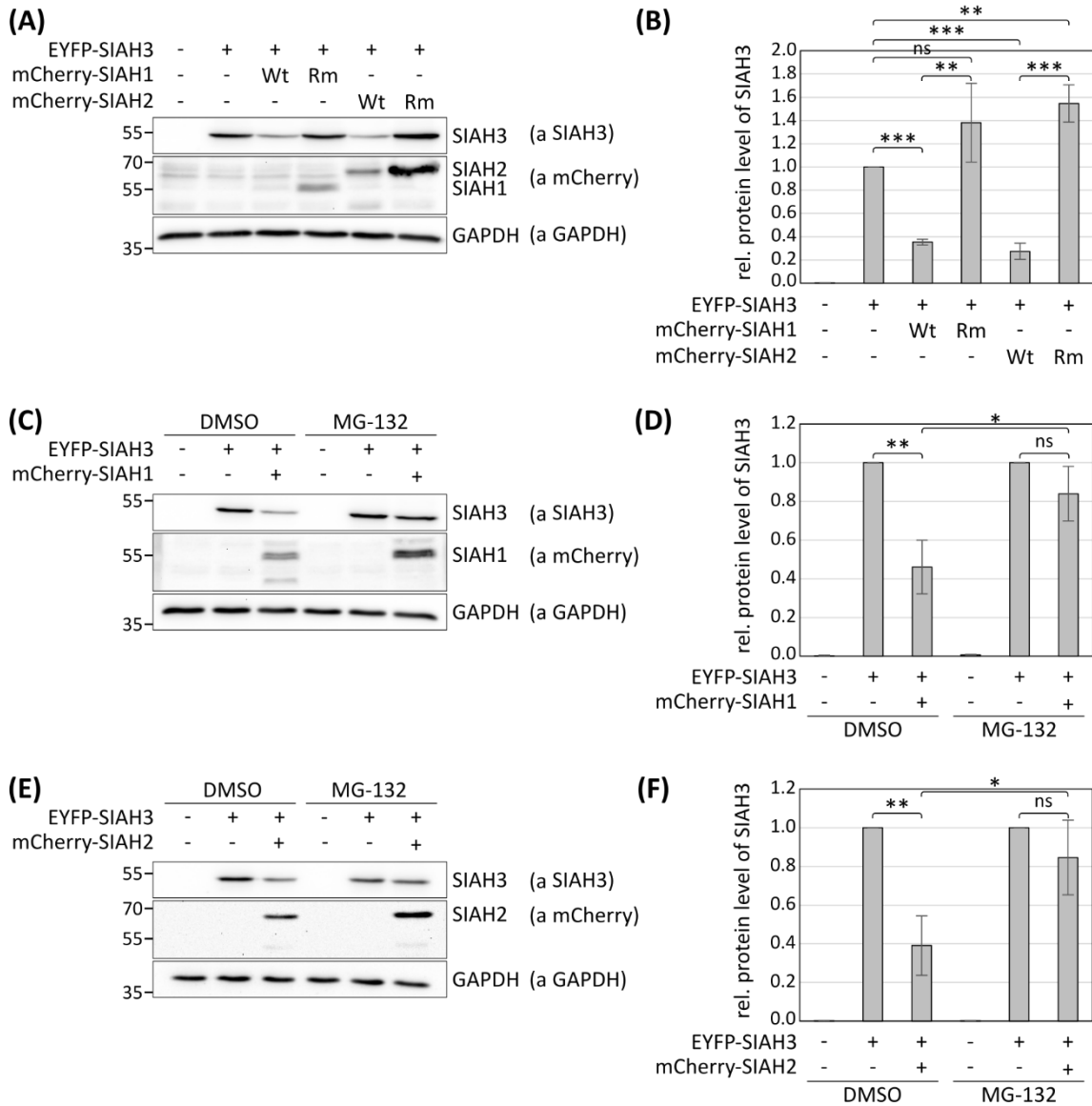


Fig. 36: Degradation of SIAH3 is mediated by the UPS.

(A) SIAH1 and SIAH2 Wt but not Rm decrease SIAH3 protein level. EYFP-SIAH3 was coexpressed with either mCherry-SIAH1 or SIAH2 (Wt or Rm; or corresponding controls) in HEK293T cells, followed by SDS-PAGE and WB. SIAH1 Wt is hardly detectable due to autoubiquitination. **(B)** Corresponding SIAH3 protein levels of three independent experiments (normalised to GAPDH), quantified by *ImageJ*. **(C)–(F)** Proteasome inhibitor MG-132 inhibits SIAH3 degradation by SIAH1 and SIAH2. **(C & E)** WB analyses of EYFP-SIAH3, coexpressed with either mCherry-SIAH1 Wt (D) or mCherry-SIAH2 Wt (E) (or corresponding controls) in HEK293T cells and treated with MG-132 (10 μ M). **(D & F)** Corresponding SIAH3 protein levels of three independent experiments (normalised to GAPDH), quantified by *ImageJ*.

ABs used in WB are indicated in parentheses. */**/** indicate the significance levels based on Student's t-test. Figure adapted from Deutschmeyer *et al.*, 2025.

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Additionally, ubiquitination assays of SIAH3 were performed by overexpressing SIAH2 Wt or SIAH2 Rm together with ubiquitin and SIAH3. Normalised SIAH3 ubiquitin levels increased when the SIAH2 Wt was present. Addition of SIAH2 Rm did not show an effect (Fig. 37). Enrichment of ubiquitin by Ni-NTA after overexpressing it together with SIAH3 and SIAH2 Wt or Rm also showed a SIAH2 Wt-dependent increased ubiquitination of SIAH3 (Fig. 38). Even though these experiments need further refinement (e.g. adjustment of the ubiquitin level and improvement of the purification), these findings indicate that the SIAH2 E3 ubiquitin ligase harbouring the intact RING domain restricts SIAH3 expression levels by ubiquitin-proteasome-dependent degradation. (Passage adapted from Deutschmeyer *et al.*, 2025.)

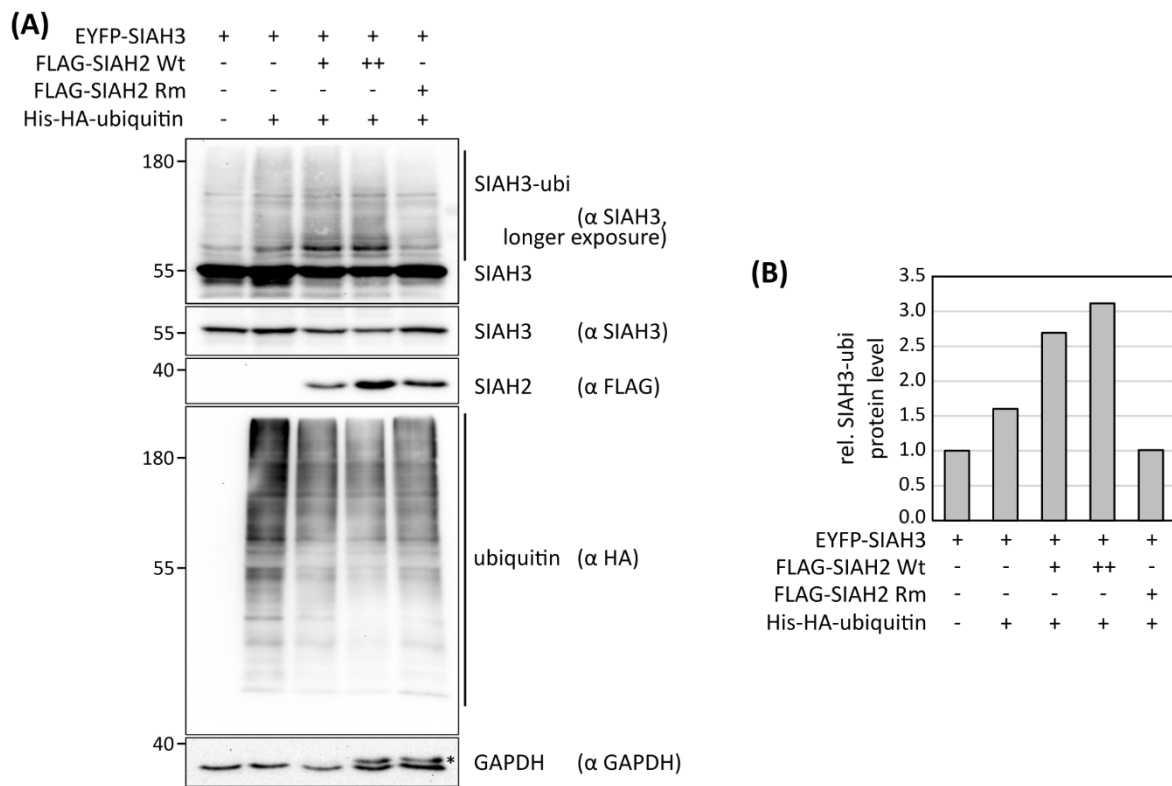


Fig. 37: SIAH2 induces the ubiquitination of SIAH3.

(A) EYFP-SIAH3 (2.5 µg) and either FLAG-SIAH2 Wt (1.25 µg or 2.5 µg) or FLAG-SIAH2-Rm (1 µg) and His-HA-ubiquitin (5 µg) were overexpressed in HEK293T cells for 24 h. 12 h prior to harvest, 10 µM MG-132 was added. Proteins were lysed in 1x SDS sample buffer, followed by sonification. SDS-PAGE and WB were performed. Used ABs are indicated in parentheses. * remains from initial SIAH2 detection. **(B)** Corresponding quantification of SIAH3-ubiquitin protein level (longer exposure) normalised to SIAH3 (shorter exposure). ubi = ubiquitin. Panel A adapted from Deutschmeyer *et al.*, 2025.

RESULTS

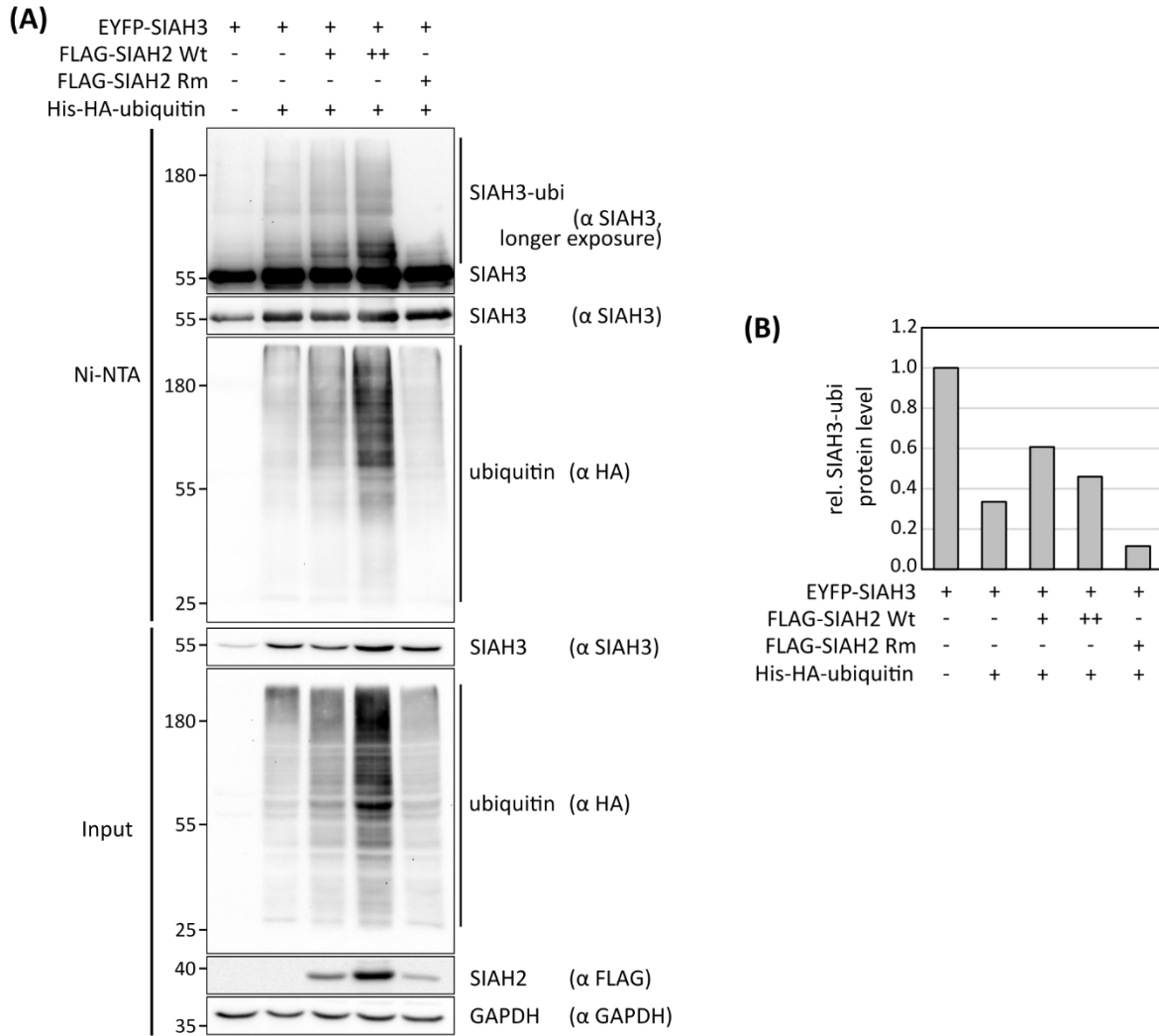


Fig. 38: Ubiquitination analysis of SIAH3 after Ni-NTA purification.

(A) EYFP-SIAH3 (2.5 μ g) and either FLAG-SIAH2 Wt (1.25 μ g or 2.5 μ g) or FLAG-SIAH2-Rm (0.5 μ g) and His-HA-ubiquitin (5 μ g) were overexpressed in HEK293T cells for 24 h. 12 h prior to harvest, 10 μ M MG-132 was added. Proteins were isolated and ubiquitin was enriched by Ni-NTA purification. SDS-PAGE and WB were performed. Used ABs are indicated in parentheses. **(B)** Corresponding quantification of SIAH3-ubiquitin protein level (Ni-NTA, longer exposure) normalised to Input SIAH3.

ubi = ubiquitin

Although it is known that proteasomal degradation is mainly mediated by the ubiquitination of lysines (e.g. Pickart, 2001), mutating any of the five lysines within the SIAH3 protein sequence to arginine did not remarkably reduce its degradation (Fig. 39A and B). Even combinational mutations of these lysines did not abolish the degradation (Fig. 39C and D). These observations led assume that the ubiquitination of SIAH3 did not (only) follow the canonical mechanism but rather occurs on other than lysine residues.

Taken together, these experiments indicate that the tumour-promoting restriction of SIAH3 is not only attributable to DNA methylation, as demonstrated in the first chapters, but also to SIAH1/2-mediated decay on protein level.

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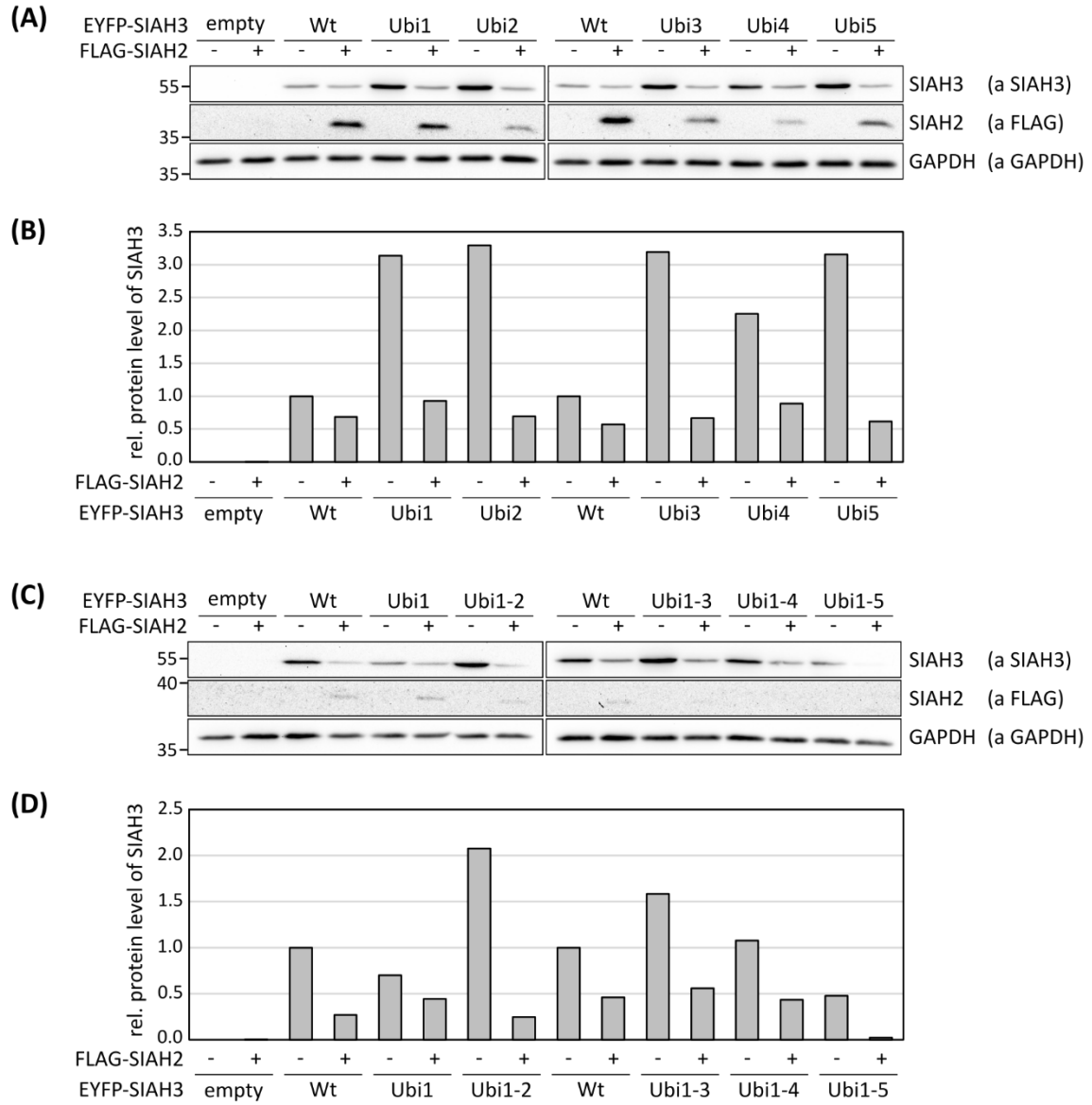


Fig. 39: SIAH3 degradation did not depend on its lysine residues.

(A) WB analysis of EYFP-SIAH3 Wt or single ubiquitin-site mutants (Ubi1 = K23R, Ubi2 = K25R, Ubi3 = K45R, Ubi4 = K173R, Ubi5 = K213R) that were coexpressed with FLAG-SIAH2 (or the empty vector) in HEK293T cells for 24 h. **(B)** Quantification of SIAH3 protein levels (normalised to GAPDH), corresponding to the experiment in A. **(C)** WB analysis of EYFP-SIAH3 Wt or multiple ubiquitin-site mutants, coexpressed with FLAG-SIAH2 (or the empty vector) in HEK293T cells for 24 h. **(D)** Quantification of SIAH3 protein levels (normalised to GAPDH), corresponding to the experiment in C.

ABs used in WB are indicated in parentheses.

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3.9 SIAH3 regulates mitochondrial respiration

3.9.1 SIAH3 deregulates proteins associated with both the mitochondrial respiratory chain and Parkinson's disease.

To further unravel the molecular mechanisms underlying SIAH3's influence on cell proliferation, the proteomes of T-REx SIAH3 (induced and uninduced) and control cells (induced) were investigated by MS. The corresponding WB demonstrating SIAH3 induction and a Coomassie-stained blot confirming an even distribution of protein amounts are shown in supplementary Fig. S22B and C. Among the 5,641 protein groups detected and included in the analysis, 806 were significantly deregulated ($FDR < 0.05$; 1,801 with $p < 0.05$) regarding the contrast between induced and uninduced T-REx SIAH3. Of these, only 28 proteins were upregulated more than two-fold and 26 were at least reduced to half, showing that the majority of the deregulation upon SIAH3 induction occurs within a narrow range. However, looking at groups of proteins (GO terms/KEGG categories) revealed several overrepresented annotation terms. An ORA for GO “cellular component” and KEGG terms, considering proteins with negative differential expression, identified terms linked to (mitochondrial) respiratory chain and oxidative phosphorylation (OXPHOS) as enriched (Fig. 40A and B, marked blue). Additionally, KEGG groups regarding the following diseases were over-represented: non-alcoholic fatty liver disease (NAFLD), Parkinson's, Alzheimer's and Huntington's disease (Fig. 40B, marked green) – all diseases in which mitochondrial dysfunction plays an important role (Lin & Beal, 2006; Dabravolski *et al.*, 2021). Considering the positively deregulated proteins, the over-represented groups were less strongly significant. Among them, the GO term “preribosome” and the KEGG group “Ribosome biogenesis in eukaryotes” were the most significantly over-represented groups (Fig. 40A and B, marked in purple).

To account for a potential side effect of Dox, the described findings were compared with the results obtained by contrasting induced T-REx SIAH3 with the Dox-induced T-REx control cells. Here, 1,621 protein groups were significantly deregulated ($FDR < 0.05$). ORA considering the negatively deregulated proteins yielded a comparable result: many terms associated with mitochondrial respiratory chain and the previously named diseases like Parkinson's disease were significantly over-represented (supplementary Fig. S23, marked in blue and green). However, the terms associated with (pre-)ribosome were found when considering the down-, not the upregulated proteins as described above (supplementary Fig. S23, marked in purple). Thus, the deregulation of proteins connected to (pre-)ribosome cannot be attributed to SIAH3, whereas the downregulation of proteins related to the mitochondrial respiratory chain as well as to NAFLD, Parkinson's and the other neurodegenerative diseases appears to be SIAH3-dependent.

RESULTS

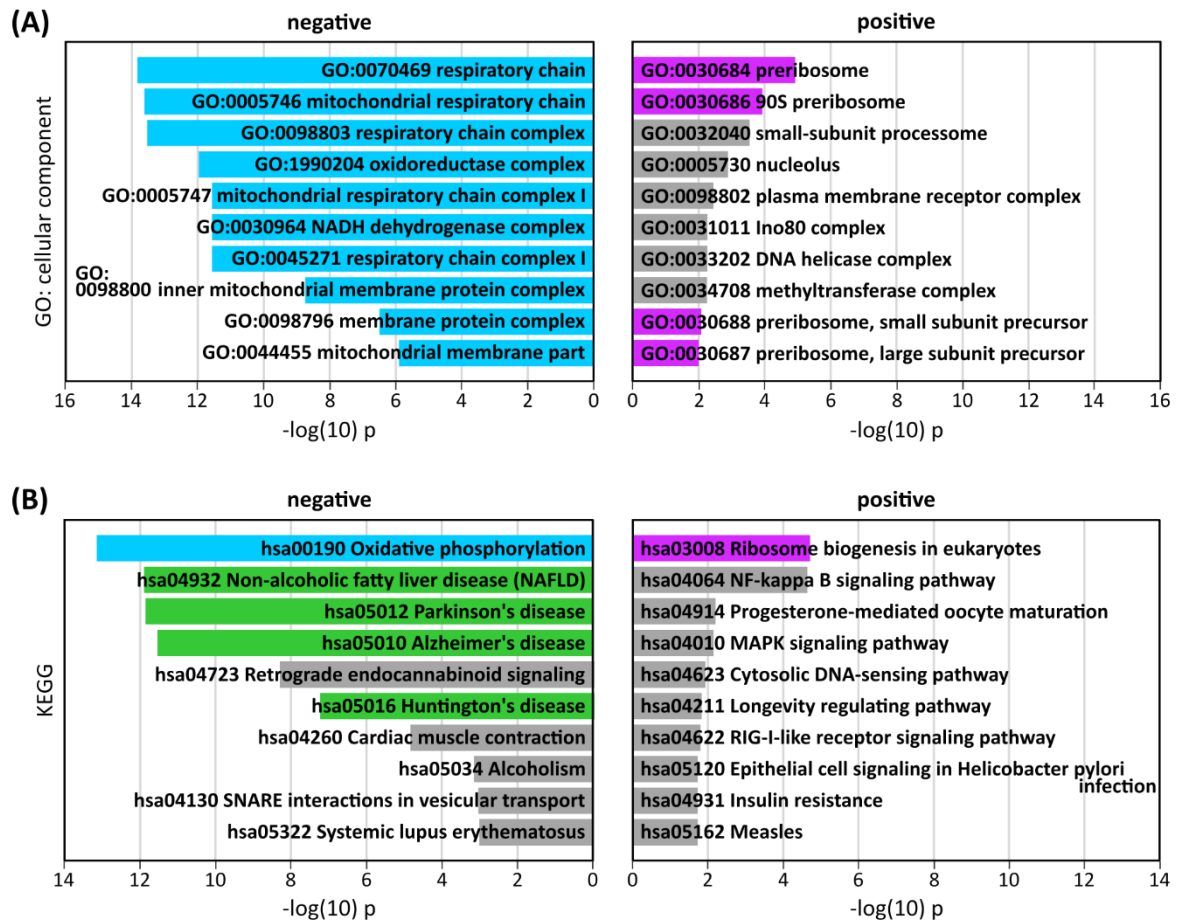


Fig. 40: SIAH3 levels correlate with reduced OXPHOS and neurodegenerative diseases.

MS analysis of the whole proteome in T-REx SIAH3 cells (pool of 3 clones, induced/uninduced with Dox for 24 h). **(A)** ORA of SIAH3 cells, induced vs. uninduced, regarding the GO database “cellular component”. The 10 most significantly over-represented terms (positively and negatively regulated) are depicted in p-values ($-\log_{10}$). **(B)** ORA using the Kyoto Encyclopedia Genes and Genomes (KEGG) database. The 10 most significantly over-represented groups (positively and negatively regulated) are depicted in p-values ($-\log_{10}$). Terms associated with the mitochondrial respiratory chain are coloured in blue, terms of diseases in which mitochondrial dysfunction plays an important role are coloured in green and terms related to the (pre-)ribosome are marked purple.

Figure adapted from Deutschmeyer *et al.*, 2025.

Regarding the different respiratory chain complexes, proteins mainly belonging to complex I, III, and IV and the mitochondrial proton-transporting ATP synthase complex were negatively regulated upon SIAH3 induction (Fig. 41A). Examination of proteins assigned to “Oxidative phosphorylation” and “Parkinson's disease” revealed that nearly all proteins associated with both categories were downregulated, whereas proteins associated with only one category were not (Fig. 41B).

RESULTS

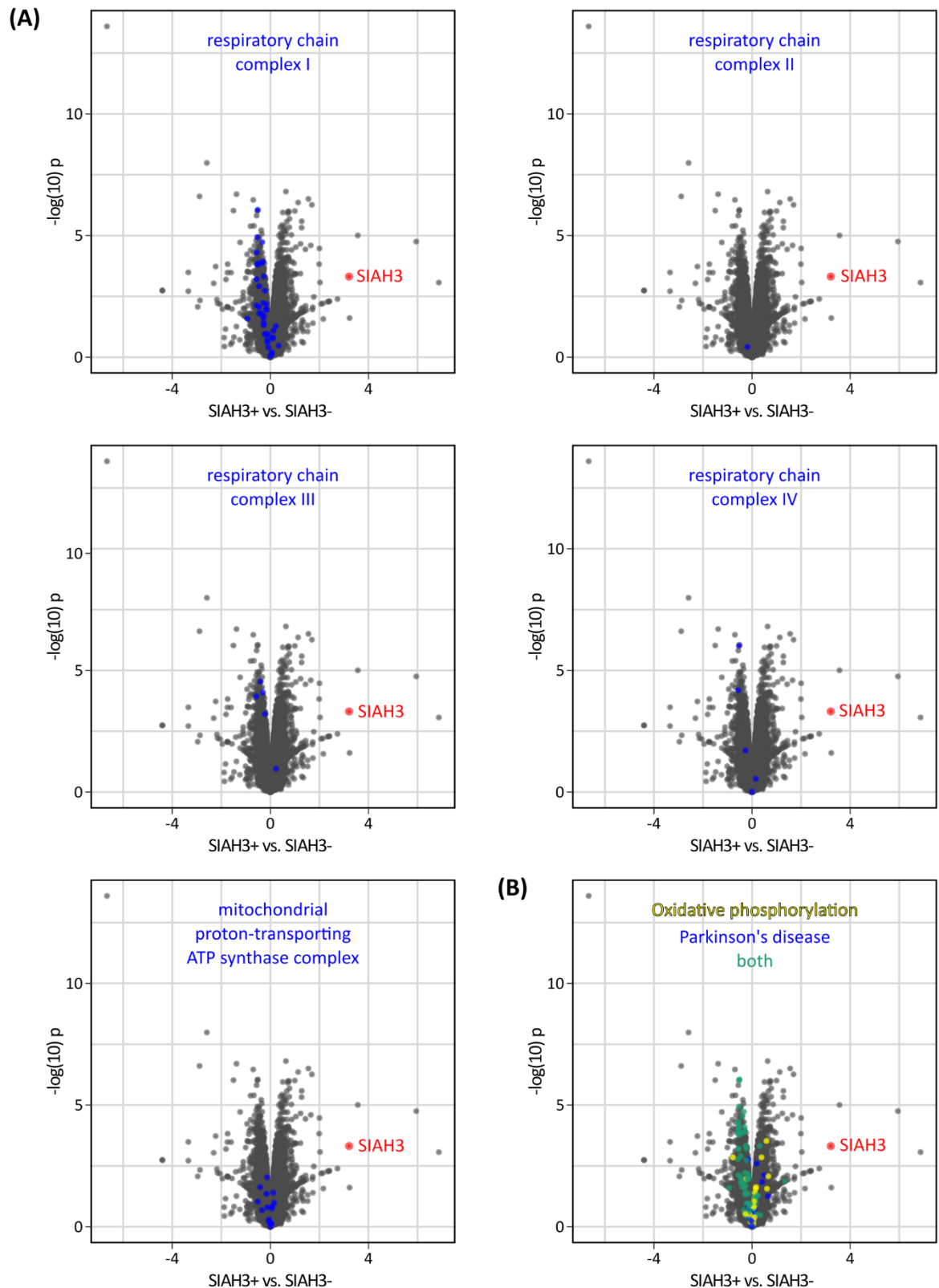


Fig. 41: The effect of SIAH3 on proteins of the respiratory chain.

MS analysis of the whole proteome in T-REx SIAH3 cells (induced vs. uninduced). **(A)** Volcano plots showing the coefficient of SIAH3 induced vs. uninduced in relation to the p-values ($-\log_{10}$). Proteins with the annotations “mitochondrial respiratory chain complex I, II, III or IV” and “mitochondrial proton-transporting ATP synthase complex” of the GO term “cellular component” (blue) as well as SIAH3 (red) are coloured. **(B)** Volcano plot showing the coefficient of SIAH3 induced vs. uninduced in relation to the p-values ($-\log_{10}$). Proteins with the KEGG annotation “Oxidative phosphorylation” (yellow), “Parkinson’s disease” (blue), or both (green) as well as SIAH3 (red) are coloured. Figure adapted from Deutschmeyer *et al.*, 2025.

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3.9.2 SIAH3 shifts cells from OXPHOS to glycolysis

To explore the functional consequences of these changes of proteins involved in the mitochondrial respiratory chain, the impact of SIAH3 on cellular metabolism in T-REx cells was investigated.

While monitoring the cell cultures, I noticed that induced T-REx SIAH3 cells changed the medium from pink to orange more rapidly than uninduced cells. Since a shift in cellular metabolism toward glycolysis increases lactate production and thereby lowers the pH, and as the colour of the culture medium reflects its pH, I measured the absorbance of the medium to assess acidification as an indicator of glycolytic activity. The absorbance spectrum showed two peaks, one at around 425 nm, reflecting the “orange colour” and one near 560 nm, reflecting the “pinkish colour” of the medium. The more the medium has been acidified, the higher the “orange peak” and the smaller the “pink peak”. The longer the cells grow within the same medium, the more shifts the colour from pink to orange, as it can be seen in Fig. 42A comparing the spectra after two and three days. Two days after induction, the medium of induced T-REx SIAH3 cells showed a smaller pink peak compared to the peak from the uninduced T-REx SIAH3 cells or the T-REx control cells and after three days, this difference got even larger. And while the pink peak decreased, the induced SIAH3 cells showed the highest increase in the orange peak (Fig. 42A). Even though Dox seemed to have an effect on the acidification, too, the influence of SIAH3 appears higher. Moreover, overexpression of SIAH3 in T-REx cells (all samples induced with Dox) also yielded a remarkable decrease in the pink peak whereas the orange peak increased (Fig. 42B). These experiments indicate that SIAH3 expression rises acidification, presumably via the lactate produced during glycolysis.

In order to test this hypothesis, the mitochondrial respiration, glycolysis and ATP production rate of T-REx cells (SIAH3/controls) were investigated using the Seahorse real-time cell metabolic analysis system (Seahorse XF96 Analyzer). Preliminary experiments (supplementary Fig. S24) demonstrated that the optimal cell count is 30,000 cells per well, and that the effective concentration of FCCP ranges from 0.75 to 1 μ M.

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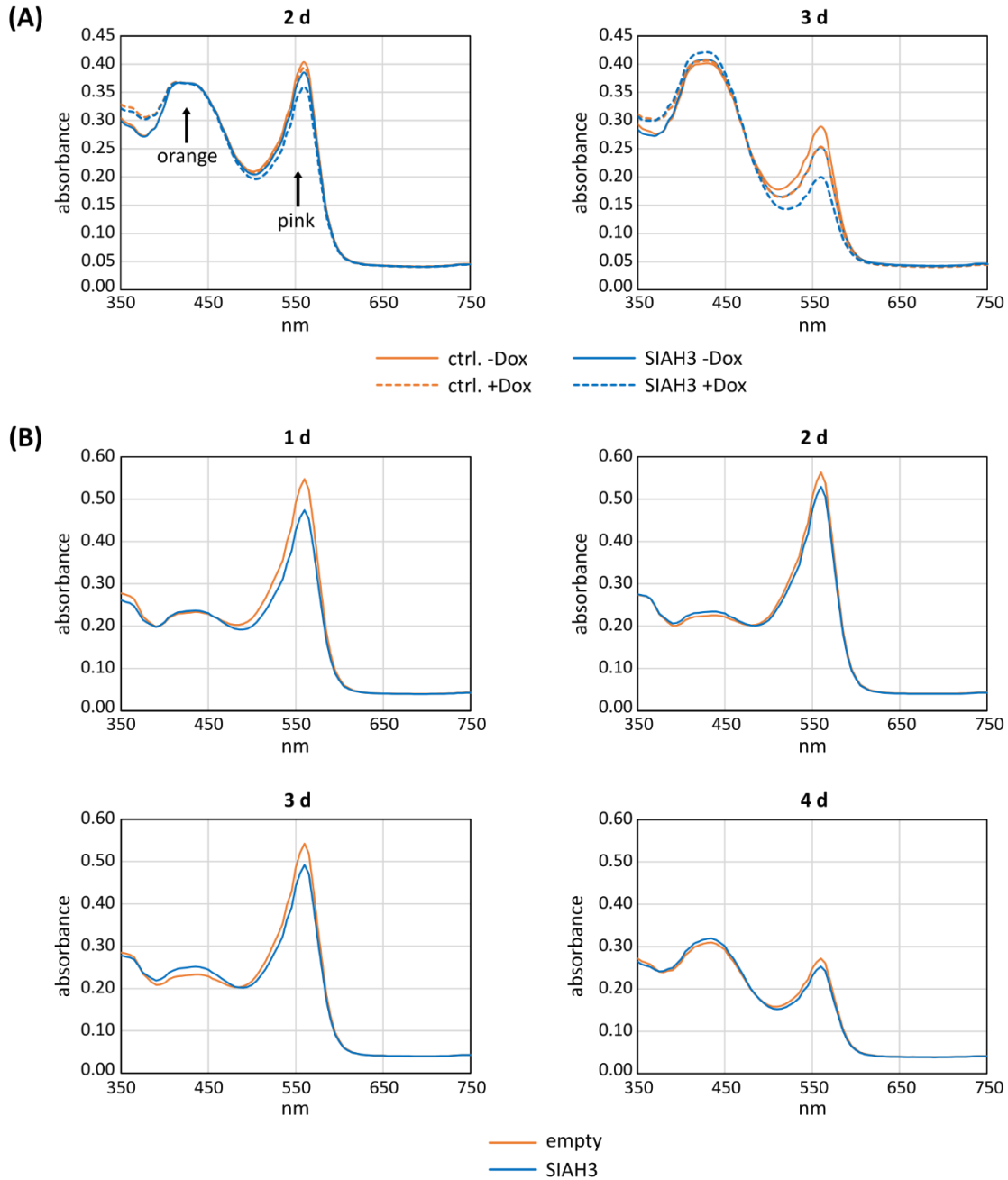


Fig. 42: SIAH3 expression increases the acidification of cell culture medium.

(A) Absorbance spectra of the cell culture medium of T-REx SIAH3 (pool of 3 clones) or control cells, induced or uninduced with Dox for 2 and 3 days. **(B)** Absorbance spectra of the cell culture medium of T-REx control cells after overexpression of SIAH3 or the corresponding empty vector for 1-4 days (all samples were induced with Dox to inhibit the repression by the stably expressed tetracycline repressor).

Afterwards, mitochondrial and glycolysis stress tests were conducted, followed by an ATP rate assay, all comparing T-REx SIAH3 Wt and T-REx control cells (each induced and uninduced with Dox) (Fig. 43). For the mitochondrial stress test, OCR was measured. ATP-linked respiration reflects the decrease in OCR upon injection of the ATP synthase inhibitor oligomycin, representing the portion of basal respiration that was being used to drive ATP production. FCCP was used as uncoupling agent. Rot/AA, complex I and III inhibitors, respectively, were used to shut down mitochondrial

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respiration. The ATP-linked respiration was significantly decreased in SIAH3-induced cells as compared to uninduced SIAH3 cells and control cells. The maximal respiration, too, was lowest in induced SIAH3 cells. Spare respiratory capacity was highest in uninduced T-REx SIAH3 cells and in general higher in the SIAH3 cells compared to control cells. The mitochondrial stress test profile showed no significant difference between induced and uninduced control cells (Fig. 43A). To analyse the glycolytic activity, ECAR was measured. Oligomycin and 2-DG were used to inhibit ATP synthase and glycolysis, respectively. The glycolytic stress test profile (Fig. 43B) indicated that glycolysis is significantly increased in SIAH3-expressing cells. The glycolytic activity level from T-REx SIAH3 cells was significantly higher than in the control cells and induced T-REx SIAH3 cells had the highest glycolytic activity. The glycolytic capacity was also increased in SIAH3-expressing cells, whereas the glycolytic reserve decreased. Taken together, these results revealed that SIAH3 expression decreases ATP-linked respiration and increases glycolysis, demonstrated by the shift from aerobic energy production towards glycolytic metabolism in the energy map (Fig. 43C). Total ATP production, however, remained unchanged as only the ratios of glycolysis and OXPHOS changed in favour of glycolysis (Fig. 43D). (Passage adapted from Deutschmeyer *et al.*, 2025.)

Next, the metabolism of C918 cells – cells expressing endogenous SIAH3 – was compared to SIAH3-knockdown C918 cells (Fig. 44A). However, depletion of SIAH3 had no significant effect on total ATP level or the metabolic production ratio (only in one of the four conditions, a slight increase in glycolysis was observed). The energy map, too, only showed a minimal shift of the knockdown cells towards glycolysis (Fig. 44B). Nevertheless, the missing all over significance let assume, that the observed small shift into the opposite direction to what was expected after the initial experiment in T-REx cells, did not reflect a meaningful effect of the SIAH3 knockdown in these cells.

In the next experiment, the effect of deleting the S3UNS domain, the N-terminal domain that is unique for SIAH3 and missing in SIAH1 and SIAH2, was analysed by an ATP rate assay. This deletion did not abolish SIAH3's ability to shift the cells towards glycolysis in T-REx cells (Fig. 45). Comparable to the Wt, the total ATP production was not altered after induction of SIAH3 Δ S3UNS cells, but the ratio between glycolysis and OXPHOS changed in favour of glycolysis (Fig. 45A). The energy map shows this shift from aerobic energy production towards glycolytic metabolism, too (Fig. 45B). However, the total ATP production of SIAH3 Δ S3UNS cells is slightly higher than in the SIAH3 Wt cells. These results suggest that that expression of SIAH3 modifies cell metabolism of T-REx cells, shifting it from OXPHOS towards glycolysis, and that this effect seems to be independent of its S3UNS domain.

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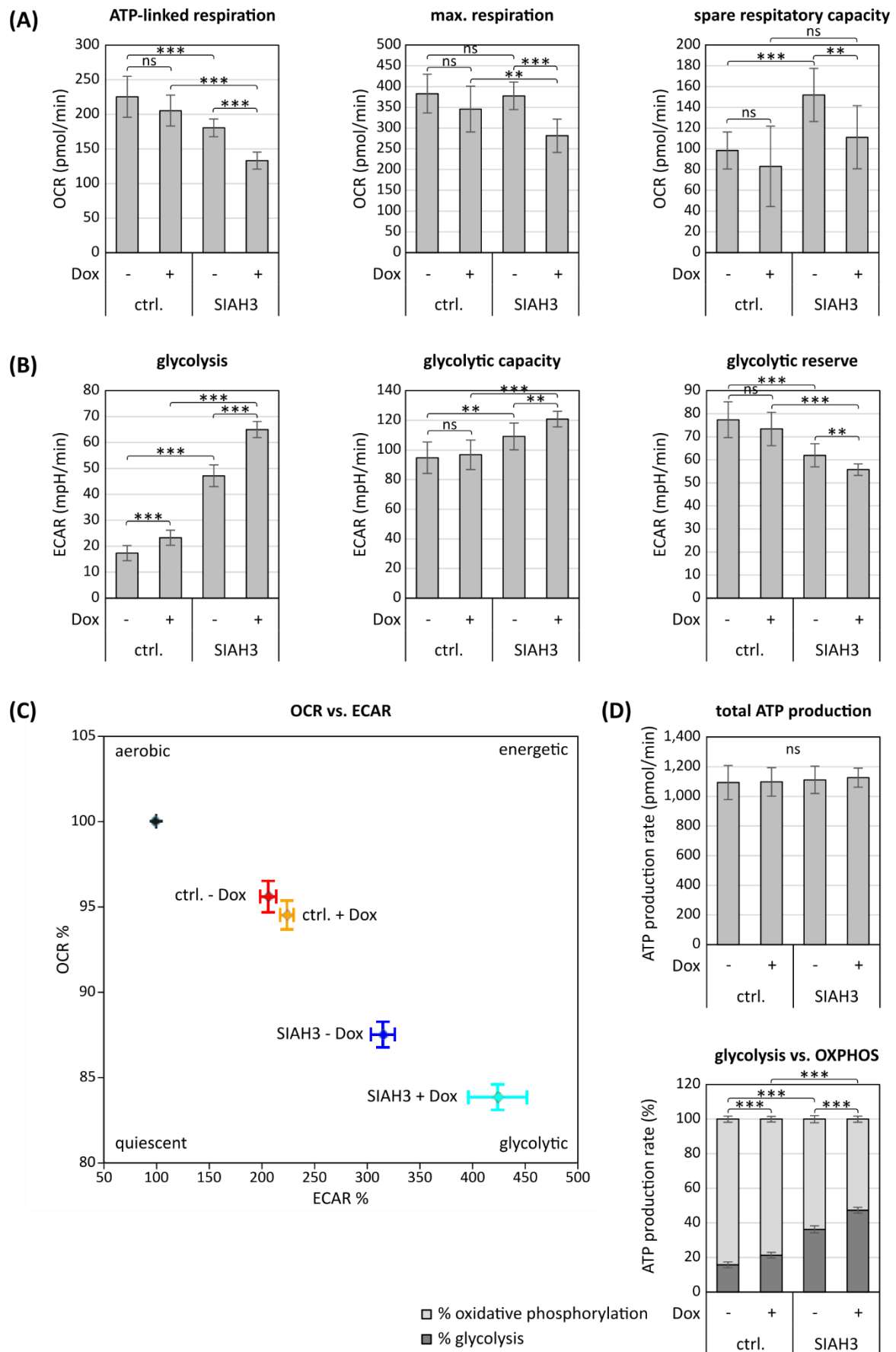


Fig. 43: SIAH3 effects metabolism and shifts cells from OXPHOS to glycolysis.

Metabolism of T-REx SIAH3 Wt (pool of 3 clones) and control cells was measured 24 h after induction ($\pm$ Dox).

(A) Mitochondrial stress test. OCR was plotted in respect to ATP-linked respiration, maximal respiration and

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spare respiratory capacity. FCCP (1 μ M) was used as uncoupling agent. Rot/AA were used to shut down mitochondrial respiration. **(B)** Glycolytic stress test. ECAR was plotted in respect to glycolysis, glycolytic capacity and glycolytic reserve. Oligomycin and 2-DG were used to inhibit ATP synthase and glycolysis, respectively. **(C)** Energy map. OCR (induced level after glucose injection) versus ECAR relation at measurement point 6 (after glucose addition), baseline set at measurement point 3 (before glucose addition) from the mitochondrial stress test. **(D)** ATP production of mitochondrial respiration and glycolysis. Total ATP production (pmol/min) of the 4 cell types and the ATP production as ratio between OXPHOS and glycolysis (in %) was plotted. Oligomycin and Rot/AA were used to inhibit ATP synthase and complex I/III, respectively. */**/** indicate the significance levels (Student's t-test). Figure modified from Deutschmeyer *et al.*, 2025.

And the finding that SIAH3 shifts cells from OXPHOS towards glycolysis is directly supported by the presented result of the MS proteome analysis where an enrichment of terms linked to (mitochondrial) respiratory chain and OXPHOS was found among the downregulated protein groups. This implies that SIAH3 is involved in the regulation of energy metabolism by influencing proteins of the mitochondrial respiratory chain. But how SIAH3 affects glycolysis, or whether this is a secondary effect, requires further investigation.

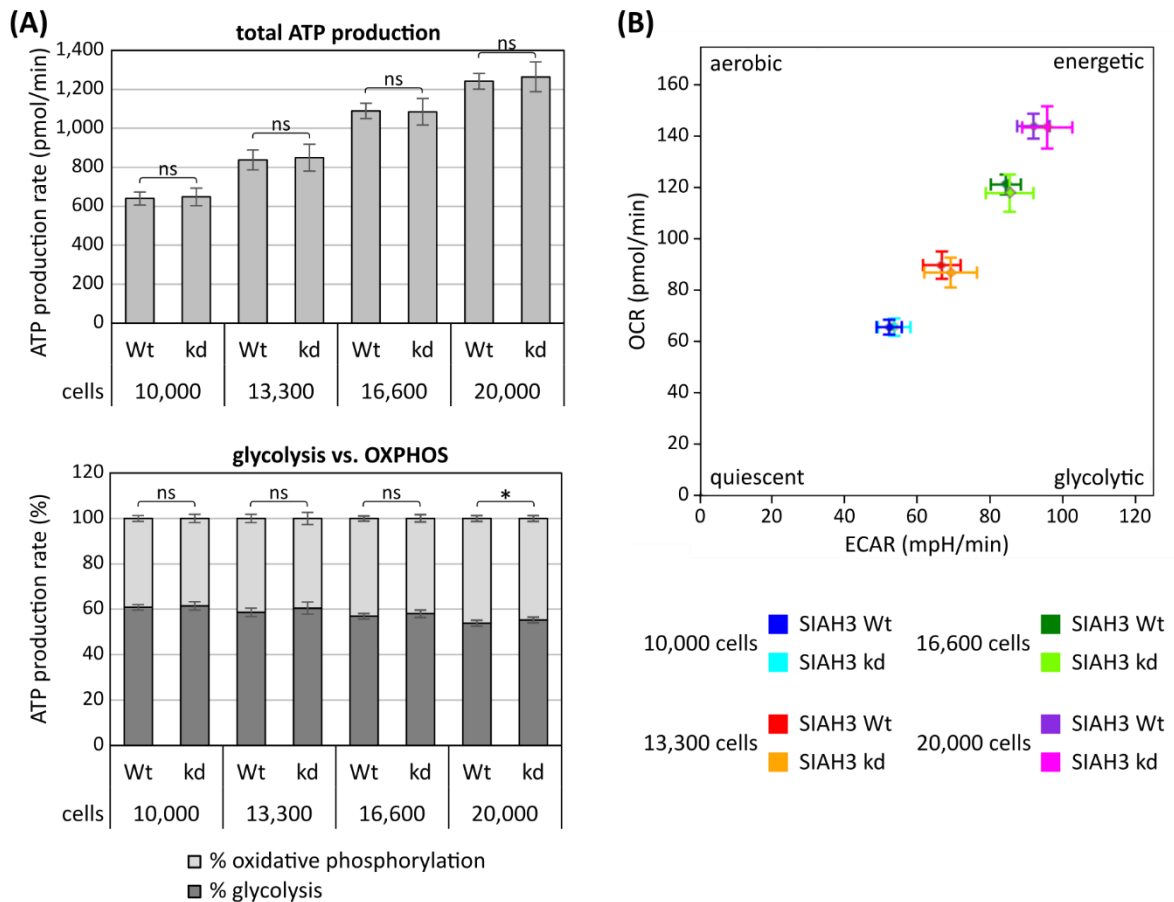


Fig. 44: Effect of SIAH3 knockdown on energy metabolism

(A) Knockdown of SIAH3 in C918 did not affect energy production. After knockdown (kd) of SIAH3 by siRNA for 3 days, an ATP rate assay for 4 different cell densities was conducted using Seahorse XF Analyzer. Total ATP production (pmol/min) and the ATP production rate as ratio between OXPHOS and glycolysis in % are plotted. **(B)** Corresponding energy map for C918. OCR versus ECAR relation at measurement point 3 (before oligomycin addition, no baseline set) is shown for Wt and SIAH3 kd C918 cells at 4 different cell densities. */**/** indicate the significance levels (Student's t-test). Figure adapted from Deutschmeyer *et al.*, 2025.

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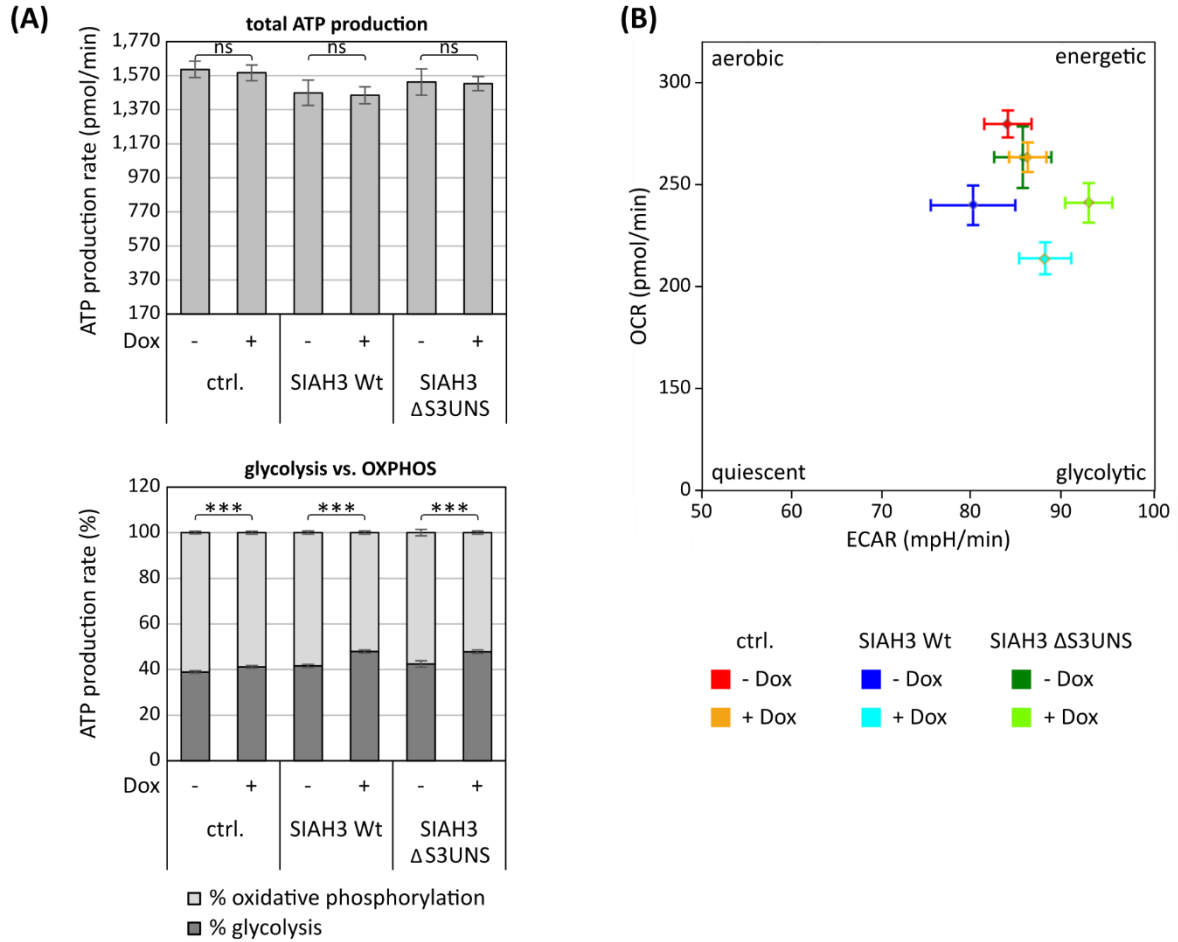


Fig. 45: Deletion of the S3UNS domain did not abolish SIAH3's effect on energy metabolism.

(A) ATP rate assay of T-REx SIAH3 Wt, ΔS3UNS and control cells (ctrl.) 24 h after induction with Dox (±Dox). Total ATP production (pmol/min) and the ATP production rate as ratio between OXPHOS and glycolysis in % are plotted for SIAH3 Wt, the S3UNS deletion mutant and control cells. **(B)** Corresponding energy map. OCR versus ECAR relation at measurement point 3 (before oligomycin addition, no baseline set) is shown for SIAH3 Wt, the S3UNS deletion mutant and control cells.

*/**/** indicate the significance levels (Student's t-test). Figure adapted from Deutschmeyer *et al.*, 2025.

The OCR and ECAR profiles corresponding to the Seahorse experiments regarding T-REx SIAH3 Wt are shown in supplementary Fig. S25 (mitochondrial stress test in A, glycolytic stress test in B) and S26A (real-Time ATP rate assay). Fig. S26 also shows the energy maps derived from the glycolytic stress test and the ATP rate assay, forming replicates of the one shown in Fig. 43C. The profiles corresponding to the experiments featuring T-REx SIAH3 ΔS3UNS and knockdown in C918 are shown in supplementary Fig. S27. WBs and qPCRs corresponding to all Seahorse experiments are presented in supplementary Fig. S28, proofing successful SIAH3 induction or knockdown.

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3.9.3 SIAH3 interacts with proteins of the mitochondrial translational system

To investigate the mechanism of SIAH3's interference with OXPHOS and shift to glycolysis, the interactome of SIAH3, as analysed by MS-based proteomics, was investigated again. ORA regarding the GO subontology "biological process" showed highest significance for "mitochondrial translation" and ORA for the GO subontology "cellular component" revealed significant enrichment for "ribonucleoprotein complex" and "mitochondrial/organellar ribosome" (Fig. 46A).

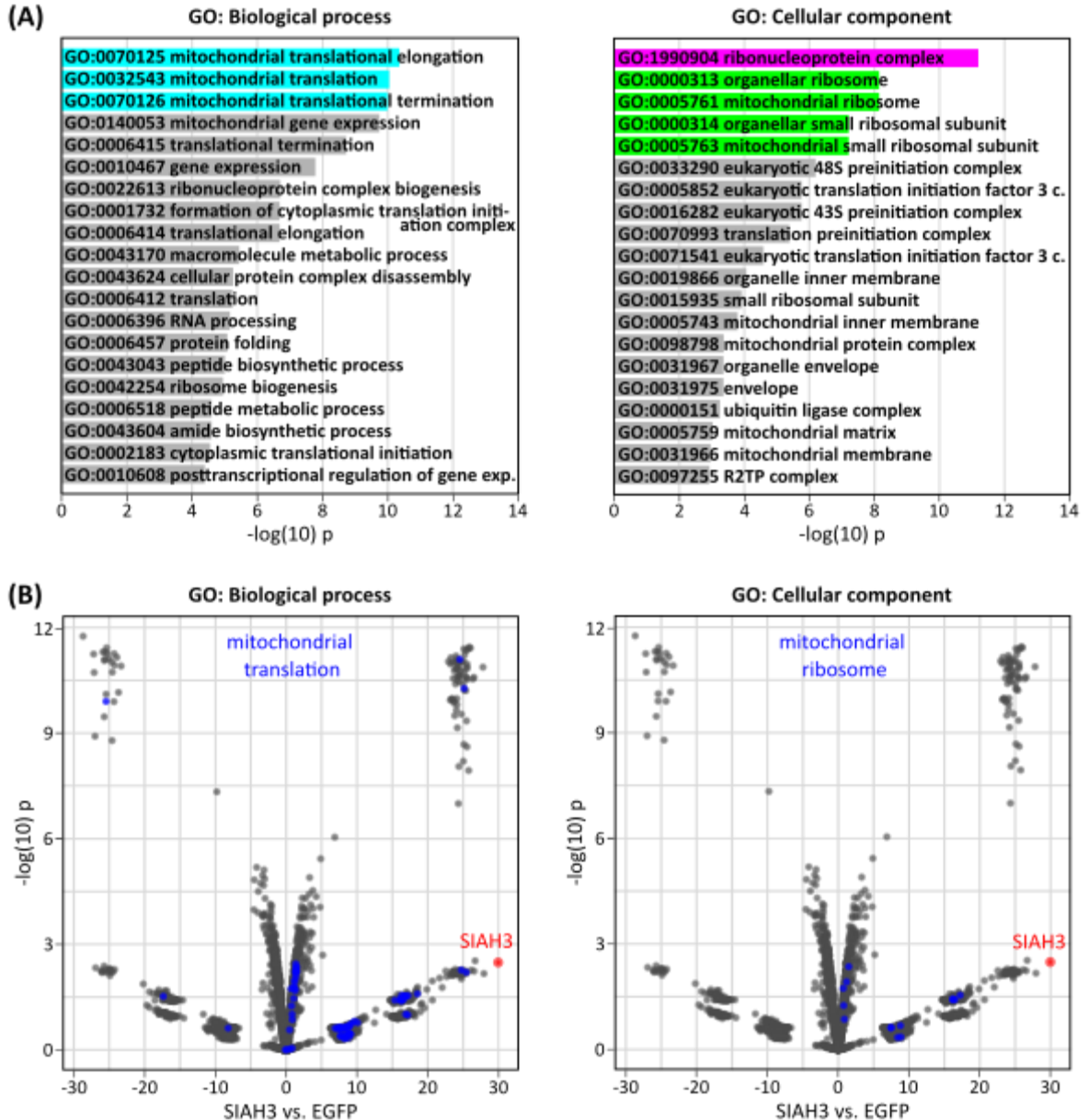


Fig. 46: SIAH3 interacts with proteins of the mitochondrial translational system.

(A) ORA of the coefficient EGFP-SIAH3 vs. EGFP-empty regarding the GO types "biological process" and "cellular component". The 20 terms with the highest significance are depicted. Analysis is based on the data of the MS analysis after IP of EGFP-SIAH3. Terms associated with "mitochondrial translation" are coloured in blue, "ribonucleoprotein complex" is purple and terms associated with "mitochondrial/organellar ribosome" are marked in green. c. = complex, exp. = expression. **(B)** Volcano plots showing the coefficient of EGFP-SIAH3 vs. EGFP-empty in relation to the p-values ($-\log_{10}$). In plot one, the proteins with the annotation "mitochondrial translation" (GO "biological process") are marked in blue, the second plot shows proteins with the annotation "mitochondrial ribosome" (GO "cellular component") in blue. In each, SIAH3 is marked in red. Figure adapted from Deutschmeyer *et al.*, 2025.

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The corresponding volcano plots also show that several interaction partners of SIAH3 are either involved in mitochondrial translation and/or are components of the mitochondrial ribosome (Fig. 46B). This result suggests that SIAH3 could influence cell metabolism through its effect on the mitochondrial translational system, which in turn may affect the production of proteins of the respiratory chain.

3.9.4 SIAH3 interacts with mitochondrial import proteins

Beyond its interaction with proteins of the mitochondrial translation system, the SIAH3 interactome also identified components of the mitochondrial import machinery as binding partners: coiled-coil helix coiled-coil helix (CHCH) domain containing 4 (CHCHD4) and translocase of the inner mitochondrial membrane 50 (TIMM50) (Fig. 47A). These proteins were also identified in the whole proteome analysis, where the expression level of TIMM50 did not change significantly, while CHCHD4 was upregulated by 1.4-fold (log2 value: 0.49) upon SIAH3 induction (Fig. 47B). Pulldown assays confirmed the interaction between SIAH3 and CHCHD4 (Fig. 48A and B). Furthermore, they showed that the SIAH3 domain deletion mutant SIAH3 Δ SBS was pulled down less strongly by CHCHD4 than the wild-type protein, suggesting that the substrate binding domain of SIAH3 is involved in this interaction (Fig. 48B). Even though the GST-pulldown for TIMM50 is not completely reliable because empty EGFP was also slightly pulled down, it showed an enhanced signal for TIMM50 when SIAH3 was present (Fig. 48C), thereby support the statement of the MS analysis.

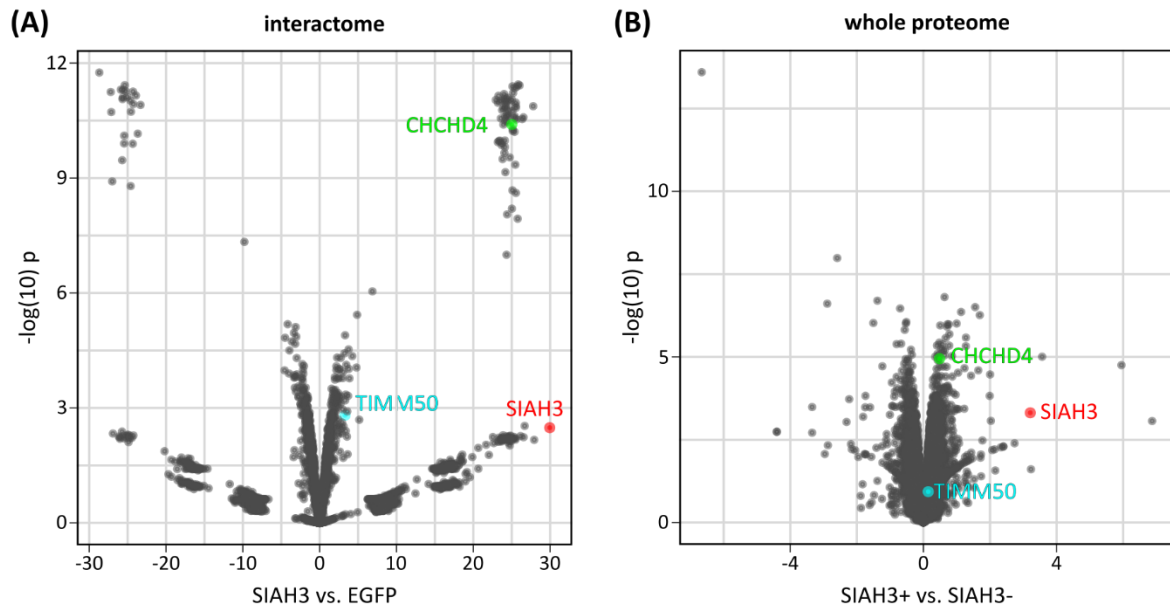


Fig. 47: SIAH3 interacts with proteins of the mitochondrial import machinery.

(A) Volcano plot showing the MS interactome data of the SIAH3-EGFP pulldown. The coefficient (log2) of SIAH3 vs. EGFP in relation to the p-values ($-\log_{10}$) is displayed. **(B)** Volcano plot showing the MS whole proteome data of T-REx SIAH3 cells (induced/uninduced). The coefficient (log2) of induced SIAH3 (SIAH3+) vs. uninduced SIAH3 (SIAH3-) in relation to the p-values ($-\log_{10}$) is displayed.

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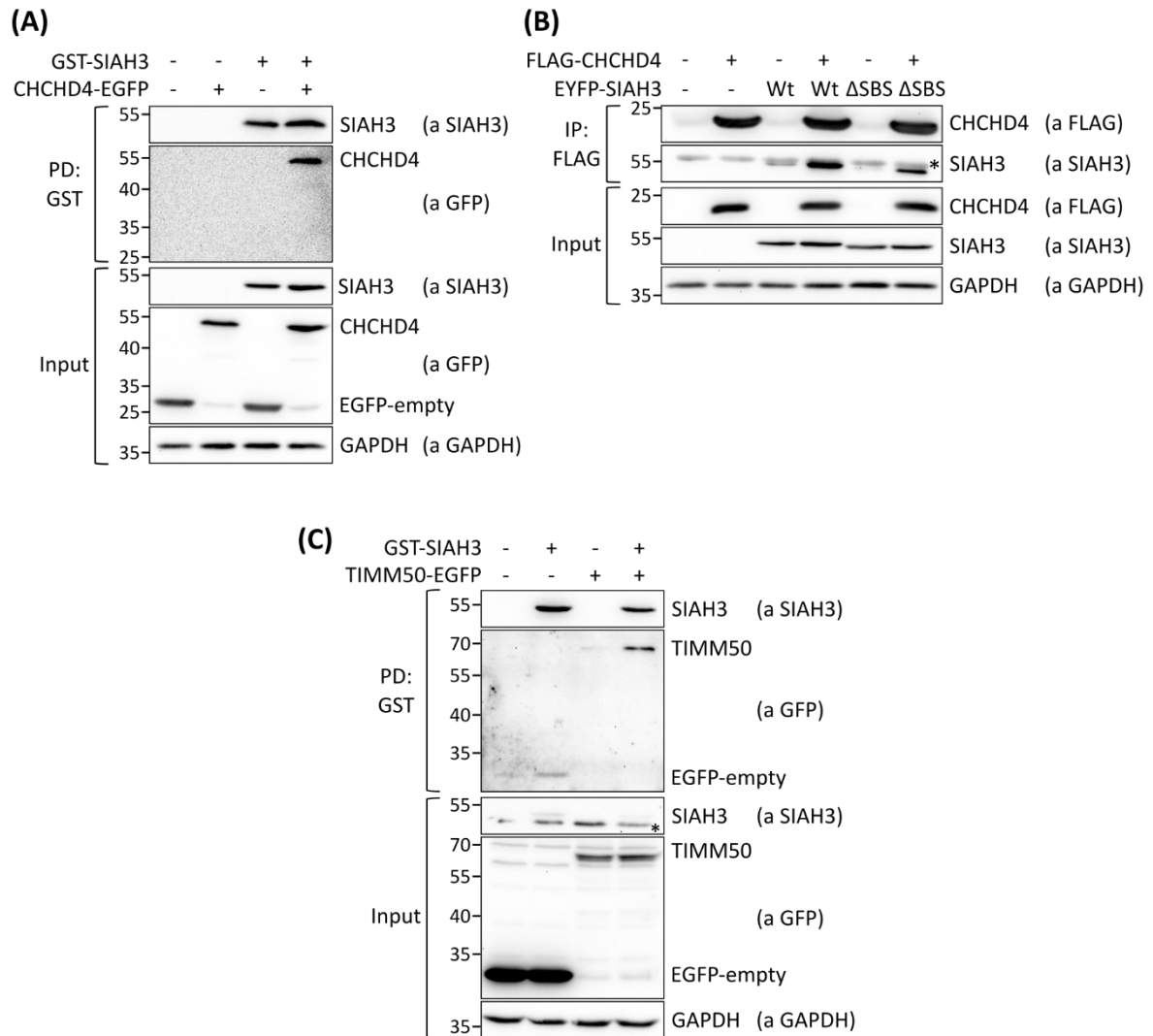


Fig. 48: Interaction of SIAH3 with CHCHD4 and TIMM50.

(A) Interaction between SIAH3 and CHCHD4. GST-pulldown analysis of GST-SIAH3, coexpressed with CHCHD4-EGFP (or the corresponding empty vectors) in HEK293T cells for 24 h. **(B)** Interaction between SIAH3 SBS-domain deletion mutant and CHCHD4. FLAG-IP of FLAG-CHCHD4, coexpressed with EYFP-SIAH3 Wt or SIAH3ΔSBS mutant (or the corresponding empty vectors) in HEK293T cells for 24 h. **(C)** Interaction between SIAH3 and TIMM50. GST-pulldown analysis of GST-SIAH3, coexpressed with TIMM50-EGFP (or the corresponding empty vectors) in HEK293T cells for 24 h.

Used ABs are indicated in parentheses. * unspecific band

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3.9.5 SIAH3 colocalises with mitochondria

The hypothesis that SIAH3 plays a role in mitochondrial metabolism was also supported by the finding that SIAH3 is localised at or in the mitochondria, as revealed by in silico-analysis using *DeepLoc* (Almagro Armenteros *et al.*, 2017) (Fig. 49A) and fluorescence microscopy (Fig. 49B). Single SIAH3 spots overlap with the signal of mitochondrial-targeted GFP, whereas SIAH3 clusters – presumably overexpression artefacts – do not coincide with the GFP signal, resulting in partial colocalisation of SIAH3 with mitochondria.

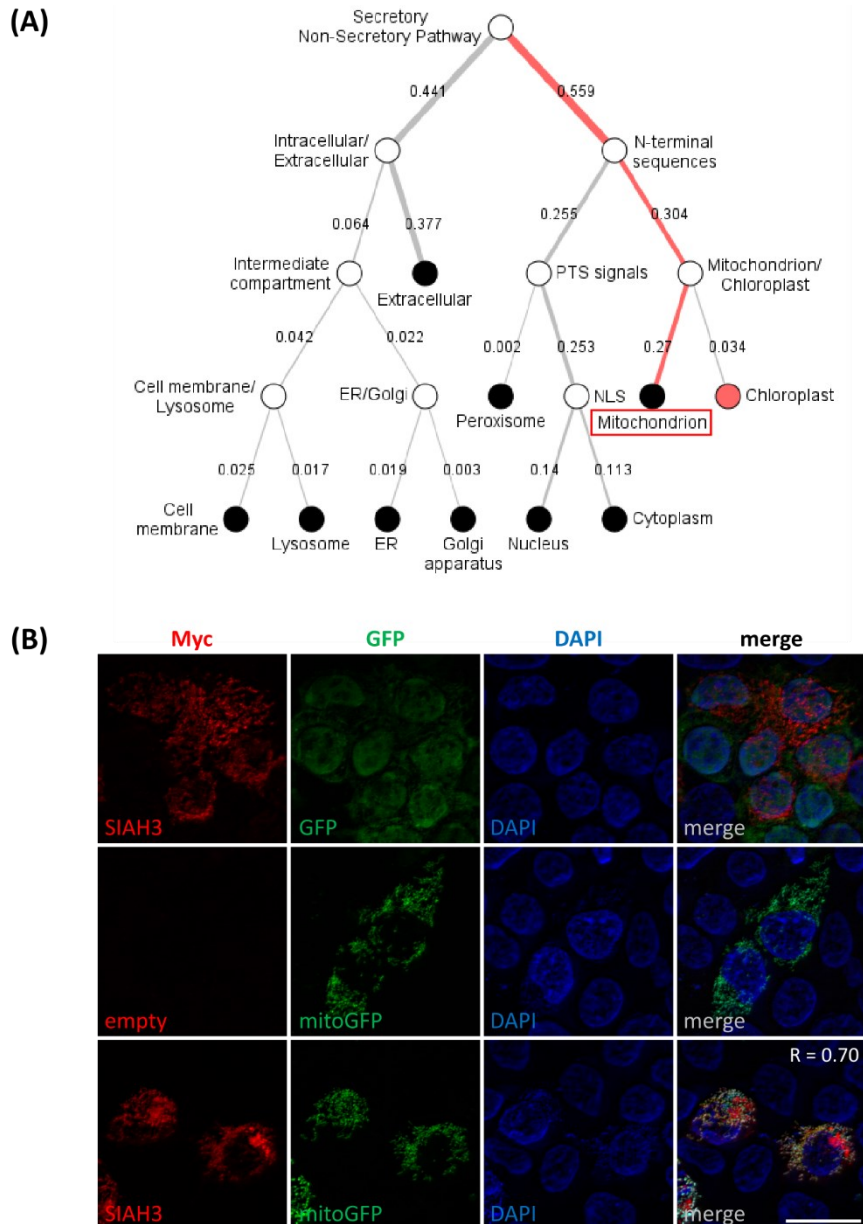


Fig. 49: SIAH3 colocalises with mitochondria.

(A) Decision tree of subcellular localisation of SIAH3, leading into mitochondria. Prediction was performed with *DeepLoc*. (B) Colocalisation of SIAH3 with mitochondrial GFP (mitoGFP, containing COX8 targeting sequence). SIAH3-Myc and mitoGFP (or the corresponding empty vectors) were coexpressed in HEK293T cells. After 24 h cells were fixed, immunostained using Myc-AB and the nuclei were marked with DAPI. Pearson's correlation coefficient (R) as a statistic for quantifying colocalisation is indicated. Scale bar: 20 μ m. Figure adapted from Deutschmeyer *et al.*, 2025.

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The DeepLoc prediction indicated further, that the highest impact on subcellular localisation of SIAH3 derives from its N-terminus (Fig. 50A), which was confirmed by deletion of the N-terminal S3UNS domain that altered the subcellular localisation from mitochondria into the nucleus (Fig. 50B). I therefore assume the S3UNS domain to be responsible for mitochondrial targeting of SIAH3. (Passage adapted from Deutschmeyer *et al.*, 2025.)

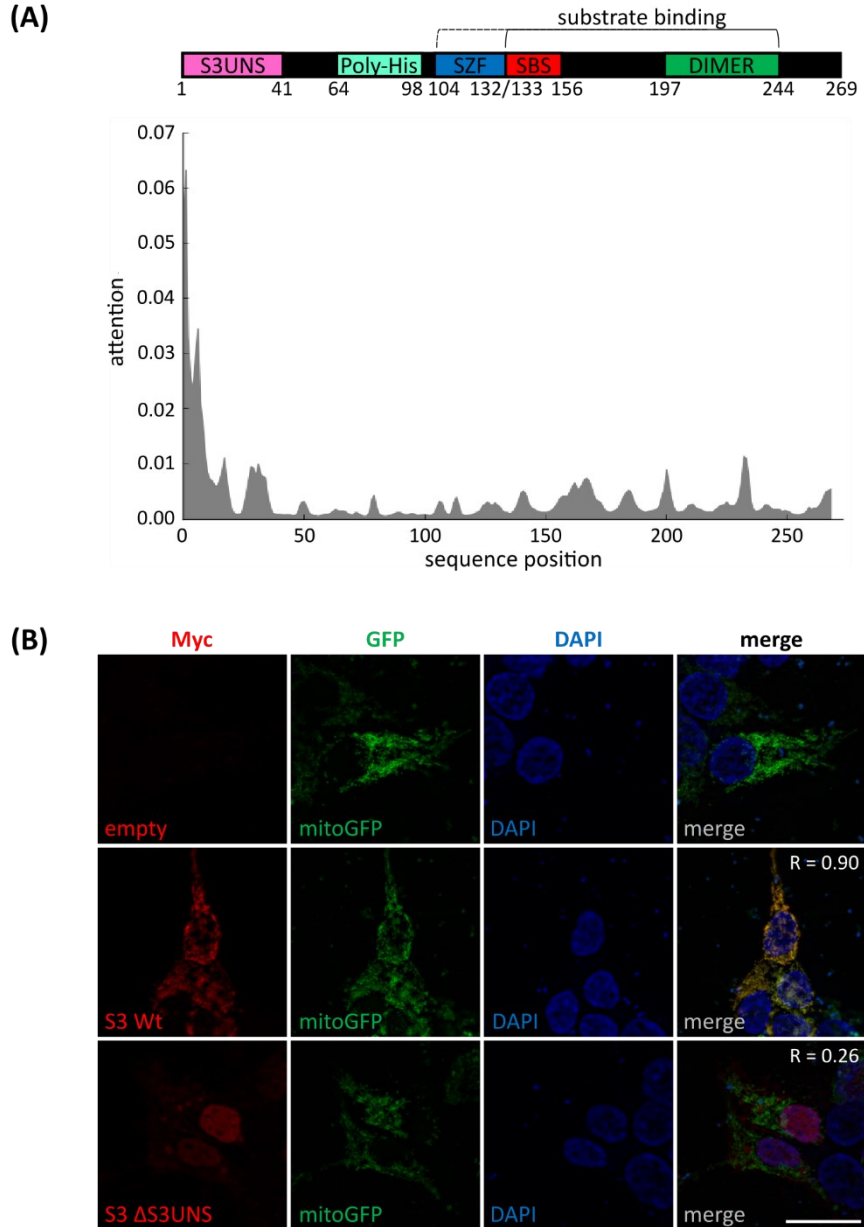


Fig. 50: SIAH3's S3UNS domain is responsible for mitochondrial targeting.

(A) Domain structure of SIAH3 (after Pepper *et al.* (2017) and Zhang *et al.* (2017)). Prediction of position information of the amino acid sequence showing the highest impact near the N-terminus. Prediction was performed with *DeepLoc*. **(B)** Colocalisation of SIAH3 Wt and the domain mutant Δ S3UNS with mitochondria (marked by GFP containing COX8 targeting sequence) in HEK293T cells. Myc-tagged SIAH3 Wt or Δ S3UNS and mitoGFP were overexpressed in HEK293T cells and after 24 h cells were fixed, immunostained using Myc-AB and the nuclei were marked with DAPI. Pearson's correlation coefficient (R) as a statistic for quantifying colocalisation is indicated. Scale bar: 20 μ m.

Figure adapted from Deutschmeyer *et al.*, 2025.

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3.10 Reactivation of SIAH3 using epigenetic editing

Taking all described results into account, it is now known that SIAH3 regulates the proteins of the respiratory chain complexes, thereby shifting energy metabolism from OXPHOS to glycolysis. It was shown that SIAH3 acts as a tumour suppressor by restricting cell proliferation. SIAH3 itself is regulated at two levels: at the protein level via the UPS, mediated by SIAH1/2, and at the transcriptional level by DNA methylation. In cancer, for example in melanoma, head and neck and lung cancer, *SIAH3* is epigenetically silenced by DNA methylation and is thus limited in its cancer-protective role. In order to restore its tumour suppressive function, the last step focused on the reactivation of SIAH3 expression using epigenetic editing.

Epigenetic editing refers to a technique based on the CRISPR/Cas9 system. Instead of initiating double-strand breaks, a catalytically inactive Cas9 (dCas9) is used to target epigenetic modifiers to a specific genomic locus, thereby altering the epigenetic landscape and influencing gene transcription (Hilton *et al.*, 2015; Gjaltema & Rots, 2020).

The used CRISPR/dCas9 system was based on the Cas9 protein of *Streptococcus pyogenes*. The modifiers, fused to the dCas9, were recruited to the *SIAH3* promoter region by six sgRNAs, spanning a region of around 650 bp upstream of the *SIAH3* CDS. Five guides (II-VI) are located upstream of the TSS, one (guide I) is located downstream of the TSS, in front of the TLS. Their positions are shown in Fig. 51.

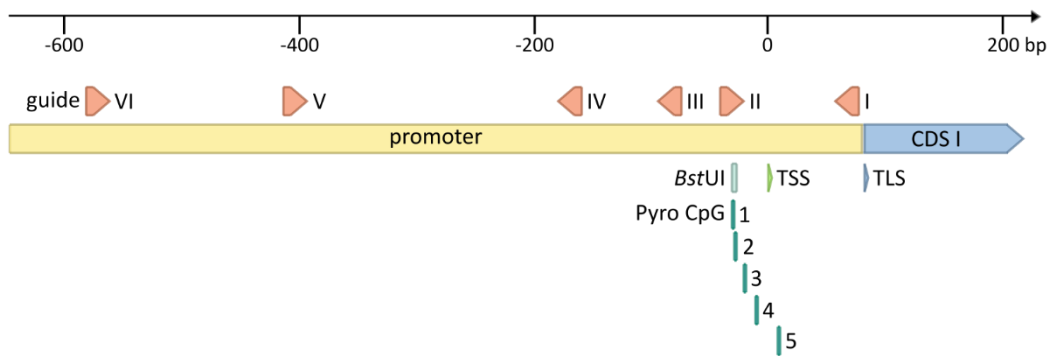


Fig. 51: Position of the sgRNAs and analysed CpGs in the *SIAH3* promoter region.

Schematic genomic locus of the *SIAH3* promoter region. The positions of the sgRNAs (guide I-VI, orange triangles), the CpGs analysed by pyrosequencing (Pyro CpGs, dark green vertical lines) and the *Bst*UI restriction site (turquoise rectangle), as well as the TSS and TLS are depicted.

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3.10.1 Epigenetic editing of an artificial promoter construct

The first tests for the modulation of *SIAH3* expression were conducted using an artificial promoter construct in combination with a reporter system. This enables the quantification of transcriptional activity by measuring the luminescent signal produced by the reporter.

The recruitment of the histone methyltransferase EZH2 (enhancer of zeste homolog 2) repressed *SIAH3* expression, while the histone-acetyltransferase p300 (core catalytic domain) activated it (Fig. 52A and supplementary Fig. S29). The DNA methyltransferase 3 (DNMT3) significantly reduces *SIAH3* expression, showing higher efficiency for DNMT3A alone than in combination with its cofactor DNMT3L (Fig. 52B). The artificial viral transcription activator VP160, however, did not significantly enhance *SIAH3* expression (Fig. 52C). These preliminary experiments suggest that it is, in principle, possible to modify *SIAH3* transcription by modulating histone and DNA marks.

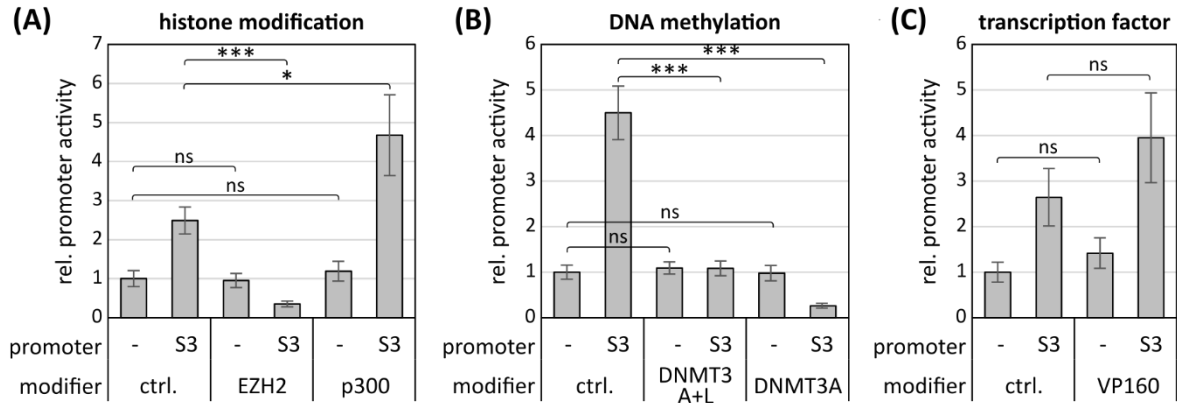


Fig. 52: Epigenetic regulation of an artificial *SIAH3* promoter construct.

SIAH3 promoter activity was quantified using the Dual-Luciferase Reporter Assay. The indicated modifiers, coupled to an inactive Cas9 (dCas9), were recruited by a mix of 3 sgRNAs (guide I, II, III) to the artificial *SIAH3* promoter-reporter-construct. Experiments were conducted in HEK293T. **(A)** Histone methyltransferase EZH2 represses, while histone acetyltransferase p300 (core) activates the *SIAH3* promoter. **(B)** DNA methyltransferase DNMT3 represses the *SIAH3* promoter activity. **(C)** The viral-derived transcription activator VP160 did not significantly alter *SIAH3* promoter activity.

*/**/** indicate the significance levels (Student's t-test).

3.10.2 Epigenetic editing of the endogenous *SIAH3* promoter

Next, the effect of three different activating modifiers on endogenous *SIAH3* expression was tested. All three modifiers – VP160 as a common transcription activator, p300 that activates through the acetylation of histones, and a new demethylation enzyme, here called DME (under non-disclosure), that promotes transcription through demethylation of DNA – succeeded in activating *SIAH3* expression (Fig. 53A and B). The recruitment of p300 yielded the highest *SIAH3* mRNA level, followed by VP160. With p300, the mRNA level increased to more than six times the level found in C918, the cell line that naturally expresses *SIAH3*. Considering the corresponding methylation status, it was found that only DME led to a small decrease in the methylation of CpG 5, whereas the

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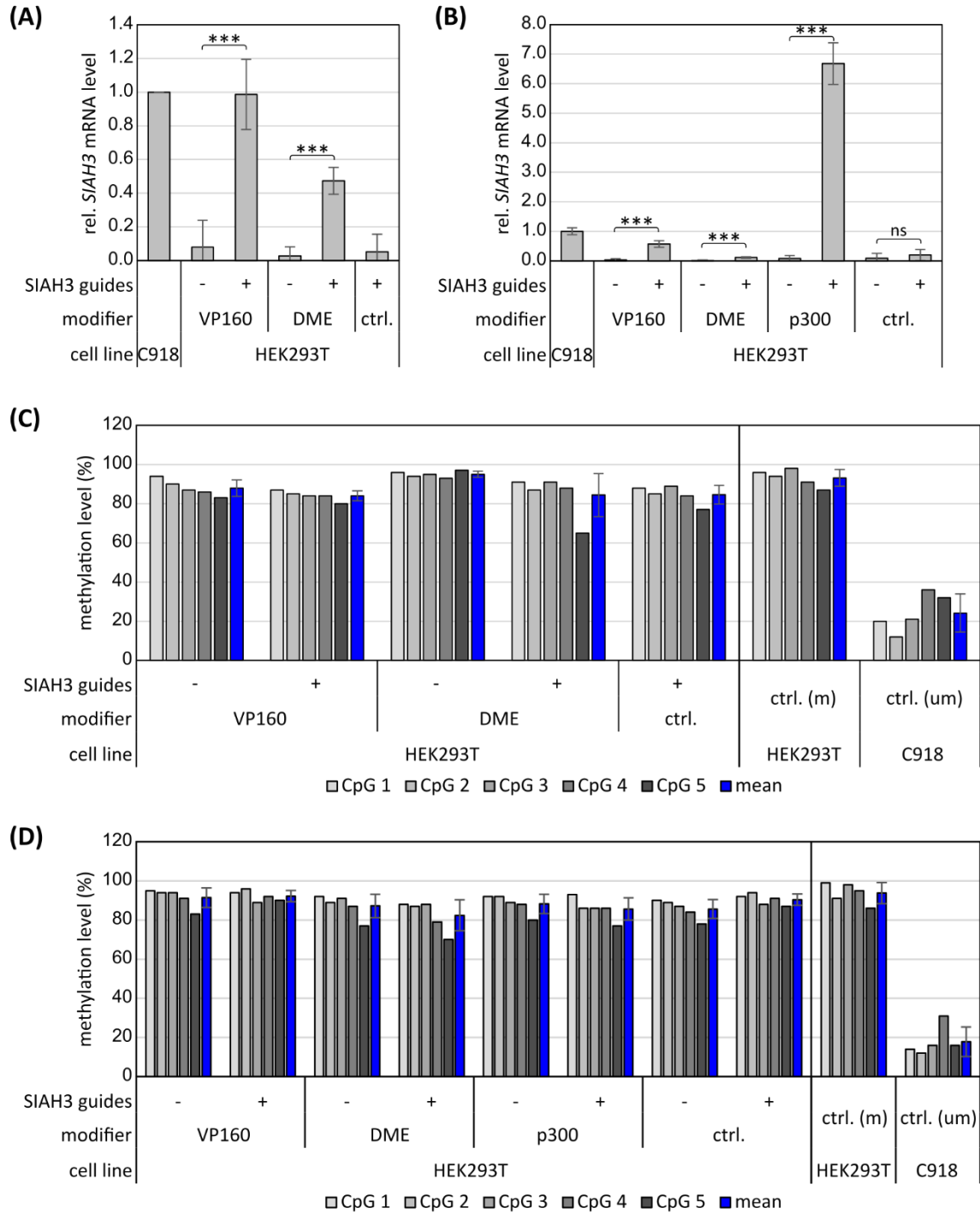


Fig. 53: Epigenetic modifiers reactivate endogenous *SIAH3*.

The transcription activator VP160, the novel demethylation enzyme (DME) and the histone acetyltransferase p300 (core), coupled to an inactive Cas9 (dCas9), were guided towards the *SIAH3* promoter by a mixture of 6 sgRNAs (or by a non-targeting sgRNA as negative control). Experiments were conducted in HEK293T. **(A)** Quantitative expression analysis shows a *SIAH3* reexpression with VP160-dCas9 and DME-dCas9. RNA was isolated 144 h after transfection and RT-qPCR was performed. The values were normalised to *ACTB*. **(B)** Quantitative expression analysis shows the highest *SIAH3* reexpression with p300-dCas9. RNA was isolated 96 h after transfection and RT-qPCR was performed. The values were normalised to *ACTB*. **(C)** DME-dCas9 led to a slight demethylation in 1 of the 5 analysed CpGs. Pyrosequencing of the experiment corresponding to (A). Untreated HEK293T served as methylated control, C918 as unmethylated control. **(D)** The same small demethylation of CpG 5, initiated by DME-dCas9, was observed in the methylation analysis (pyrosequencing of five CpGs) corresponding to the experiment shown in (B). The positions of the sgRNAs and the analysed CpGs are shown in Fig. 51. */**/* indicate the significance levels (Student's t-test). Experiments were performed by my Master's student Julia Bender.

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other two modifiers did not change the methylation (Fig. 53C and D). The activating effect of DME and p300 is independent of the used cell line, as the experiment in C918 showed that is depicted in Fig. 54A. Fig. 54B shows the CoBRA analysis of untreated C918 and HEK293T cells, revealing that these two cell lines have different methylation states: C918 is unmethylated, while HEK293T is methylated.

These experiments provide evidence that it is possible to turn on *SIAH3* expression by editing the epigenetic landscape. In this context, it was shown that histone acetylation has a greater effect than DNA demethylation, suggesting that although *SIAH3* is repressed by DNA methylation, additional mechanisms such as histone modifications also play a crucial role and must be considered when attempting to reactivate *SIAH3*.

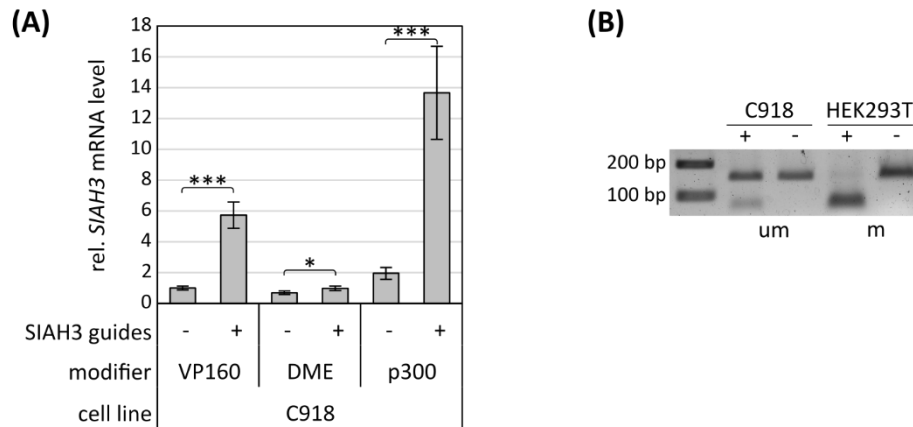


Fig. 54: Activation of *SIAH3* expression in C918.

(A) VP160, DME and p300 (core), coupled to an inactive Cas9 (dCas9), were guided towards the *SIAH3* promoter by a mixture of 6 sgRNAs (or by a non-targeting sgRNA as negative control) in C918 cells. RNA was isolated 120 h after transfection and RT-qPCR was performed. *SIAH3* expression was normalised to *ACTB*. */**/** indicate the significance levels (Student's t-test). **(B)** The *SIAH3* promoter is unmethylated in C918, whereas it is methylated in HEK293T. CoBRA analysis of untreated C918 and HEK293T cells. + digested with *Bst*UI, - mock digest without enzyme, um = unmethylated, m = methylated. Experiments were performed by my Master's student Julia Bender.

Since p300 had the highest impact on *SIAH3* expression, the conditions underlying this activation were investigated further in the next step. A time-course experiment showed that the expression peaked 120 h after transfection (Fig. 55A). It also revealed that a co-treatment (under non-disclosure) did not significantly enhance the activating effect of p300. In the experiments presented so far, a mix of multiple guides was used. Analysis of the individual guides revealed that guide IV yielded the greatest effect, comparable to the mixture of all 6 guides (Fig. 55B). The sgRNAs I, II and III, the ones nearest to the TSS showed the smallest effects, whereas sgRNAs IV to VI, the ones further upstream, had larger impacts. Number IV, the most effective guide, binds from 179 to 159 bp upstream of the TSS, which seems to be the best position for p300 to activate *SIAH3* transcription.

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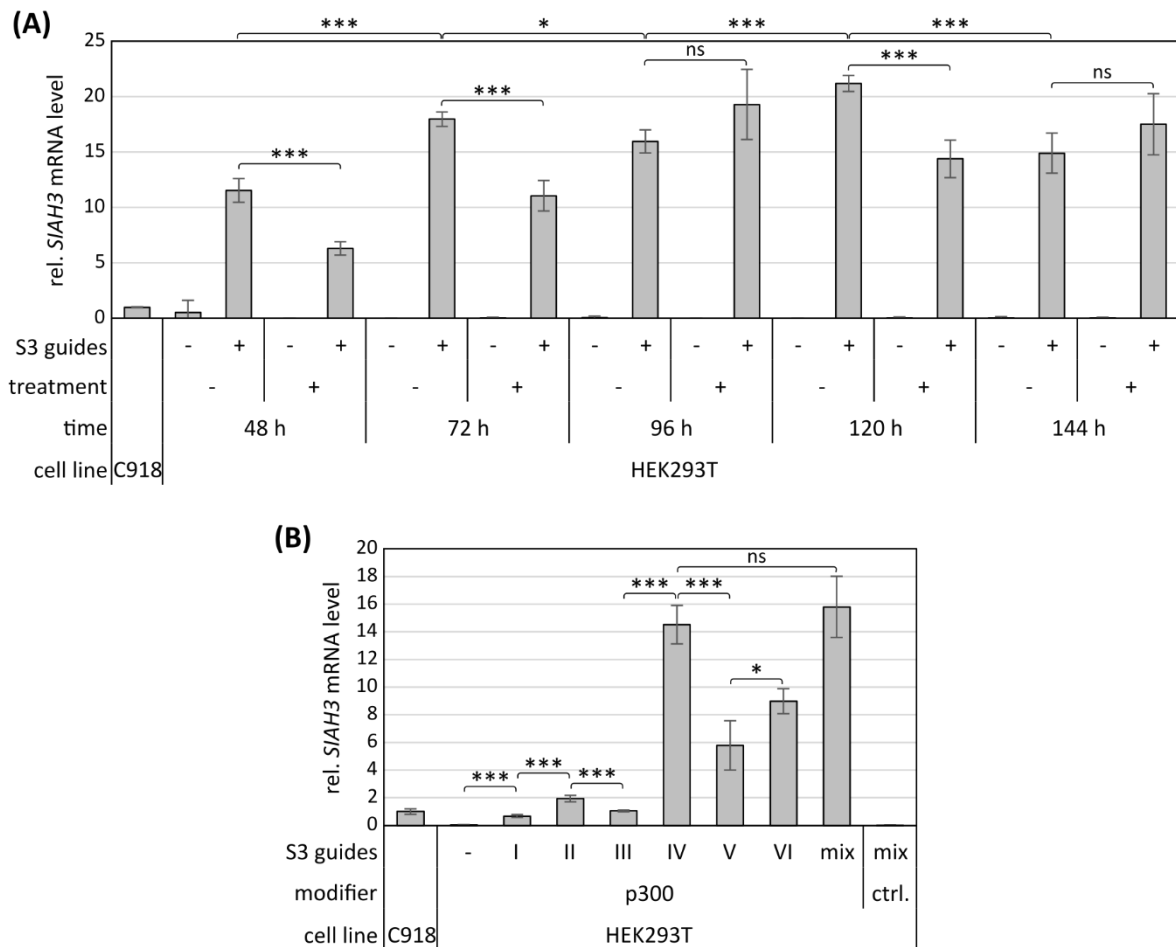


Fig. 55: 120 h of overexpression and single guide IV yielded the highest *SIAH3* expression.

(A) Time-course experiment. HEK293T cells were cotransfected with p300(core)-dCas9 and either a mixture of all 6 *SIAH3* sgRNAs (+) or the non-targeting control guide (-). Additionally, the effect of a chemical co-treatment (under non-disclosure) was investigated. RNA was isolated at 48, 72, 96, 120 or 144 h after transfection and RT-qPCR was performed. *SIAH3* expression was normalised to *ACTB*. C918 served as control sample that showed an endogenous *SIAH3* expression. **(B)** Single guide test. HEK293T cells were cotransfected with p300(core)-dCas9 and either a mixture of all *SIAH3* sgRNAs (mix), one of the six single guides (I, II, III, IV, V or VI) or the non-targeting control guide (-). RNA was isolated 120 h after transfection and RT-qPCR was performed. *SIAH3* expression was normalised to *ACTB*. C918 served as control sample that showed an endogenous *SIAH3* expression. pcDNA3 was used as a negative control without modifier.

*/**/** indicate the significance levels (Student's t-test). Experiments were performed by my Master's student Julia Bender.

Even though promoter demethylation did not have the strongest effect on the reexpression of *SIAH3*, as initially expected based on the observed hypermethylation, these experiments nevertheless provide important insights into the mechanisms underlying the reactivation of *SIAH3* by modulating the epigenetic landscape.

4 DISCUSSION

SIAH1 and SIAH2, the first two SIAH family members, are known players in tumorigenesis. SIAH1 is mostly described as tumour suppressor (e.g. summarised in Zhang *et al.*, 2022), whereas SIAH2 mainly acts as oncogene (e.g. summarised in Li *et al.*, 2022). Our results suggest that SIAH3, too, plays a role in tumorigenesis.

4.1 *SIAH3* is an epigenetically silenced tumour suppressor gene

The analysis of the DNA methylation status of *SIAH3* showed that its promoter region is hypermethylated in various tumour entities including cutaneous melanomas, head and neck cancer and lung adenocarcinomas. Additionally, pharmacological inhibition of DNA methyltransferases was associated with reexpression and demethylation of *SIAH3* in melanoma cancer cells, showing the regulatory relevance of DNA methylation for the transcription of the *SIAH3* gene. Promoter hypermethylation of TSG, leading to their silencing, has been described for many tumours (Dammann *et al.*, 2000; Esteller, 2002) and may serve as a potential cancer biomarker (Baylin & Jones, 2011; Küster *et al.*, 2020; Woods *et al.*, 2022). While hypermethylation of the *SIAH3* promoter has not been described in tumorigenesis yet, hypomethylation of *SIAH3* in cadmium rich placentas of female infants (Mohanty *et al.*, 2015) and in persons with early life adversity (Wiegand *et al.*, 2021) has been reported, albeit with respect to CpG sites located in exons instead of in promoter regions and it remains unclear whether they impact expression levels. In this study, a significant correlation between CpG hypermethylation upstream of the *SIAH3* TSS and downregulation of the *SIAH3* transcript has been observed. Additionally, pharmacological inhibition of DNA methyltransferases was associated with reexpression and demethylation of *SIAH3* in melanoma cancer cells. The results further showed a reduced *SIAH3* expression associated with an unfavourable prognosis in melanoma, head and neck and lung cancer patients. Accordingly, lower methylation levels were linked to improved prognosis in melanoma as well as lung cancer patients. These characteristics propose that *SIAH3* may be an epigenetically silenced TSG. In contrast to epigenetics, genetic alterations of *SIAH3*, however, have already been described in tumorigenesis. López de Maturana *et al.* (2021) identified a genetic variant within the *SIAH3* locus in pancreatic cancer and Robbins *et al.* (2011) analysed metastatic prostate tumours and found a focal homozygous deletion of a genetic region containing *SIAH3*. These discoveries suggest that lack of correct SIAH3 seems to be favourable for cancer cells. Although the functional significance of these findings remained undiscussed, they support the hypothesis that SIAH3 is involved in tumorigenesis. This study now could provide evidence that SIAH3 indeed acts as a functional tumour suppressor by inhibiting cell proliferation. As assessed by growth curves, overexpression of SIAH3 led to a reduced cell number compared to control cells and increased the number of dead

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cells. Flow cytometry analysis, though, did not show an impact of *SIAH3* on the cell cycle distribution. A scratch assay, however, showed, that *SIAH3* expressing cells closed the gap significantly slower than control cells. If this observation is a result of a reduced migration ability of the *SIAH3* cells or only due to the reduced cell proliferation and enhanced dying process needs further investigation, for example using trans-well migration assays (Boyden chamber assay). But the observed effects show *SIAH3*'s ability to interfere with the cancer cell's characteristics of "sustaining proliferative signaling" and "resisting cell death" (Hanahan, 2022), thereby executing its tumour suppressive function. And to avoid this, the hypermethylation of the *SIAH3* promoter appears to be part of a cell's "nonmutational epigenetic reprogramming", a characteristic enabling its transformation into a cancer cell (Hanahan, 2022).

(Parts of this chapter are adapted from Deutschmeyer *et al.*, 2025.)

4.2 *SIAH3* gene expression is regulated by additional mechanisms

Beside the finding that *SIAH3* gene expression is negatively correlated with methylation in cancers such as melanoma, HNSC and kidney cancer, it is positively correlated with methylation in some other entities like THYM and UCEC. This suggests that there are additional mechanisms regulating *SIAH3* gene expression beyond methylation. I found the *SIAH3* mRNA level to be dependent on cell density and on the amount of oxygen. The denser the cells, the higher was the *SIAH3* expression. This behaviour reminds of genes that are involved in cell cycle regulation and contact inhibition, for example p16 (Yamashiro *et al.*, 2011) and VHL (Baba *et al.*, 2001). Thereby, *SIAH3* is higher expressed when cell proliferation needs to be slowed down to avoid overgrowths of cells – which is also the task of TSG during cancer growth. Furthermore, *SIAH3* expression was found to be reduced under hypoxic conditions. Due to high proliferation, an imbalance between oxygen supply and consumptions occurs, making hypoxia an important feature of solid tumours (Vaupel & Harrison, 2004). It is known that *SIAH2* mRNA levels increase under hypoxic conditions and that *SIAH2* stabilises HIF1 α through the degradation of PHD3, promoting vascularisation and the adaption of the cancer cell to the hypoxic microenvironment (Nakayama *et al.*, 2009). It needs further investigation whether *SIAH3* acts, contrary to *SIAH2*, in an antiproliferative manner – for example by destabilising HIF1 α and thereby inhibiting the hypoxic response –, as its expression is reduced by hypoxia. Next to methylation, cell density and hypoxia, *SIAH3* expression is regulated by the melatonin-induced microRNA miR4516, as described by Yoon *et al.* (2021). Thus, at least four mechanisms are known to be involved in the regulation of *SIAH3* expression.

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4.3 The interplay between SIAH1/2 and SIAH3

MS analysis as well as WB-pulldown experiments revealed an interaction between SIAH3 and many ubiquitin ligases – among them SIAH1 and SIAH2. Microscopy analysis also showed that SIAH3 is located in the mitochondria and attracts SIAH1 to its own loci. While I was writing the manuscript for publication, Abd Elghani *et al.* (2022) published that SIAH3 interacts with SIAH1/2 and recruits SIAH1 to mitochondria, which confirmed my findings. Additionally, I showed that SIAH3 and SIAH2, too, affect each other's subcellular localisation, diminishing SIAH3 occurrence at the mitochondria, and that the interactions depend on SIAH3's substrate binding domain (SBS/DIMER). I also demonstrated the N-terminal S3UNS domain to be responsible for the mitochondrial localisation of SIAH3, which is in line with the findings of Abd Elghani *et al.*'s study that identified the first 30 amino acids to be responsible for mitochondrial localisation in the outer membrane (Abd Elghani *et al.*, 2022). Besides the heterodimerisation with SIAH1 and SIAH2, I found SIAH3 to form homodimers. For SIAH1 and SIAH2 homo- and heterodimerisation has already been described (Polekhina *et al.*, 2002; Depaux *et al.*, 2006). Depaux *et al.* (2006) demonstrated SIAH2 to regulate the steady-state level of itself as well as the level of SIAH1 through dimerisation, depending on the RING domain. And House *et al.* (2006) showed SIAH to bind to its substrate in a dimeric form. Since SIAH1 and 2 are E3 ubiquitin ligases, they mediate the proteasomal degradation of a number of substrates, for example PHDs (Nakayama *et al.*, 2004), Axin (Ji *et al.*, 2017) and HIPK2 (Calzado *et al.*, 2009). I further showed that SIAH3 as well undergoes proteasomal degradation induced by SIAH2 and SIAH1. The degradation depended, on the one hand, on SIAH1/2's functional RING domain and, on the other hand, on the presence of the binding domains of SIAH3. While elimination of the SZF and SBS domains (and S3UNS for the effect mediated by SIAH1) abolished the degradation, eliminating the DIMER domain even resulted in an increase of SIAH3 protein levels. And since the SBS and DIMER domain are also relevant for the protein interaction, this suggests that binding between SIAH1/2 and SIAH3 is necessary for promoting the degradation, presumably to transfer ubiquitin to SIAH3. House *et al.* (2003; 2006) described PxAxVxP as binding motif for SIAH. However, this consensus sequence does not occur in SIAH3. Only the most conserved VxP exists three times, but not within the SBS or DIMER domain, indicating that the binding to SIAH3 is not dependent on this special binding motif. Furthermore, deletion of none of the five lysines within the SIAH3 protein, nor the deletion of all lysines together, resulted in a reduced protein degradation. Next to the canonical lysine ubiquitylation, ubiquitin can also be bound to the N-terminal amino-group of a protein or to the nucleophilic amino acid side chains of cysteine, serine or threonine (Kellsall, 2022; Akizuki *et al.*, 2024). However, for SIAH1 and SIAH3 only the ubiquitination of lysine residues is known. For example, Calzado *et al.* (2009) described that mutation of the eleven lysines in the previously mapped relevant region impaired ubiquitination by SIAH2 and Chen *et al.* (2022) and

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Gao *et al.* (2022) showed that RPS3 is ubiquitinated at K214 and YBX1 at K204 lysine residues by SIAH1, respectively. This suggests that the binding and degradation of SIAH3 works differently to the common substrates of SIAH1/2 and the detailed mechanism is still to be revealed.

Thus, additionally to the regulation of *SIAH3* gene expression, SIAH3 is also controlled on protein level. Since increased levels of *SIAH2* are described in cancer, for example in lung carcinomas (Moreno *et al.*, 2015), degradation of SIAH3 by the proteasomal pathway may also contribute to the downregulation of SIAH3 during carcinogenesis.

(Parts of this chapter are adapted from Deutschmeyer *et al.*, 2025.)

4.4 SIAH3 may play a role in Hippo signalling via its interaction with LATS2

The first idea about the mechanism behind the tumour-suppressive function of SIAH3 referred to its missing RING domain and the resulting presumed lack of ligase activity while other protein regions are similar to SIAH1 and SIAH2. Maybe SIAH3 acts as a negative counterpart to SIAH1 and/or SIAH2 in their regulatory pathways. An important pathway, in which SIAH2 plays a role, is the Hippo signalling (Ma *et al.*, 2015). The Hippo pathway is an evolutionary conserved regulator of organ size and tissue growth (Harvey *et al.*, 2013; Yu *et al.*, 2015). Its core consists of a kinase cascade that finally phosphorylates YAP and TAZ. Phosphorylated YAP and TAZ are thus retained in the cytoplasm and finally degraded by ubiquitin-mediated proteolysis, being therefore no longer able to activate transcription of their target genes (Harvey *et al.*, 2013). Two of the main players are LATS1 and 2. LATS2, the kinase that phosphorylates YAP, is destabilised by SIAH2 (Ma *et al.*, 2015), thus SIAH2 turns Hippo signalling off and YAP/TAZ can act as transcription coactivator. One experiment showed a stabilising effect of SIAH3 on LATS2 degradation, but this could not be confirmed in other experiments. Thus, it needs further investigation under which conditions SIAH3 influences LATS2 stability. Nevertheless, an interaction between SIAH3 and LATS2 was observed and overexpression of SIAH3 led to a shift of the YAP signal in WB. This small downshift may reflect a weak dephosphorylation, but this should be further analysed using phospho-specific antibodies for YAP. SIAH3 may inhibit LATS2 by binding to it, thereby preventing LATS2 from phosphorylating its substrates, which then leads to reduced phosphorylation of YAP. Through this potential dephosphorylation, YAP can enter the nucleus and activate transcription. This would hint towards the assumption that SIAH3 acts, likewise or maybe together with SIAH2, oncogenic. But besides the growth promoting function of YAP via the activation of CTGF (Zhao *et al.*, 2008) or cyclin D1 (Mizuno *et al.*, 2012), YAP has also been described to have a tumour suppressive function due to its binding to p73 and the activation of proapoptotic genes like PUMA (Matallanas *et al.*, 2007) and GADD45A (Wang *et al.*, 2018). Another Hippo target is the transcription factor ANKRD1 (Stein *et al.*, 2015), that is known as co-activator of p53 (Kojic *et al.*, 2010) and plays a role in apoptosis (Shen *et al.*,

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2015), but it has also been described to participate in tumour progression (Xu *et al.*, 2024). The microarray analysis in this study revealed *ANKRD1* as the second most upregulated gene that could also be verified by qPCR. Furthermore, *GADD45A* was found to be upregulated. In total, four Hippo targets were identified to be upregulated by SIAH3: *ANKRD1*, *GADD45A*, *CCN1* (*CYR61*) and *CCN2* (*CTGF*). While *GADD45A* is mostly described as tumour suppressor (Palomer *et al.*, 2024), *ANKRD1* and the matricellular proteins *CCN1* (Lau, 2011) and *CCN2* (Kubota & Takigawa, 2015) carry both oncogenic and tumour suppressive functions. Since the transcription factors, that YAP is binding, decide which target genes are induced, it would be interesting to identify YAP's binding partners after SIAH3 overexpression. Together with TEAD, the expression of pro-proliferative genes is induced whereas switching to p73 or RUNX as partner activates pro-apoptotic genes (Jho, 2018). Another decisive player is RASSF1A. RASSF1A enhances the interaction of MST1 with LATS1 that in turn phosphorylates YAP, allowing it to translocate to the nucleus, to associate with p73 and induce the transcription of apoptotic genes like *PUMA* (Matallanas *et al.*, 2007). Furthermore, Jiménez *et al.* (2017) described that RASSF1A counteracts the oncogenic function of YAP and that coexpression of RASSF1A and YAP induces the expression of *ANKRD1*, which then stabilises p53 by reducing the protein levels of MDM2. It is therefore possible that SIAH3 executes its tumour suppressive effect via modelling the Hippo pathway. To figure out how SIAH3 is involved in this complex regulatory process, next to the binding partners of YAP, SIAH3's interplay with RASSF1A and the resulting effect on further Hippo target genes should be analysed. Furthermore, the phosphorylation status and a potential subcellular translocation of YAP upon SIAH3 overexpression could give important information on the possible mechanism through which SIAH3 can execute its tumour suppressive function.

4.5 SIAH3 regulates cell metabolism by reducing OXPHOS-related proteins

Another possibility for how SIAH3 could exert its tumour-suppressive function derives from its involvement in cellular metabolism. The data suggest that SIAH3 shifts the cells from aerobic energy production towards glycolytic metabolism and that SIAH3 expression reduces the level of proteins involved in OXPHOS. As revealed by MS-based interactomic analysis, SIAH3 interacts with proteins involved in mitochondrial translation, including many of the mitoribosome. I hypothesise that these interactions may lead to the inhibition of the translation of proteins of the ETC. A closer look at proteins deregulated by SIAH3 revealed that mainly complex I, but also complexes III and IV, as well as the ATP synthase are negatively regulated. The mitochondrial DNA (mtDNA) encodes 13 proteins belonging to complexes I, III, IV and V, but no protein of complex II (Buneeva *et al.*, 2020). Thus, SIAH3 appears solely involved in the regulation of OXPHOS complexes that are partly encoded in mtDNA. However, SIAH3 not only reduced the levels of mtDNA encoded proteins, but also other

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ETC proteins of these complexes expressed from the nuclear DNA. Thus, additionally to mitochondrial translation, SIAH3 also appears to selectively influence cytoplasmic translation and/or protein stability. The fact that SIAH3 is localised in the mitochondria (see chapters 9.3.5 & 4.3 and Abd Elghani *et al.*, 2022) supports the proposition that SIAH3 is involved in cell respiration, since function and location match.

Interactome analyses further showed that SIAH3 interacts with several ubiquitin ligases. Thus, besides the interaction with mitoribosomes, the interaction with ubiquitin ligases could represent another mechanism to decrease the level of proteins involved in cellular respiration. Because SIAH3 lacks intrinsic ligase activity, it cannot catalyse ubiquitination on its own. However, several E3 RING ligases – including SIAH1/2 – are known to function as dimers (Depaux *et al.*, 2006; House *et al.*, 2006), and SIAH3 can bind to these proteins. Through such interactions, SIAH3 may act as a decoy molecule or an adaptor that initiates (or maybe inhibit) ubiquitination by recruiting or modulating the catalytic activity of other E3 ligases. And for SIAH1, microscopy analysis showed that it is indeed attracted by SIAH3 to the mitochondria. This was also published recently by Abd Elghani *et al.* (2022). Matching my results, they showed that SIAH3 promotes the translocation of SIAH1 to the mitochondria and prevents its ubiquitin-ligase activity towards PINK1. Additionally, SIAH3 interacts and aggregates with PINK1 in mitochondria and inactivates it. And Abraham *et al.* (2024) published, that (in mice) Siah3 forms complexes with parkin, a ubiquitin ligase and the second important player in mitophagy. Thereby SIAH3 impedes the elimination of dysfunctional mitochondria by mitophagy. Previously, however, Szargel *et al.* (2016) presented that SIAH1 is recruited to the mitochondria by synphilin-1 and can induce Parkin-independent mitophagy, letting assume that SIAH3 may recruit SIAH1 in a same manner. These findings propose different possibilities how SIAH3 could encroach in mitophagy, either increase or decrease it, but in both ways, affect mitochondrial homeostasis. SIAH2, the second binding partner of SIAH3, has been associated with mitochondria, too. Upregulation of Siah2, by modulation of the cellular levels of AKAP1, significantly affects mitochondrial activity assessed as mitochondrial membrane potential and oxidative capacity (Carlucci *et al.*, 2008). Furthermore, SIAH2 is involved in the regulation of mitochondrial fission and fusion via the degradation of NCX3 in hypoxic neurons, worsening mitochondrial oxidative capacity and ATP production (Sisalli *et al.*, 2020). From the group of identified ubiquitin ligases, STUB1 was the third protein verified to interact with SIAH3. The U-box dependent E3 ubiquitin ligase STUB1 was shown to interact with Parkin and to enhance its activity (Imai *et al.*, 2002). It is although thought to compensate for mutated Parkin, regulate mitochondrial morphology and is translocated to the mitochondria upon neurologic stress (Palubinsky *et al.*, 2015; Lizama *et al.*, 2018). Next to these ubiquitin ligases, the mitochondrial import proteins CHCHD4 and TIMM50 were identified as binding partners of SIAH3. TIMM50 is part of the TIMM23 complex, the main translocator through

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the inner mitochondrial membrane, recognising the N-terminal mitochondrial targeting signal of proteins to be imported (Guo *et al.*, 2004; Chaudhuri *et al.*, 2021). If the binding of SIAH3 to TIMM50 occurs only because of the import of itself or if SIAH3 regulates the function of TIM50 needs to be examined. The oxidoreductase CHCHD4 is a key component of the disulfide relay system, an import mechanism within the mitochondrial intermembrane space, that introduces intramolecular disulfide bonds into its substrates, thereby folding and stabilising them, functioning as “sorting hub” (Finger & Riemer, 2020; Al-Habib & Ashcroft, 2021). Among its substrates are subunits and assembly factors of complex I, III and IV of the ETC (Thomas *et al.*, 2019) – the same complexes that are downregulated after SIAH3 overexpression. And a study in zebrafish showed that a loss of *chchd4a* resulted in a reduced OCR (Sokol *et al.*, 2018). The interaction with and a possible blocking of CHCHD4 represents a promising mechanism through which SIAH3 may carry out its function. Thus, through the interaction with SIAH1/2 and STUB1 or with mitochondrial importers, further mechanisms come into play by which SIAH3 could influence mitochondrial function. The precise mechanisms behind are worth further examination.

Extending the published function of SIAH3 in PINK1 dependent mitochondrial homeostasis (Hasson *et al.*, 2013; Yoon *et al.*, 2021; Abd Elghani *et al.*, 2022), I now propose additional possible mechanisms underlying SIAH3’s involvement in mitochondrial function: SIAH3 could either affect mitochondrial translation through its interaction with mitoribosomes or mediate the import inside the mitochondria by its interaction with TIMM50 or CHCHD4, reducing the formation of proteins of the ETC complexes, or bind to and attract ubiquitin ligases to mitochondria. And these may directly degrade proteins involved in mitochondrial respiration or indirectly affect mitochondrial homeostasis, which in turn might result in disturbed mitochondrial function, leading to the observed effects on OXPHOS. However, the finding that neither the anti-proliferative effect (except the increase in dead cells) nor the metabolic shift from OXPHOS to glycolysis is (completely) dependent on the mitochondrial localisation of SIAH3, creates a puzzle. One explanation could be that SIAH3 may bind its interaction partners (e.g. the ubiquitin ligases like SIAH1/2, STUB1 or Parkin) not only near the mitochondria but also in the cytosol. For example, STUB1 mainly locates in the cytosol. There it forms a complex with HSP70, a chaperone involved in proper protein folding, linking it with the UPS (Liu *et al.*, 2018; Zhao *et al.*, 2023). Most mitochondrial proteins, including the ones of mitoribosomes, are encoded in nuclear genes and translated in the cytoplasm (Kim *et al.*, 2017). By acting through this translation mechanism, SIAH3 may influence mitochondrial function without the need to enter the mitochondria. Nonetheless, more research is needed to unravel the mystery of SIAH3’s mechanism of action and the responsibility of its domains.

Most available information about the SIAHs and mitochondria derives from studies in a neurological context, where mitochondrial dysfunction is a known contributor to the pathogenesis of diseases.

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For example, SIAH3 was found to be upregulated in Parkinson's disease brains and to promote proteotoxicity (Abd Elghani *et al.*, 2022) and that it is involved in the degeneration of sensory neuron axons by repressing mitophagy in order to regulate target tissue innervation (Abraham *et al.*, 2024).

But what are the consequences for tumorigenesis and how could the described effect of SIAH3 on cell proliferation be explained? Evading apoptosis is a key feature of cancer cells (Hanahan & Weinberg, 2000). And mitochondria play a pivotal role in the initiation of apoptosis. Many apoptotic signals converge on the mitochondria, leading to the release of Cytochrome C, which activates a caspase cascade that arranges the death of the cell (Green & Reed, 1998). Thus, I think microscopy imaging or WB analyses using fractionation of mitochondrial and cytosolic phase at different time points after SIAH3 transfection/induction could provide valuable information if regulation of this mechanism is part of SIAH3's tumour suppressive effect. The next thought that enters one's mind regarding metabolism and cancer is the Warburg effect. It describes the tendency of tumour cells to enhance glycolysis even in the presence of oxygen (Warburg & Minami, 1923; Warburg, 1925). The observed increase in glycolysis together with a decrease in OXPHOS upon SIAH3 overexpression resembles the Warburg effect, but in this case, it was associated with tumour suppressive characteristics, not oncogenic ones, as Warburg has described. And since the proteome analyses showed an effect of SIAH3 on mitochondrial respiration but not on proteins linked to glycolysis, I assume, that this effect could be a compensatory response to the decline in OXPHOS. And since upon SIAH3 overexpression OXPHOS was decreased to the same degree as glycolysis was increased, total ATP production stayed the same. Therefore, a mere shortage in energy supply does not explain the described growth inhibition, neither. Impairment of the ETC also results in an increase in ROS production (Badrinath & Yoo, 2018) and ROS can trigger apoptosis (Perillo *et al.*, 2020). As ETC is coupled with the TCA cycle, impaired ETC prevents TCA from going round and impedes proliferation due to a shortage of needed building blocks (Vasan *et al.*, 2020). These effects need further investigation but may explain the observed slowdown in cell growth and thereby the tumour suppressive effect of SIAH3 and why it is downregulated in cancer.

(Parts of this chapter are adapted from Deutschmeyer *et al.*, 2025.)

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4.6 Silenced *SIAH3* can be reactivated using epigenetic editing

SIAH3 transcription is suppressed by promoter methylation in different cancers, including melanoma, head and neck cancer and lung carcinomas. This epigenetic silencing prevents *SIAH3* from functioning as tumour suppressor. Therefore, it would be valuable to reactivate *SIAH3* in order to regain a protein that could help to oppose tumorigenesis.

Epigenetic editing via the CRISPR/dCas9 system combined with various effector domains produced the greatest transcriptional activation when p300 was used. DME, a novel demethylation enzyme, only resulted in a slight transcriptional activation and a small demethylation of CpG 5. Although the initial findings of this thesis revealed clear hypermethylation of the promoter, this additional data suggests that DNA methylation is not the sole mechanism mediating *SIAH3* silencing. The recruited catalytic core of the acetyltransferase p300 catalyses the acetylation of histone H3 lysine 27 (H3K27) at its target site (Hilton *et al.*, 2015). Histones are post-translational modified mainly by acetylation, methylation or phosphorylation. Acetylation neutralises the positive charge of lysines, weakening the interaction between histones and DNA, opening the chromatin, which leads to a better accessibility of the DNA, that enables, for example, the binding of transcription factors, and can therefore promote transcription (Bannister & Kouzarides, 2011; Nitsch *et al.*, 2021). Despite the unchanged DNA methylation indicated by pyrosequencing, *SIAH3* expression rises following p300 recruitment. This demonstrates that the actively introduced histone modification can enable transcription even in the presence of repressive DNA methylation. The same effect has been observed by Arroyo Villora *et al.* (2024) concerning the tumour suppressor RIPK3 and Raynal *et al.* (2012) showed that DNA methylation could not prevent gene reactivation by drug-induced resetting of the chromatin state. And vice-versa, Si *et al.* (2010) described that DNA hypomethylation alone is not always enough and additional chromatin remodelling is required for gene reactivation. Having a look at the UCSC Genome Browser and the ChIP-seq information from around the *SIAH3* TSS site shows that there are nearly no peaks for H3H27 acetylation, but high signals for H3K27 trimethylation, with H3K27 trimethylation being a mark associated with repressed promoters (Black *et al.*, 2012). These pieces of information suggest that the *SIAH3* promoter is silenced by DNA methylation as well as by histone modification. Raynal *et al.* (2012) described furthermore that gene reactivation via HDAC inhibitors (leading to active chromatin) was detectable for only a few weeks, whereas DNA demethylation drugs induced a permanent change. Thus, further experiments that combine histone modifying enzymes – such as p300 – with (subsequent) DNA demethylation by DME or TET1 could be successful in the reactivation of *SIAH3* in a sustained manner.

Moreover, this work presents that the best position for the sgRNA to recruit p300 lies around 170 bp upstream of the TSS, which matches with the best position for targeting ZAR1

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(being -130 bp), what we published before (Deutschmeyer *et al.*, 2019) and that the best activation occurs after 120 h of overexpression. Even though reactivation through DNA demethylation was not yet highly effective and requires refinement, these data provide important information about the experimental CRISPR/dCas9 procedure and indicate that reactivation of silenced *SIAH3* is possible, encourage the notion that it is worthwhile to keep working on it. And since it is now known that *SIAH3* is additionally controlled on protein level by the other two family members, it would be promising to simultaneously epigenetically silence *SIAH1* and *SIAH2* to inhibit proteasomal degradation of the *SIAH3* protein. These achievements could finally help restore the tumour-suppressive effect that *SIAH3* is able to mediate.

4.7 Conclusion

In this work I described that *SIAH3* is hypermethylated in melanoma, lung cancer and head and neck tumours and its higher expression is correlated with improved patient survival. Furthermore, *SIAH3* induction reduces cell proliferation and induced cell death. In addition to the epigenetic silencing via DNA methylation, *SIAH3* expression is regulated by cell density and hypoxia. And a yet another control mechanism occurs on protein level, where *SIAH3* is degraded via the UPS mediated by *SIAH1* and *SIAH2*. Even though the detailed process behind its anti-tumour function needs further investigation, the experimental data suggest different mechanisms. A first option derives from its interaction with *LATS2* and the transcriptional upregulation of *ANKRD1* and *GADD45A*, showing an involvement of *SIAH3* Hippo signalling, an important pathway that controls organ size and tissue growth. Secondly, I showed *SIAH3* to localise in mitochondria and to be involved in metabolism. I propose *SIAH3* to influence mitochondrial function by negatively regulating proteins involved in mitochondrial respiration. My experiments suggest that *SIAH3* inhibits (mitochondrial) translation, possibly through interaction with mitoribosomes or ubiquitin ligases, which may, in turn, degrade proteins involved in translation or OXPHOS subunits directly. *SIAH3*s interaction with mitochondrial importers could present another option to reduce the level of the OXPHOS subunits, leading to a reduction of cellular respiration. And the negative regulation of mitochondrial function interferes with cell growth, which may be the mechanism by which *SIAH3* suppresses proliferation. And with the aim to regain this tumour suppressive function, first experiments using epigenetic editing were done and showed that it is in principle possible to epigenetically reactivate *SIAH3* by recruiting p300 or DME. (Parts of this paragraph are adapted from Deutschmeyer *et al.*, 2025.)

A schematic overview integrating the new findings and assumptions is shown in Fig. 56.

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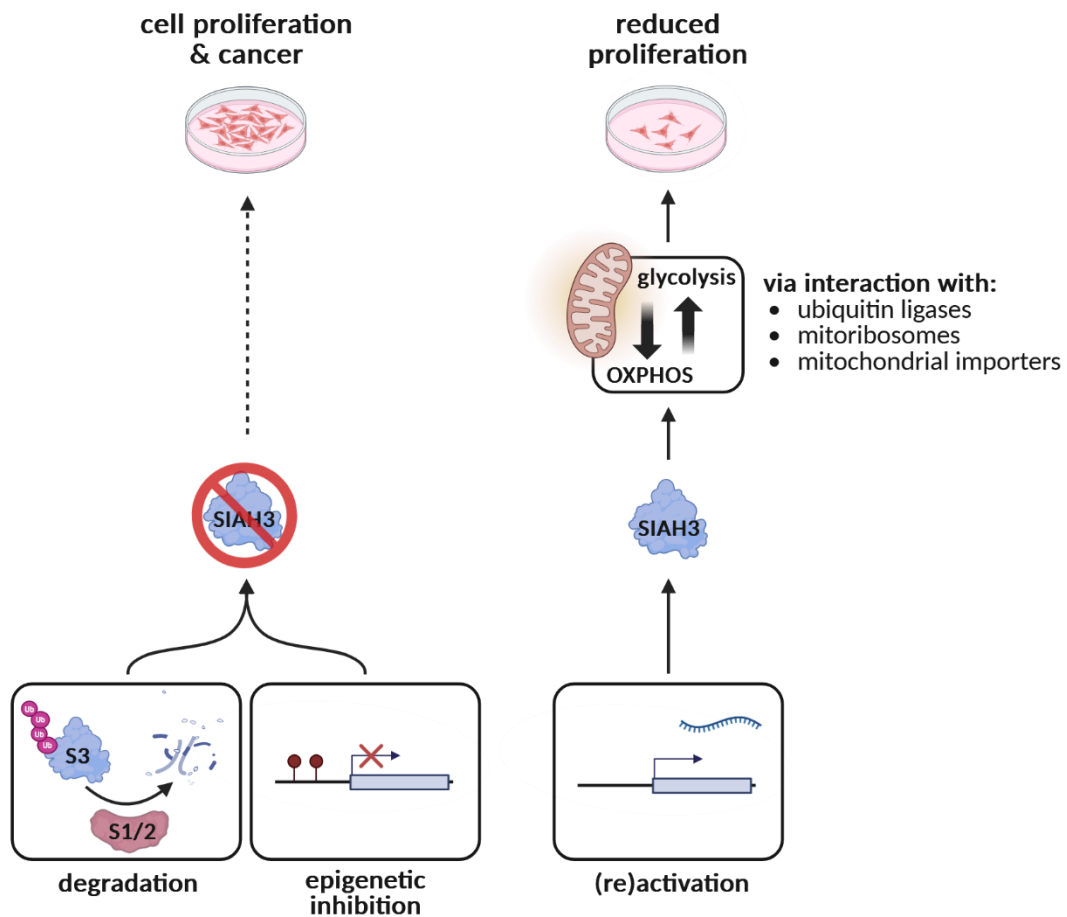


Fig. 56: Proposed model of the regulation and function of SIAH3.

SIAH3 (S3) is controlled both by epigenetic inhibition and proteasomal degradation mediated by SIAH1 and SIAH2 (S1/2). Reduced *SIAH3* expression was found in several cancer entities. And overexpression of SIAH3 shifts cells from OXPHOS to glycolysis and reduces cell proliferation.

Coming back to the hallmarks of cancer (Hanahan & Weinberg, 2000, 2011; Hanahan, 2022), I can now say that SIAH3 is involved in different aspects that characterise the nature of tumours. Its hypermethylation in different cancer types makes *SIAH3* a victim of the cancer cell's "nonmutational epigenetic reprogramming". By reducing cellular respiration, SIAH3 affects the hallmark of "deregulated cellular metabolism". And since SIAH3 reduces cell growth and increased the number of dead cells, it impedes the cancer cell's ability to "resist cell death and sustain chronic proliferation". These features characterise SIAH3 as a potential tumour suppressor whose role in tumorigenesis is worth further exploration.

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5 REFERENCES

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6 SUPPLEMENTARY MATERIAL

6.1 Sequences and plasmid maps

6.1.1 SIAH3 sequences

Protein sequence of SIAH3 (269 amino acids (aa))

MLFFTQCFCGAVLDLIHLRFQHYKAKRVFSAAGQLVCVNP^THN^LKYVSSRRRAVTQSAPEQGSFHPHLSHHHC
HHRHHHHLRHHAPHHLHHQEAGLHANPVT^PCLCM^PLFSCQWEGRLVVPHLRQIHR^VDILQGA^EIVFLA^T
TDMHLPAPADW^IIMHSLGHHFLLVLR^KQERHEGHPQFFATMMLIGTPTQAD^CFTYRLELN^RNHRR^LKWEAT^R
PRSVLECVDSVITDGDCLV^LNTSLAQLFSDNGSLAIGIAITATEVLPSEAEM

DNA sequence of SIAH3 (819 bp)

ATGCTTTTCTTTACCCAGTGCTTTGGGGCTGTATTAGATCTCATTCTCTCCGGTTTCAGCACTAC^{AAGGCT}^{AAA}CGGG
TTTTCTCCGCTGCCGGGCAACTTGTCTGTGTCGTAACCCACACACAACCTA^{AAG}TATGTGTCCAGTCGGCGCGCCGT
CACTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACCCTCACCATCTCTCCACCACCACTGCCACCACCGCCACCACCAC
CACCTCCGCCACCACGCCCCACCCACCCACCTTCACCACCAGGAGGCGGGGCTGCACGCCAACCCGGTGAC
GCCCTGCCTGTGCATGTGTCCCTTGTCTCCTGCCAGTGGGAAGGCCGCTGGAGGTGGTGGTGCCCCACC
TGCGGCAGATCCATAGG^{GTT}GACATCCTCCAGGGAGCCGAGATCGTCTTCTTGCCACGGACATGCACCTC
CCCGCGCCGGCTGATTGGATCATCATGCACTCCTGCCTTGGCCACCACTTTCTGTTGGTGCTGAGG^{AAACA}
GGAGAGGCATGAAGGGCACCCCCAGTTCTTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGCCGACT
GCTTCACCTATCGCCTGGAGCTCAACAGAAACCATCGGCGCCTC^{AAG}TGGGAGGCCACGCCCGGTCTGTT
CTTGAGTGCCTGGACTCGGTGATTACGGACGGGACTGCCTCGTCCTCAACACCTCGCTGGCACAGCTCTT
CTCTGACAACGGCAGCCTTGCCATTGGGATTGCCATCACCGCGACAGAGGTCCTCCCTCAGAAGCTGAAA
TGTGA

magenta: S3UNS domain

purple: Poly-His domain

blue: SZF domain

red: SBS domain

green: DIMER domain

K: potential ubiquitination sites

(domains after Pepper *et al.*, 2017 and Zhang *et al.*, 2017)

SUPPLEMENTARY MATERIAL

6.1.2 SIAH3 plasmids

6.1.2.1 pEYFP-C2 constructs

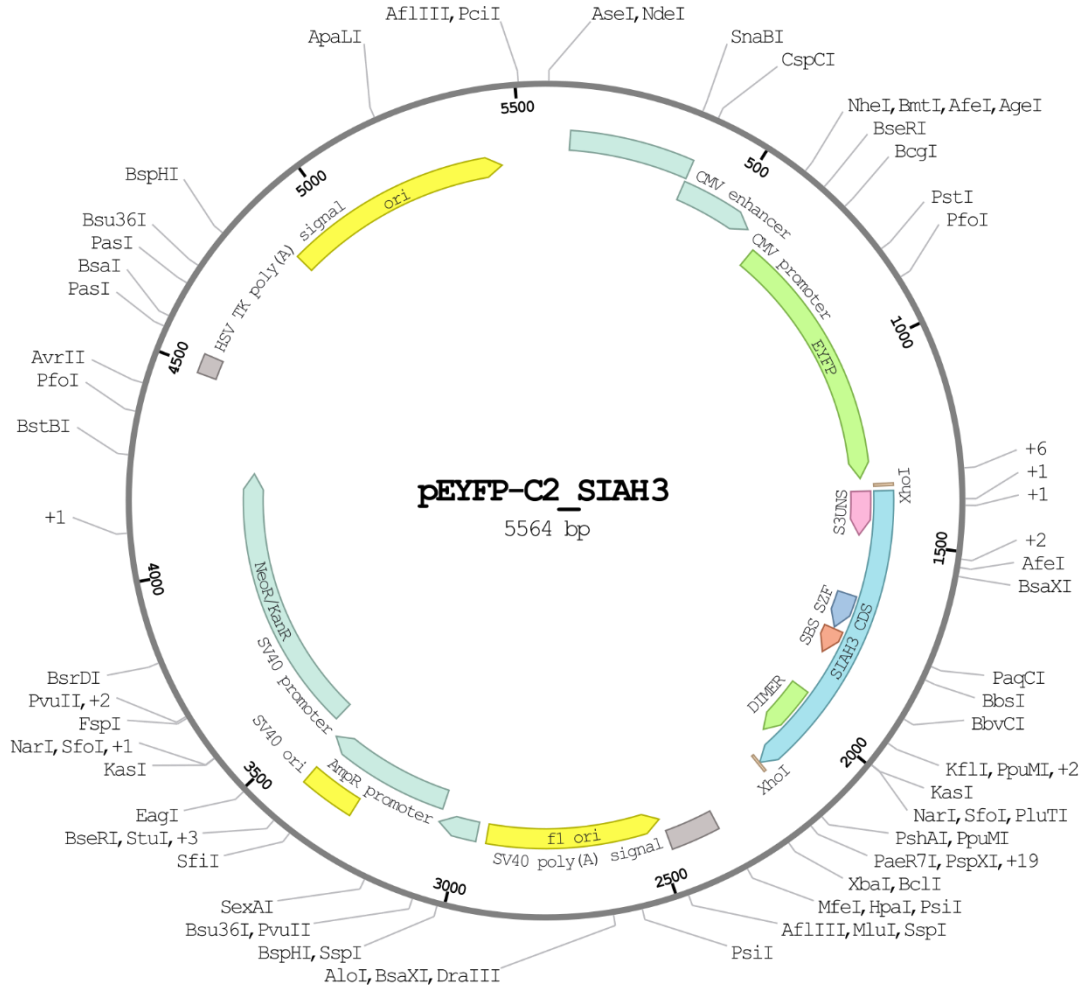


Fig. S1: pEYFP-C2 vector containing SIAH3.

Insert sequences of the different constructs (cloned via *XhoI* – *XhoI*):

SIAH3 Wt

CTCGAGTTTTCAGCAATTCATGCTTTCTTTACCCAGTGCTTTGGGGCTGTATTAGATCTCATTATCTCCGG
 TTTCAGCACTACAAGGCTAAACGGGTTTCTCCGCTGCCGGGCAACTTGCTGTGTGTCGCAACCCACA
 CAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCCTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACCCT
 CACCATCTCTCCACCACCACTGCCACCACCGCCACCACCACCTCCGCCACCACGCCACCCCAACCCAC
 CTTACCAACAGGAGGCGGGGCTGCACGCCAACCCGGTGACGCCCTGCTGTGCATGTGTCCCTTGTTCTC
 CTGCCAGTGGAAGGCCGCTGGAGGTGGTGGTGCCCACTGCGGCAGATCCATAGGGTTGACATCCT
 CCAGGGAGCCGAGATCGTCTTCTGCGCACGGACATGCACCTCCCCGCGCCGGCTGATTGGATCATCATG
 CACTCCTGCCTTGGCCACCACTTTCTGTTGGTGCTGAGGAAACAGGAGAGGCATGAAGGGCACCCCCAGT
 TCTTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGCCGACTGCTTACCTATCGCTGGAGCTCAAC
 AGAAACCATCGGCGCCTCAAGTGGGAGGCCACGCCCGGTCTGTTCTTGAGTGGTGGACTCGGTGATT
 ACGGACGGGGACTGCCTCGTCTCAACACCTCGCTGGCACAGCTTTCTCTGACAACGGCAGCCTTGCCAT
 TGGGATTGCCATACCGCGACAGAGGTCTCCCTCAGAAGCTGAAATGTGACTCGAG

CTCGAG *XhoI* restriction site

GAATTC: *EcoRI* restriction site (modified pJet1.2 sequence, originally GCAAGA)

CTCGAGTTTTCAGCAAGA: fragment of pJet1.2

SUPPLEMENTARY MATERIAL

SIAH3 Δ S3UNS

CTCGAGTTTTCAGAATTCACAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCACTCAGAGCGCTCCAG
AGCAAGGCAGCTTCCACCCTCACCATCTCTCCACCACCACTGCCACCACCGCCACCACCACCTCCGCC
ACCACGCCCCACCCACCTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGTGACGCCCTGCCT
GTGCATGTGTCCCTTGTCTCCTGCCAGTGGGAAGGCCGCTGGAGGTGGTGGTGCCCCACCTGCGGCAG
ATCCATAGGGTTGACATCCTCCAGGGAGCCGAGATCGTCTTCTGGCCACGGACATGCACCTCCCCGCGC
CGGCTGATTGGATCATCATGCACTCCTGCCTTGCCACCACCTTTCTGTTGGTGGTGGAGGAAACAGGAGAG
GCATGAAGGGCACCCCCAGTTCTTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGCCGACTGCTTC
ACCTATCGCCTGGAGCTCAACAGAAACCATCGGCGCCTCAAGTGGGAGGCCACGCCCCGGTCTGTTCTTG
AGTGCCTGGACTCGGTGATTACGGACGGGGACTGCCTCGTCTCAACACCTCGCTGGCACAGCTCTTCTC
TGACAACGGCAGCCTTGCCATTGGGATTGCCATCACCGCGACAGAGGTCTCCCCTCAGAAGCTGAAATG
TGA

SIAH3 Δ SZF

CTCGAGTTTTCAGAATTCATGCTTTTCTTACCAGTGCTTTGGGGCTGTATTAGATCTCATTATCTCCGG
TTTCAGCACTACAAGGCTAAACGGGTTTTCTCCGCTGCCGGGCAACTTGCTGTGTCTCAACCCACACA
CAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCACTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACCCT
CACCATCTCTCCACCACCACTGCCACCACCGCCACCACCACCTCCGCCACCACGCCCCACCCACAC
CTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGTGACGGTTGACATCCTCCAGGGAGCCGAGATC
GTCTTCTGGCCACGGACATGCACCTCCCCGCGCCGGCTGATTGGATCATCATGCACTCCTGCCTTGCCA
CCACTTTCTGTTGGTGGTGGAGGAAACAGGAGAGGCATGAAGGGCACCCCCAGTTCTTTGCCACCATGATG
CTGATTGGGACCCCCACCCAGGCCGACTGCTTCACCTATCGCCTGGAGCTCAACAGAAACCATCGGCGCC
TCAAGTGGGAGGCCACGCCCCGGTCTGTTCTTGAGTGCGTGGACTCGGTGATTACGGACGGGGACTGCC
TCGTCTCAACACCTCGCTGGCACAGCTCTTCTTGACAACGGCAGCCTTGCCATTGGGATTGCCATCACC
GCGACAGAGGTCTCCCCTCAGAAGCTGAAATGTGA

SIAH3 Δ SBS

CTCGAGTTTTCAGAATTCATGCTTTTCTTACCAGTGCTTTGGGGCTGTATTAGATCTCATTATCTCCGG
TTTCAGCACTACAAGGCTAAACGGGTTTTCTCCGCTGCCGGGCAACTTGCTGTGTCTCAACCCACACA
CAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCACTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACCCT
CACCATCTCTCCACCACCACTGCCACCACCGCCACCACCACCTCCGCCACCACGCCCCACCCACAC
CTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGTGACGCCCTGCTGTGTCTCAACCCACACA
CTGCCAGTGGGAAGGCCGCTGGAGGTGGTGGTGCCCCACCTGCGGCAGATCCATAGGATCATCATGCA
CTCCTGCCTTGCCACCACCTTTCTGTTGGTGGTGGAGGAAACAGGAGAGGCATGAAGGGCACCCCCAGTTC
TTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGCCGACTGCTTCACCTATCGCCTGGAGCTCAACAG
AAACCATCGGCGCCTCAAGTGGGAGGCCACGCCCCGGTCTGTTCTTGAGTGCGTGGACTCGGTGATTACG
GACGGGGACTGCCTCGTCTCAACACCTCGCTGGCACAGCTCTTCTTGACAACGGCAGCCTTGCCATTGG
GATTGCCATCACCGCGACAGAGGTCTCCCCTCAGAAGCTGAAATGTGA

SIAH3 Δ DIMER

CTCGAGTTTTCAGAATTCATGCTTTTCTTACCAGTGCTTTGGGGCTGTATTAGATCTCATTATCTCCGG
TTTCAGCACTACAAGGCTAAACGGGTTTTCTCCGCTGCCGGGCAACTTGCTGTGTCTCAACCCACACA
CAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCACTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACCCT
CACCATCTCTCCACCACCACTGCCACCACCGCCACCACCACCTCCGCCACCACGCCCCACCCACAC
CTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGTGACGCCCTGCTGTGTCTCAACCCACACA
CTGCCAGTGGGAAGGCCGCTGGAGGTGGTGGTGCCCCACCTGCGGCAGATCCATAGGTTGACATCCT
CCAGGGAGCCGAGATCGTCTTCTGGCCACGGACATGCACCTCCCCGCGCCGGCTGATTGGATCATCATG
CACTCCTGCCTTGCCACCACCTTTCTGTTGGTGGTGGAGGAAACAGGAGAGGCATGAAGGGCACCCCCAGT
TCTTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGCCCTTCTTGACAACGGCAGCCTTGCCATTGG
ATTGCCATCACCGCGACAGAGGTCTCCCCTCAGAAGCTGAAATGTGA

SIAH3 Δ bind (Δ SBS+ Δ DIMER)

CTCGAGTTTTCAGAATTCATGCTTTTCTTACCAGTGCTTTGGGGCTGTATTAGATCTCATTATCTCCGG
TTTCAGCACTACAAGGCTAAACGGGTTTTCTCCGCTGCCGGGCAACTTGCTGTGTCTCAACCCACACA

SUPPLEMENTARY MATERIAL

CAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCCTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACCCT
CACCATCTCTCCACCACCACTGCCACCACCGCCACCACCACCACTCCGCCACCACGCCACCCCCACCAC
CTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGTGACG**CCCTGCCTGTGCATGTGTCCCTTGTTCTC**
CTGCCAGTGGGAAGGCCGCTGGAGGTGGTGGTGCCCCACCTGCGGCAGATCCATAGGATCATCATGCA
CTCCTGCCTTGCCACCACCTTTCTGTTGGTGCTGAGGAAACAGGAGAGGCATGAAGGGCACCCCCAGTTC
TTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGCCTTCTCTGACAACGGCAGCCTTGCCATTGGGAT
TGCCATCACCGCGACAGAGGTCTCCCTCAGAAGCTGAAATGTGA**CTCGAG**

SIAH3 Ubi1-5 (potential ubiquitination sites: lysine to arginine)

CTCGAGTTTTTCAG**GAATTC**ATGCTTTTCTTTACCCAGTGCTTTGGGGCTGTATTAGATCTCATTATCTCCGG
TTTCAGCACTAC**AGGGCTAGA**CGGGTTTTCTCCGCTGCGGGCAACTTGCTGTGTCTGTCACCCCCACA
CAACCTA**AGG**TATGTGTCCAGTCGGCGCGCCGTCCTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACCCT
CACCATCTCTCCACCACCACTGCCACCACCGCCACCACCACCACTCCGCCACCACGCCACCCCCACCAC
CTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGTGACG**CCCTGCCTGTGCATGTGTCCCTTGTTCTC**
CTGCCAGTGGGAAGGCCGCTGGAGGTGGTGGTGCCCCACCTGCGGCAGATCCATAGG**GTTGACATCCT**
CCAGGGAGCCGAGATCGTCTTCTGCGCACGGACATGCACCTCCCCGCGCCGGCTGATTGGATCATCATG
CACTCCTGCCTTGCCACCACCTTTCTGTTGGTGCTGAGG**AGA**CAGGAGAGGCATGAAGGGCACCCCCAGT
TCTTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGCC**GACTGCTTACCTATCGCTGGAGCTCAAC**
AGAAACCATCGGCGCCTCAGGTGGGAGGCCACGCCCGGTCTGTTCTTGAGTGCGTGGACTCGGTGATT
ACGGACGGGGACTGCCTCGTCCTCAACACCTCGCTGGCACAGCTTTCTCTGACAACGGCAGCCTTGCCAT
TGGGATTGCCATCACCGCGACAGAGGTCTCCCTCAGAAGCTGAAATGTGA**CTCGAG**

Ubi 1: A68G -> K23R (AAG -> AGG)

Ubi 2: A74G -> K25R (AAA -> AGA)

Ubi 3: A134G -> K45R (AAG -> AGG)

Ubi 4: A518G -> K173R (AAA -> AGA)

Ubi 5: A638G -> K213R (AAG -> AGG)

Ubi 1+2: A68G + A74G

Ubi 1-3: A68G + A74G + A134G

Ubi 1-4: A68G + A74G + A134G + A518G

Ubi 1-5: A68G + A74G + A134G + A518G + A638G

SUPPLEMENTARY MATERIAL

6.1.2.3 pCMV-Tag1 construct

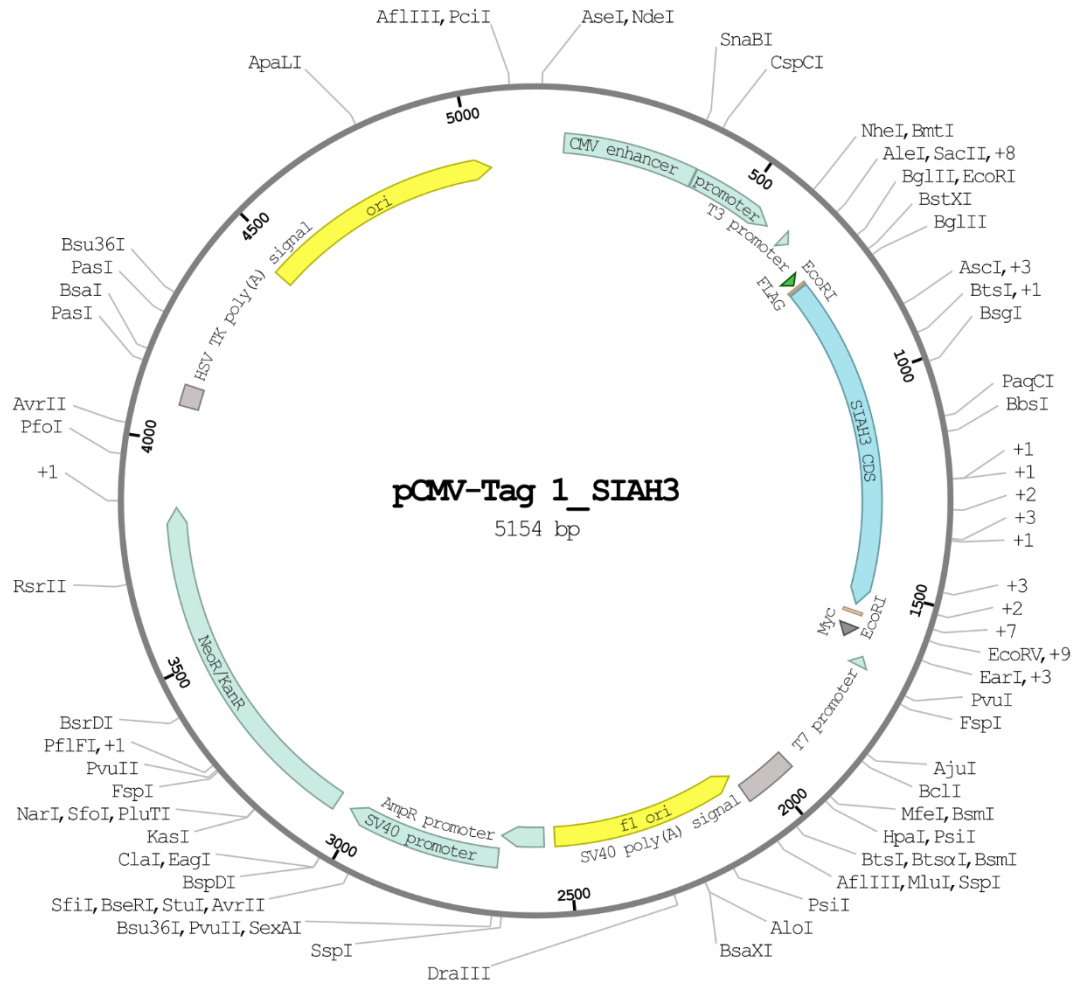


Fig. S3: pCMV-Tag1 vector (mod. by A. Richter) containing SIAH3. FLAG N-terminal in frame with SIAH3. No open reading frame with myc.

Insert sequence SIAH3 Wt (cloned via *EcoRI* – *EcoRI*)

GAATTCATGCTTTCTTTACCCAGTGCTTGGGGCTGTATTAGATCTCATTCACTCCGGTTTCAGCACTAC
AAGGCTAAACGGGTTTTCTCCGCTGCCGGGCAACTTGCTGTGTCGTCACCCACACACAACCTAAAGT
ATGTGTCCAGTCGGCGCGCCGCTCACTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACCCTCACCATCTCTCC
CACCACCACTGCCACCACCGCCACCACCACCTCCGCCACCACGCCACCCCAACCTTACCACCAG
GAGGCGGGGCTGCACGCCAACCCGGTGACGCCCTGCTGTGCATGTGTCCCTTGTCTCTGCCAGTGGG
AAGGCCGCTGGAGGTGGTGGTGCCCCACCTGCGGCAGATCCATAGGGTTGACATCTCCAGGGAGCCG
AGATCGTCTTCTGGCCACGGACATGCACCTCCCCGCGCCGGCTGATTGGATCATCATGCACTCTGCCTT
GGCCACCACTTTCTGTTGGTGTGCTGAGGAAACAGGAGAGGCATGAAGGGCACCCCAAGTTCTTTGCCACC
ATGATGCTGATTGGGACCCCAACCCAGGCCGACTGCTTACCTATCGCTGGAGCTCAACAGAAACCATC
GGCGCTCAAGTGGGAGGCCACGCCCGGTCTGTTCTTGAGTGCGTGGACTCGGTGATTACGGACGGGG
ACTGCTCTGCTCTCAACACCTCGCTGGCACAGCTTCTCTGACAACGGCAGCCTTGCCATTGGGATTGCC
ATCACCGCGACAGAGGTCTCCCTCAGAAGCTGAAATGTGACTCGAGCTCAAGCTTCGAATTC

GAATTC: *EcoRI* restriction site

CTCGAGCTCAAGCTTC: fragment of pEYFP-C2

SUPPLEMENTARY MATERIAL

SIAH3 ΔS3UNS

GGATCC ACCATG GATTACAAGGATGACGACGATAAG ATCTTAGAATTC CACAACCTAAAGTATGTGTCCA
GTCGGCGCGCCGTCACTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACCCTCACCATCTCTCCACCACCAC
TGCCACCACCGCCACCACCACCACCTCCGCCACCACGCCCCACCACCTTACCACCAGGAGGCGGG
GCTGCACGCCAACCCGGTGACG CCTGCGCTGTGCATGTGTCCCTGTTCTCTGCCAGTGGGAAGGCCG
CTGGAGGTGGTGGTGCCCCACCTGCGGCAGATCCATAGG GTTGACATCCTCCAGGGAGCCGAGATCGTC
TTCTGGCCACGGACATGCACCTCCCCGCGCCGGCTGATTGG ATCATCATGCACTCCTGCCTTGCCACCA
CTTTCTGTTGGTGCTGAGGAAACAGGAGAGGCATGAAGGGCACCCCCAGTTCTTGCCACCATGATGCTG
ATTGGGACCCCCACCCAGGCC GACTGCTTCACCTATCGCCTGGAGCTCAACAGAAACCATCGGCGCCTCA
AGTGGGAGGCCACGCCCCGGTCTGTTCTTGAGTGCGTGGACTCGGTGATTACGGACGGGGACTGCCTCG
TCCTCAACACCTCGCTGGCACAGCTCTTCTCTGACAACGGCAGCCTTGCCATTGGGATTGCCATCACCGCG
ACAGAGGTCTCCCTCAGAAGCTGAAATGTGA CTCGAGCTCAAGCTTCGAATTCGATATCAAGCTTATCG
ATACCGTCGACCTCGAGCAGAAACTCATCTCTGAAGAGGATCTGTA GGTACC

SIAH3 ΔSZF

GGATCC ACCATG GATTACAAGGATGACGACGATAAG ATCTTAGAATTC ATGCTTTTCTTTACCCAGTGCTT
TGGGGCTGTATTAGATCTCATTATCTCCGGTTTCAGCACTACAAGGCTAAACGGGTTTTCTCCGCTGCCG
GGCAACTTGTCTGTGTCTCAACCCCA CACAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCACTCA
GAGCGCTCCAGAGCAAGGCAGCTTCCACCCTCACCATCTCTCCACCACCCTGCCACCACCGCCACCACC
ACCACCTCCGCCACCACGCCCCACCCACCACCTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGT
GACG GTTGACATCCTCCAGGGAGCCGAGATCGTCTTCTGGCCACGGACATGCACCTCCCCGCGCCGGCT
GATTGG ATCATCATGCACTCCTGCCTTGCCACCACCTTTCTGTTGGTGCTGAGGAAACAGGAGAGGCATG
AAGGGCACCCCCAGTTCTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGCC GACTGCTTCACCTAT
CGCCTGGAGCTCAACAGAAACCATCGGCGCCTCAAGTGGGAGGCCACGCCCCGGTCTGTTCTTGAGTGC
GTGGACTCGGTGATTACGGACGGGGACTGCCTCGTCTCAACACCTCGCTGGCACAGCTCTTCTCTGACA
ACGGCAGCCTTGCCATTGGGATTGCCATCACCGCGACAGAGGTCTCCCTCAGAAGCTGAAATGTGA C
TCGAGCTCAAGCTTCGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGCAGAAACTCATCTCTGAA
GAGGATCTGTA GGTACC

SIAH3 ΔSBS

GGATCC ACCATG GATTACAAGGATGACGACGATAAG ATCTTAGAATTC ATGCTTTTCTTTACCCAGTGCTT
TGGGGCTGTATTAGATCTCATTATCTCCGGTTTCAGCACTACAAGGCTAAACGGGTTTTCTCCGCTGCCG
GGCAACTTGTCTGTGTCTCAACCCCA CACAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCACTCA
GAGCGCTCCAGAGCAAGGCAGCTTCCACCCTCACCATCTCTCCACCACCCTGCCACCACCGCCACCACC
ACCACCTCCGCCACCACGCCCCACCCACCACCTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGT
GACG CCTGCGCTGTGCATGTGTCCCTGTTCTCTGCCAGTGGGAAGGCCGCTGGAGGTGGTGGTGCC
CACCTGCGGCAGATCCATAGG ATCATCATGCACTCCTGCCTTGCCACCACCTTTCTGTTGGTGCTGAGGAA
ACAGGAGAGGCATGAAGGGCACCCCCAGTTCTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGC
CGACTGCTTCACCTATCGCCTGGAGCTCAACAGAAACCATCGGCGCCTCAAGTGGGAGGCCACGCCCCGG
TCTGTTCTTGAGTGCGTGGACTCGGTGATTACGGACGGGGACTGCCTCGTCTCAACACCTCGCTGGCAC
AGCTCTTCTCTGACAACGGCAGCCTTGCCATTGGGATTGCCATCACCGCGACAGAGGTCTCCCTCAGA
AGCTGAAATGTGA CTCGAGCTCAAGCTTCGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGCAG
AAACTCATCTCTGAAGAGGATCTGTA GGTACC

SIAH3 ΔDIMER

GGATCC ACCATG GATTACAAGGATGACGACGATAAG ATCTTAGAATTC ATGCTTTTCTTTACCCAGTGCTT
TGGGGCTGTATTAGATCTCATTATCTCCGGTTTCAGCACTACAAGGCTAAACGGGTTTTCTCCGCTGCCG
GGCAACTTGTCTGTGTCTCAACCCCA CACAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCACTCA
GAGCGCTCCAGAGCAAGGCAGCTTCCACCCTCACCATCTCTCCACCACCCTGCCACCACCGCCACCACC
ACCACCTCCGCCACCACGCCCCACCCACCACCTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGT
GACG CCTGCGCTGTGCATGTGTCCCTGTTCTCTGCCAGTGGGAAGGCCGCTGGAGGTGGTGGTGCC
CACCTGCGGCAGATCCATAGG GTTGACATCCTCCAGGGAGCCGAGATCGTCTTCTGGCCACGGACATGC
ACCTCCCCGCGCCGGCTGATTGG ATCATCATGCACTCCTGCCTTGCCACCACCTTTCTGTTGGTGCTGAGG

SUPPLEMENTARY MATERIAL

AAACAGGAGAGGCATGAAGGGCACCCCCAGTTCTTTGCCACCATGATGCTGATTGGGACCCCCACCCAG
GCCTTCTCTGACAACGGCAGCCTTGCCATTGGGATTGCCATCACC GCGACAGAGGTCCTCCCTCAGAAG
CTGAAATGTGA CTCGAGCTCAAGCTTCGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGCAGAA
ACTCATCTCTGAAGAGGATCTGTA **GGTACC**

SIAH3 Δ bind (Δ SBS+ Δ DIMER)

GGATCC ACCATG **GATTACAAGGATGACGACGATAAG** ATCTTAGAATTC **ATGCTTTTCTTTACCCAGTGCTT**
TGGGGCTGTATTAGATCTCATTATCTCCGGTTTCAGCACTACAAGGCTAAACGGGTTTTCTCCGCTGCCG
GGCAACTTGTCTGTGTCTCAACCCCA CACAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCCTCA
GAGCGCTCCAGAGCAAGGCAGCTTCCACCCTCACCATCTCTCCACCACCACTGCCACCACCGCCACCACC
ACCACCTCCGCCACCACGCCACCCCCACCACCTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGT
GACG **CCCTGCCTGTGCATGTGTCCCTTGTCTCCTGCCAGTGGGAAGGCCGCTGGAGGTGGTGGTGCCC**
CACCTGCGGCAGATCCATAGG ATCATCATGCACTCTGCCTTGGCCACCACTTTCTGTTGGTGCTGAGGAA
ACAGGAGAGGCATGAAGGGCACCCCCAGTTCTTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGC
CTTCTCTGACAACGGCAGCCTTGCCATTGGGATTGCCATCACC GCGACAGAGGTCCTCCCTCAGAAGCT
GAAATGTGA CTCGAGCTCAAGCTTCGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGCAGAAAC
TCATCTCTGAAGAGGATCTGTA **GGTACC**

6.1.2.5 pcDNA3-mCherry construct

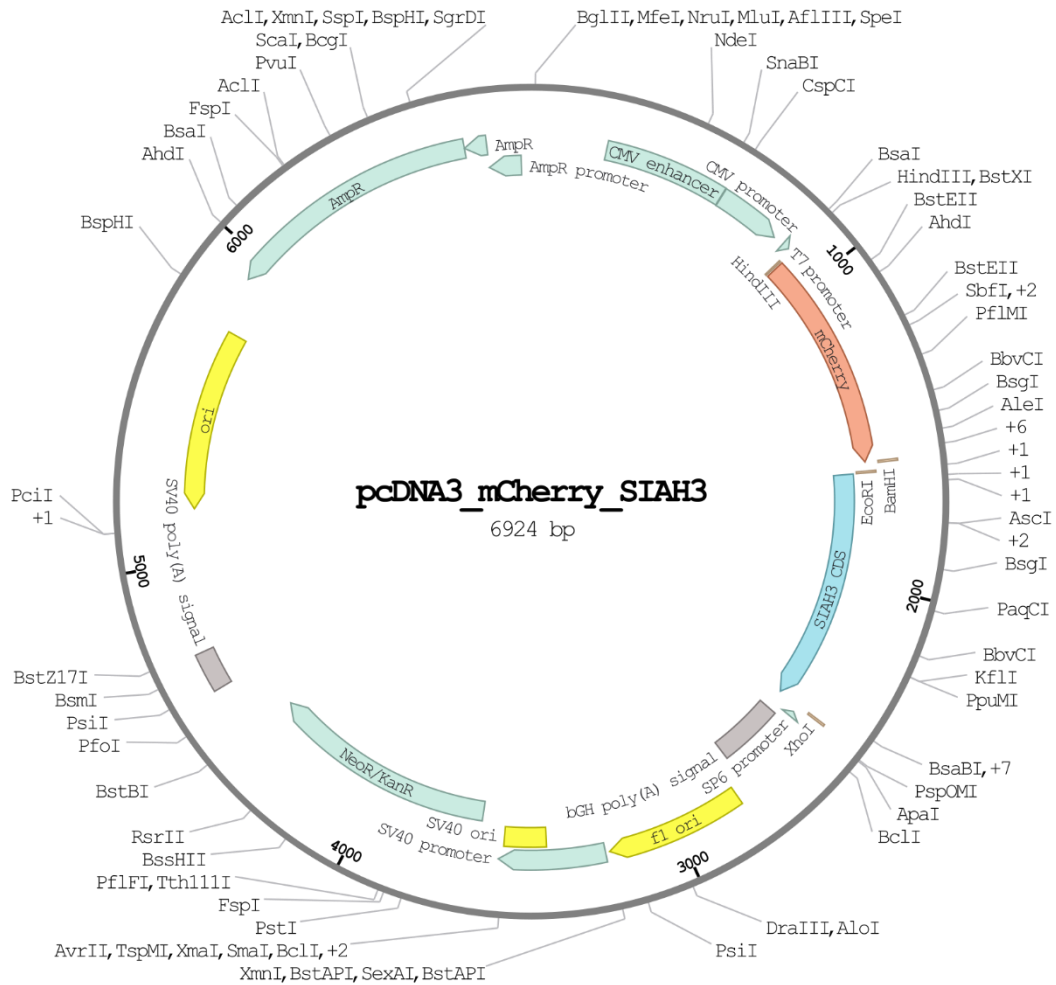


Fig. S5: pcDNA3 vector containing mCherry and SIAH3.

SUPPLEMENTARY MATERIAL

Insert sequence mCherry-SIAH3 Wt (mCherry cloned via *HindIII* – *BamHI*, SIAH3 cloned via *EcoRI* – *XhoI*):

AAGCTTATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGT
GCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACG
AGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGT
CCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTG
TCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAAGTTCGAGGACGGCGGCGTGGTGACCGTGACC
CAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCG
ACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCTCCTCCGAGCGGATGTACCCCGAGGAC
GGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGG
TCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGCGCCTACAACGTCAACATCAAGTTGG
ACATCACCTCCCACAACGAGGACTACACCATCGTGGAAACAGTACGAACGCGCCGAGGGCCGCCACTCCA
CCGGCGGCATGGACGAGCTGTACAAG**GCATCC**ACTAGTAACGGCCGCCAGTGTGCT**GAATTC**ATGCTTTT
CTTTACCCAGTGCTTTGGGGCTGTATTAGATCTCATTATCTCCGGTTTCAGCACTACAAGGCTAAACGGG
TTTTCTCCGCTGCCGGGCAACTTGTCTGTGTGTCGTAACCCACACACAACCTAAAGTATGTGTCCAGTCGG
CGCGCCGCTCACTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACCCTCACCATCTCTCCACCACTGCCA
CCACCGCCACCACCACCTCCGCCACCACGCCACCCCCACCACTTCAACACAGGAGGCGGGGCTGC
ACGCCAACCCGGTGACGCCCTGCCTGTGCATGTGTCCCTTGTCTCCTGCCAGTGGGAAGGCCGCCTGGA
GGTGGTGGTGCCCCACCTGCGGCAGATCCATAGGGTTGACATCCTCCAGGGAGCCGAGATCGTCTTCCTG
GCCACGGACATGCACCTCCCCGCGCCGGCTGATTGGATCATCATGCACTCCTGCCTTGGCCACCACTTTCT
GTTGGTGCTGAGGAAACAGGAGAGGCATGAAGGGCACCCCCAGTTCTTTGCCACCATGATGCTGATTGG
GACCCCCACCCAGGCCGACTGCTTACCTATCGCTGGAGCTCAACAGAAACCATCGGCGCCTCAAGTGG
GAGGCCACGCCCCGGTCTGTTCTTGAGTGCGTGGACTCGGTGATTACGGACGGGGACTGCCTCGTCCTCA
ACACCTCGCTGGCACAGCTCTTCTTGACAACGGCAGCCTTGCCATTGGGATTGCCATCACCGCGACAGA
GGTCCTCCCCTCAGAAGCTGAAATGTGA**CTCGAG**

AAGCTT: *HindIII* restriction site

GCATCC: *BamHI* restriction site

GAATTC: *EcoRI* restriction site

CTCGAG: *XhoI* restriction site

ATGGTGAGCAAG...: mCherry

ACTAGTAACGGC...: fragment of pcDNA3

SUPPLEMENTARY MATERIAL

GAACAAAAACTCATCTCAGAAGAGGATCTG: myc-tag

CATCATCACCATCACCAT: 6xHIS

SIAH3 ΔS3UNS with myc-tag, A frame

CTCGAG TTTTTCAGCAAGAATGCACAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCACTCAGAGCGCTC
CAGAGCAAGGCAGCTTCCACCCTCACCATCTCTCCACCACCACTGCCACCACCGCCACCACCACCACTCC
GCCACCACGCCCACCCCCACCACCTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGTGACGCCCTG
CCTGTGCATGTGTCCCTTGTCTCCTGCCAGTGGGAAGGCCGCCTGGAGGTGGTGGTGCCCCACCTGCGG
CAGATCCATAGGGTTGACATCCTCCAGGGAGCCGAGATCGTCTTCTGGCCACGGACATGCACCTCCCCG
CGCCGGCTGATTGGATCATCATGCACTCCTGCCTTGCCACCACCTTCTGTTGGTGCTGAGGAAACAGGA
GAGGCATGAAGGGCACCCCCAGTTCTTGGCCACCATGATGCTGATTGGGACCCCCACCAGGCCGACTGC
TTCACCTATCGCCTGGAGCTCAACAGAAACCATCGGCGCCTCAAGTGGGAGGCCACGCCCCGGTCTGTTC
TTGAGTGCGTGGACTCGGTGATTACGGACGGGGACTGCCTCGTCTCAACACCTCGCTGGCACAGCTCTT
CTCTGACAACGGCAGCCTTGCCATTGGGATTGCCATCACC GCGACAGAGGTCTCTCCCTCAGAAGCTGAA
ATG**CTCGAG**TCTAGAGGGGCCCTTC**GAACAAAAACTCATCTCAGAAGAGGATCTG**AATATGCATACCGGT**CA**
TCATCACCATCACCATTGAGTTTAAA...

SIAH3 Wt without tag, B frame (construct for Tet-on; cloned by S. Walesch)

CTCGAG TTTTTCAGCAAGA**ATGCTTTTCTTTACCCAGTGCTTTGGGGCTGTATTAGATCTCATTATCTCCG**
GTTTCAGCACTACAAGGCTAAACGGGTTTTCTCCGCTGCCGGCAACTTGCTGTGTCTGTAACCCACAC
ACAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCACTCAGAGCGCTCCAGAGCAAGGCAGCTTCCACC
TCACCATCTCTCCACCACCACTGCCACCACCGCCACCACCACCTCCGCCACCACGCCCACCCCCACCA
CCTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGTGACGCCCTGCCTGTGCATGTGTCCCTTGTCT
CCTGCCAGTGGGAAGGCCGCCTGGAGGTGGTGGTGCCCCACCTGCGGCAGATCCATAGGGTTGACATCC
TCCAGGGAGCCGAGATCGTCTTCTGGCCACGGACATGCACCTCCCCGCGCCGGCTGATTGGATCATCAT
GCACTCCTGCCTTGCCACCACCTTCTGTTGGTGCTGAGGAAACAGGAGAGGCATGAAGGGCACCCCCA
GTTCTTTGCCACCATGATGCTGATTGGGACCCCCACCCAGGCCGACTGCTTACCTATCGCCTGGAGCTCA
ACAGAAACCATCGGCGCCTCAAGTGGGAGGCCACGCCCCGGTCTGTTCTTGAGTGCGTGGACTCGGTGA
TTACGGACGGGGACTGCCTCGTCTCAACACCTCGCTGGCACAGCTCTTCTGACAACGGCAGCCTTGCC
ATTGGGATTGCCATCACC GCGACAGAGGTCTCTCCCTCAGAAGCTGAAATGTGA**CTCGAG**TCTAGAGGGC
CG**GCGG**TTTC**GAACAAAAACTCATCTCAGAAGAGGATCTG**AATATGCATACCGGT**CATCATCACCATCACCAT**
TGAGTTTAAA...

GCGG: 4 bp more in frame B than in frame A

SIAH3 ΔS3UNS without tag, B frame (construct for Tet-on)

CTCGAG TTTTTCAGCAAGAATGCACAACCTAAAGTATGTGTCCAGTCGGCGCGCCGTCACTCAGAGCGCTC
CAGAGCAAGGCAGCTTCCACCCTCACCATCTCTCCACCACCACTGCCACCACCGCCACCACCACCACTCC
GCCACCACGCCCACCCCCACCACCTTACCACCAGGAGGCGGGGCTGCACGCCAACCCGGTGACGCCCTG
CCTGTGCATGTGTCCCTTGTCTCCTGCCAGTGGGAAGGCCGCCTGGAGGTGGTGGTGCCCCACCTGCGG
CAGATCCATAGGGTTGACATCCTCCAGGGAGCCGAGATCGTCTTCTGGCCACGGACATGCACCTCCCCG
CGCCGGCTGATTGGATCATCATGCACTCCTGCCTTGCCACCACCTTCTGTTGGTGCTGAGGAAACAGGA
GAGGCATGAAGGGCACCCCCAGTTCTTGGCCACCATGATGCTGATTGGGACCCCCACCAGGCCGACTGC
TTCACCTATCGCCTGGAGCTCAACAGAAACCATCGGCGCCTCAAGTGGGAGGCCACGCCCCGGTCTGTTC
TTGAGTGCGTGGACTCGGTGATTACGGACGGGGACTGCCTCGTCTCAACACCTCGCTGGCACAGCTCTT
CTCTGACAACGGCAGCCTTGCCATTGGGATTGCCATCACC GCGACAGAGGTCTCTCCCTCAGAAGCTGAA
ATGTGA**CTCGAG**TCTAGAGGGCCC**GCGG**TTTC**GAACAAAAACTCATCTCAGAAGAGGATCTG**AATATGCATA
CCGGT**CATCATCACCATCACCAT**TGAGTTTAAA...

SUPPLEMENTARY MATERIAL

6.1.2.7 pRL-null_SIAH3-promoter

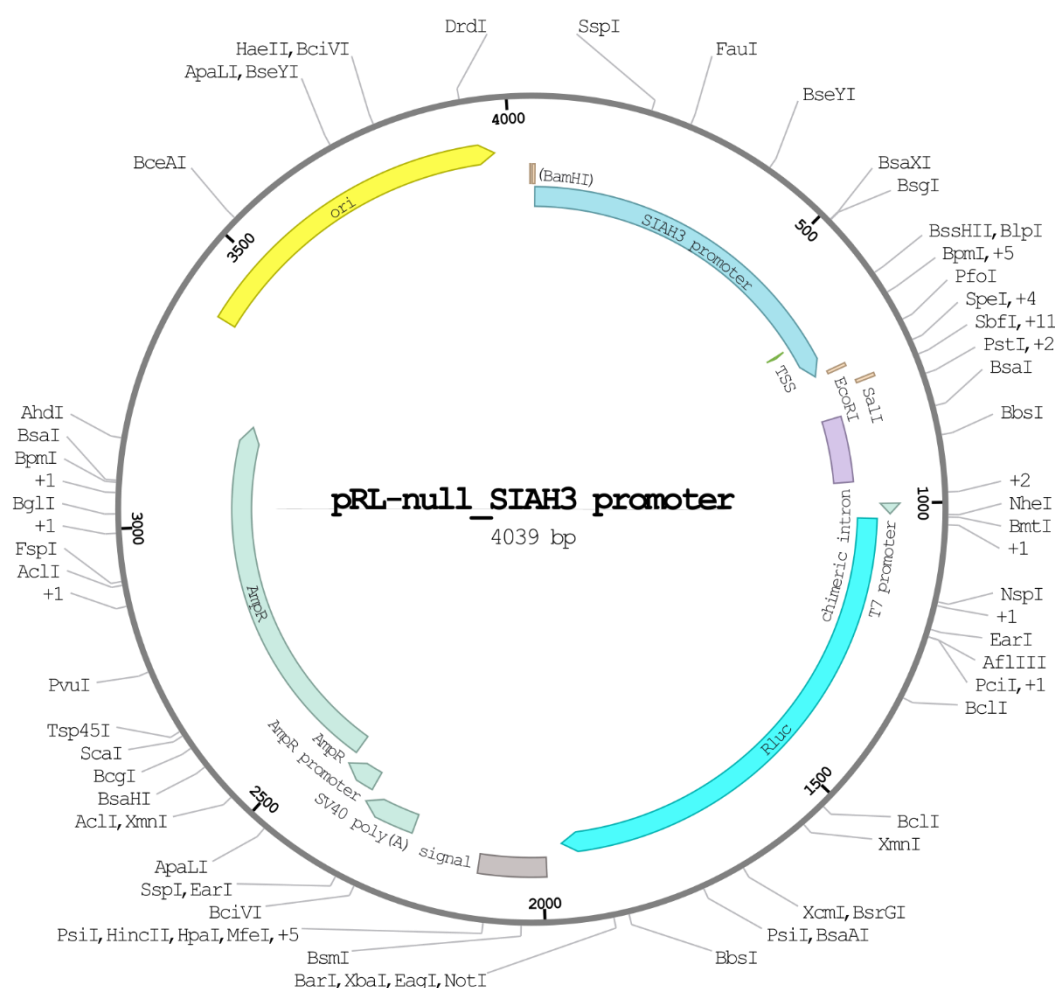


Fig. S7: pRL-null vector containing the SIAH3 promoter region.

Insert sequence SIAH3 promoter (cloned via *Bam*HI – *Sall* in *Bgl*II – *Sall*)

AGATCC TGTGCTTCCCTCCACCCCTCAACTCCAAATGAATTTGTCAGGGATTCTTTCTTTGATAAAGAGTG
GAGAGTAAGATTTATCAAGGGGCTGTAATCCAAATTGCAGAAAGTCAGAACGCAAGATTGAAAGATAC
ACATTTTTGCTGCTGCCAAGTCCTTATCTGGAATCAGAGTCTAGTAATATTTATTTCTCCCAAGCAGCTC
TGGTGAGTCGAGCCATGATCTGCGTTATCCATCTGCCTACTTTGGAGCGGGGTGTTGTACTATTTGGCGG
CTTAAACGCTTACGTTCTTGCCAGGGCATTAAAGAAAGGCTCTCATGTTAAATTAAGTCAATTAATAAATG
GAGACACATTAAGCAATCAATTTTGTGAGCCTATCTCCAGCATTAGGTATGGTTGGCTGTAATCACAA
GATAAATGCAATGTGCTGATCTATGGTAATGTTCAATAATACATTCTCCGCCAGGCTCAATTCATCAGGG
AGTTTCGGTAGGGAAGGCATTTGTTATTGGCTGTCTCTGCACCTCAAGGATAACATTTCTGTCCTTTTGAT
TGGCCGCTCACTGCTGCAAGATTCTAACCGTCTTATTTGATGATGAATATGAAGGCGCGCTAAGCCGG
GCTGGAGCGGAGTGTCTCAGAGGCTGCGTCTCTCCAGGGCCCAAGGACTCACAGCTTCTCCGCTGCCT
TCCCGGACCAACAACCCCCCAAA CAATTCAGTGAATTCGCGGCCGCTGCAG GTCGAC

AGATCC: combined *Bgl*II + *Bam*HI restriction overhangs (restriction site lost)

CAATTC: *Eco*RI restriction site

GTCGAC: *Sall* restriction site

AATCACTAGTGAATTCGCGGCCGCTGCAG: fragment of pGEM-T easy

SUPPLEMENTARY MATERIAL

6.1.2.8 pX459 constructs (SIAH3 guides)

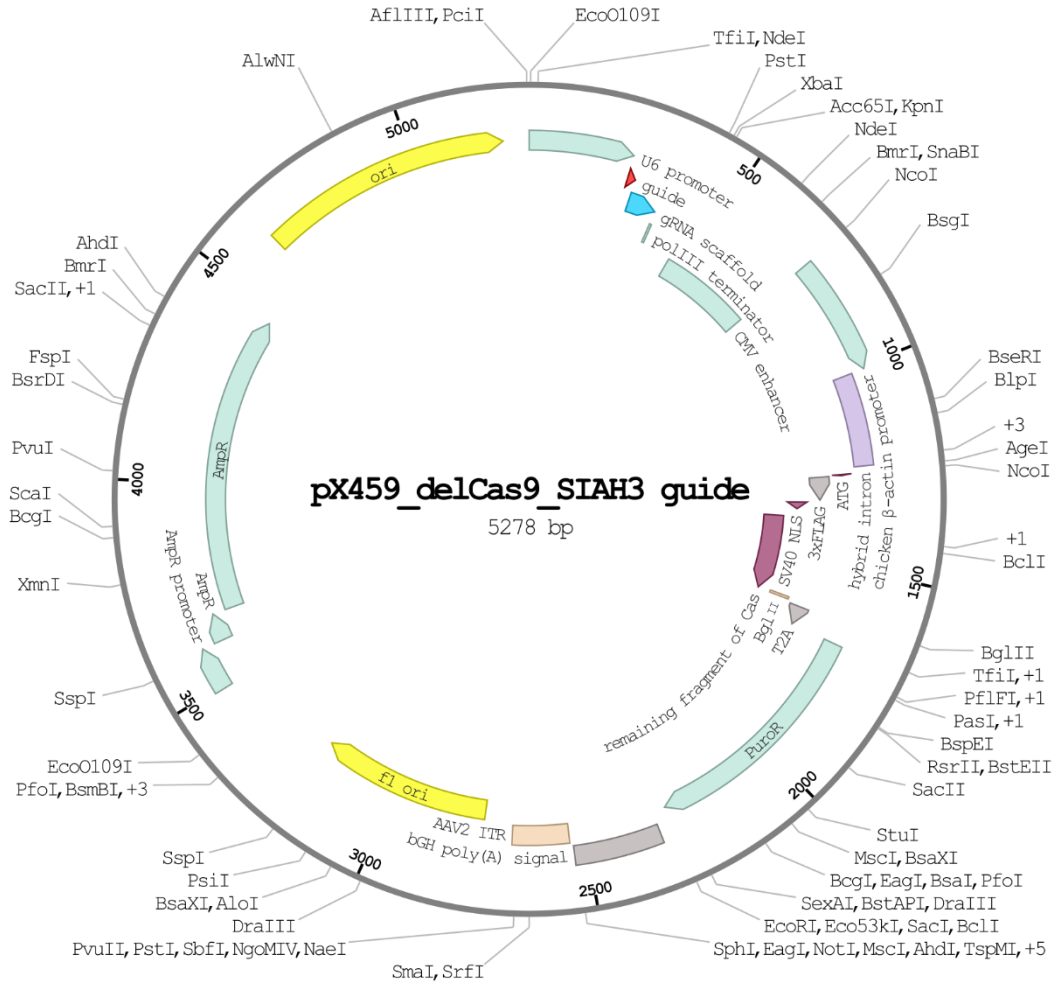


Fig. S8: pSpCas9(BB)-2A-Puro V2.0 (pX459) vector (Cas9 deleted) containing the sequence for the SIAH3-binding sgRNAs. Cloned by T. Zenz and F. Geyer.

Insert sequences for the SIAH3-binding sgRNAs (annealed oligonucleotides, inserted via *BbsI*)

Guide I: CACCG**CTTTTGGGGGTTGTTGGTC**
CGAAAACCCCCCAACAACCAGCAAA
(**GACCAACAACCCCCCAAAA**)

Guide II: CACCG**AATATGAAGGCGCGCTAAGC**
CTTATACTTCCGCGCGATTTCGCAAA
(**AATATGAAGGCGCGCTAAGC**)

Guide III: CACCG**GCAGTGAGGCGGCCAATCAAA**
CGTCACTCCGCCGGTTAGTTTCAAA
(**TTTGATTGGCCGCCTCACTG**)

Guide IV: CACCG**CCCTGATGAATTGAGCCTGG**
CGGGACTACTTAACTCGGACCAGCAAA
(**CCAGGCTCAATTCATCAGGG**)

Guide V: CACCG**TCCATCTGCCTACTTTGGAG**
CAGGTAGACGGATGAAACCTCAGCAAA
(**TCCATCTGCCTACTTTGGAG**)

red: guide sequence (T=U)
green: target sequence
(sense orientation)
CACCG/CAAA: overhangs

SUPPLEMENTARY MATERIAL

Guide VI: CACC**GGAGAGTAAGATTTATCAAG**
CCTCTCATTCTAAATAGTTC CAAA
(GGAGAGTAAGATTTATCAAG)

6.1.3 SIAH1 and SIAH2 plasmids (those cloned by me)

6.1.3.1 pCMV-Tag 1 constructs

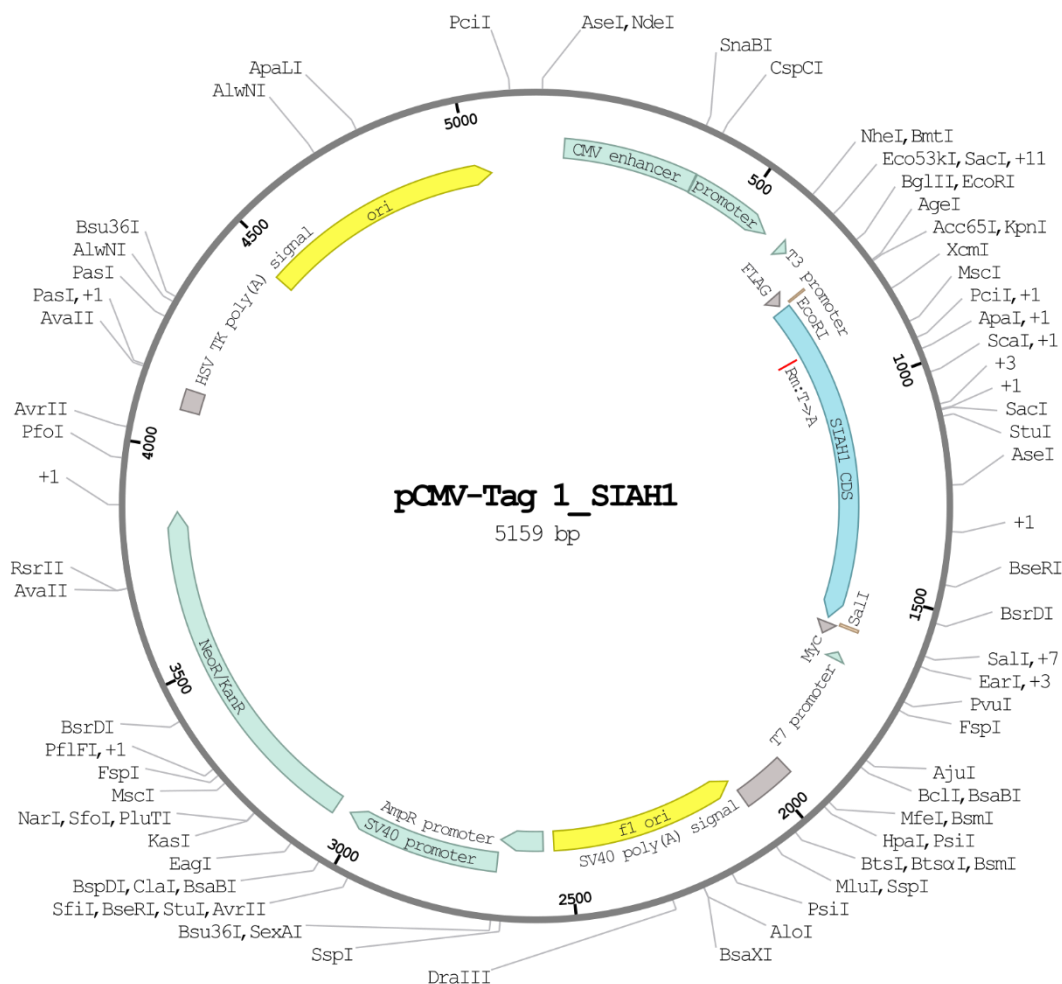


Fig. S9: pCMV-Tag1 vector (mod. by A. Richter) containing SIAH1 Wt or Rm. FLAG N-terminal in frame with SIAH3. No open reading frame with myc.

Insert sequence SIAH1 Wt and Rm (cloned via *EcoRI* – *Sall*)

GAATTCGTGGATCCGAGCCGTCAGACTGCTACAGCATTACCTACCGGTACCTCGAAGTGTCCACCATCCCCA
GAGGGTGCCTGCCCTGACTGGCACAACCTGCATCCAACAATGACTTGGCGAGTCTTTTTGAGTGTCCAGTC
TGCTTTGACTATGTGTTACCGCCATTCTTCAATGTCAGAGTGGCCATCTTGTTTGTAGCAACTGTCGCCCA
AAGCTCACATGTTGTCCAACCTGCCGGGGCCCTTTGGGATCCATTGCGAACCTGGCTATGGAGAAAGTGG
CTAATTCAGTACTTTTCCCTGTAAATATGCGTCTTCTGGATGTGAAATAACTCTGCCACACACAGAAAAA
GCAGACCATGAAGAGCTCTGTGAGTTAGGCCCTATTCTGTCCGTGCCCTGGTGCTTCTGTAAATGGCA
AGGCTCTCTGGATGCTGTAATGCCCCATCTGATGCATCAGCATAAGTCCATTACAACCCTACAGGGAGAG
GATATAGTTTTTCTTGCTACAGACATTAATCTTCTGGTGCTGTTGACTGGGTGATGATGCAGTCCTGTTTT
GGCTTTCATTCATGTTAGTCTTAGAGAAACAGGAAAAATACGATGGTCACCAGCAGTCTTCGCAATCG
TACAGCTGATAGGAACACGCAAGCAAGCTGAAAATTTTGCTTACCGACTTGAGCTAAATGGTCATAGGC
GACGATTGACTTGGGAAGCGACTCCTCGATCTATTATGAAGGAATTGCAACAGCCATTATGAATAGCG
ACTGTCTAGTCTTTGACACCAGCATTGCACAGCTTTTTGCAGAAAATGGCAATTTAGGCATCAATGTAAT
ATTTCCATGTGTTGAGCGGTCTGAC

SUPPLEMENTARY MATERIAL

GAATTC: *EcoRI* restriction site

GTCGAC: *Sall* restriction site

T: in Wt T, in Rm A

6.1.3.2 pcDNA3-mCherry constructs

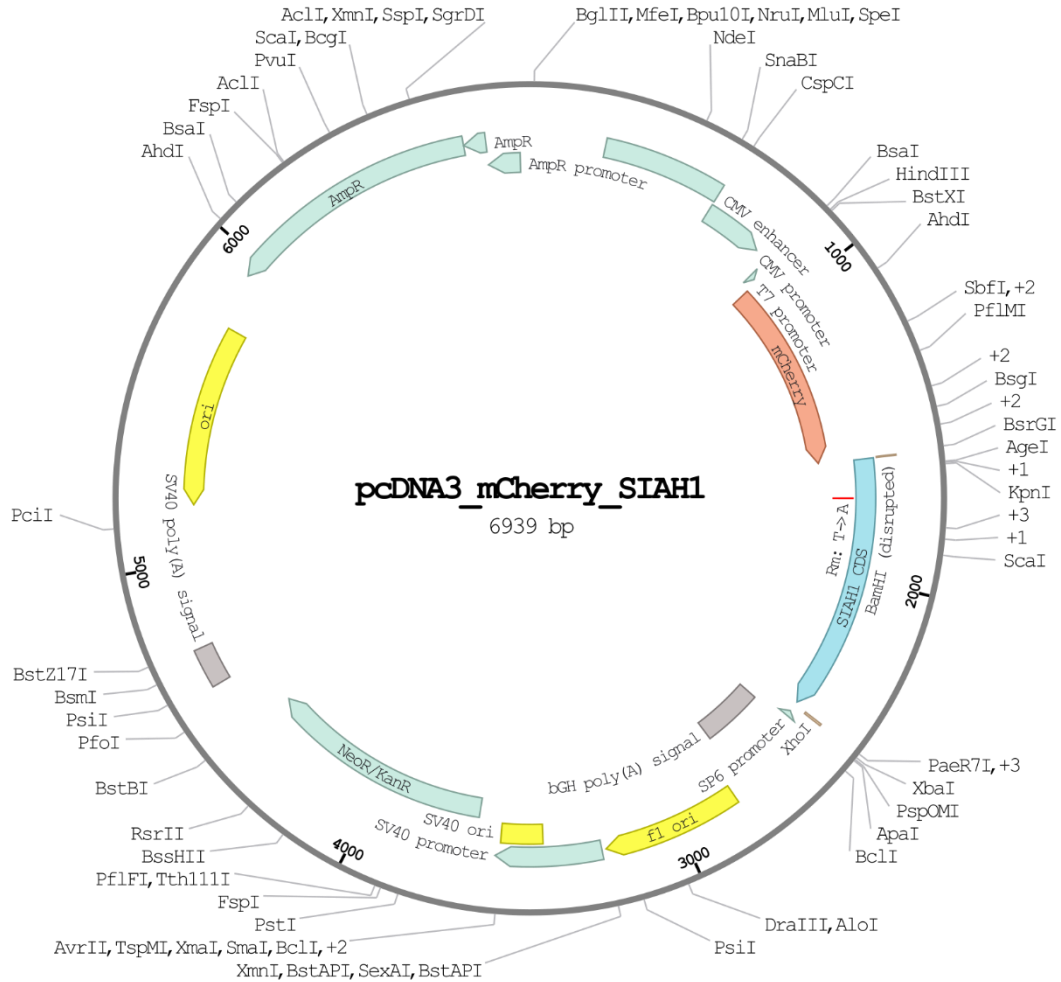


Fig. S10: pcDNA3 vector containing mCherry and SIAH1 Wt or Rm.

Insert sequence SIAH1 Wt and Rm (cloned via *BamHI* – *XhoI*)

GGAATGAGCCGTCAGACTGCTACAGCATTACCTACCGGTACCTCGAAGTGTCCACCATCCCAGAGGGTGC
CTGCCCTGACTGGCACAACATGCATCCAACAATGACTTGGCGAGTCTTTTGTAGTGTCCAGTC**T**GCTTTGAC
TATGTGTTACCGCCATTCTTCAATGTCAGAGTGGCCATCTTGTTGTAGCAACTGTCGCCCAAAGCTCAC
ATGTTGTCCAACCTGCCGGGGCCCTTTGGGATCCATTCGCAACTGGCTATGGAGAAAAGTGGCTAATTCA
GTACTTTTCCCCTGTAAATATGCGTCTTCTGGATGTGAAATAACTCTGCCACACACAGAAAAAGCAGACC
ATGAAGAGCTCTGTGAGTTTAGGCCTTATTCCTGTCCGTGCCCTGGTGCTTCTGTAAATGGCAAGGCTCT
CTGGATGCTGTAATGCCCATCTGATGCATCAGCATAAGTCCATTACAACCCTACAGGGAGAGGATATAG
TTTTTCTTGCTACAGACATTAATCTTCTGGTGCTGTTGACTGGGTGATGATGCAGTCCTGTTTTGGCTTTC
ACTTCATGTTAGTCTTAGAGAAACAGGAAAAATACGATGGTCACCAGCAGTTCTTCGCAATCGTACAGCT
GATAGGAACACGCAAGCAAGCTGAAAATTTTGCTTACCGACTTGAGCTAAATGGTCATAGGCGACGATT
GACTTGGGAAGCGACTCCTCGATCTATTCATGAAGGAATTGCAACAGCCATTATGAATAGCGACTGTCTA
GTCTTTGACACCAGCATTGCACAGCTTTTTGCAGAAAATGGCAATTTAGGCATCAATGTAACATTTCCAT
GTGTTGAG**GCGGTCGACCTCGAG**

GGA: disrupted *BamHI* restriction site

GTCGAG: *XhoI* restriction site

T: in Wt T, in Rm A

SUPPLEMENTARY MATERIAL

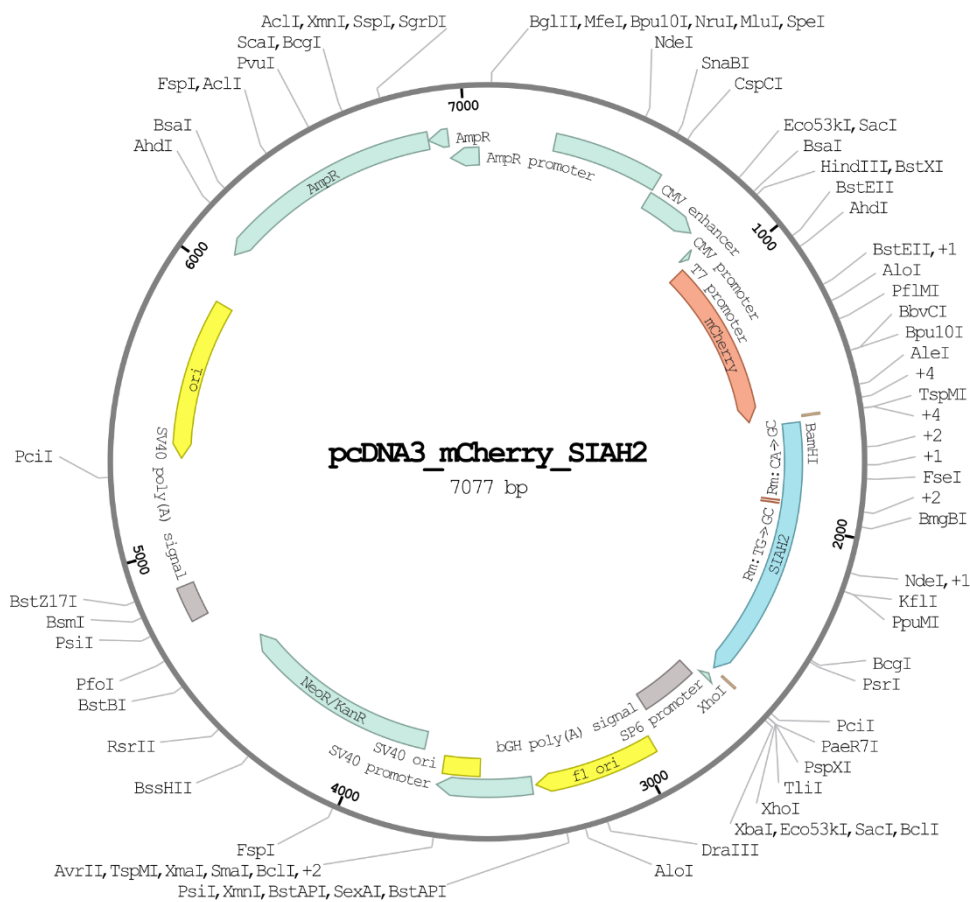


Fig. S11: pcDNA3 vector containing mCherry and SIAH2 Wt or Rm.

Insert sequence SIAH2 Wt and Rm (cloned via *Bam*HI – *Xho*I)

GGATCC CCCGGGCTGCAGGAATTCATGAGCCGCCGTCTCCACCGCCCCAGCGCTAATAAACCCCTGCA
GCAAGCAGCCGCCGCCGAGCCCCAGCACTCCGTCCCCGGCTGCGCCCCGGCCGCCACCATCTC
GGCTGCGGGCCCCGGCTCGTCCGCGGTGCCCGCCGCGGCGGCGGTGATCTCGGGCCCCGGCGGCGGCGG
CGGGGCCGGCCCGGTGTCCCCGAGCACCACGAGCTGACCTCGTCTTCGAGTGTCCGGTCTGCTTTGAC
TATGTCCTGCCTCTATTCTGCAGTGCCAGGCCGGGCACTGGTGTGTAACCAATGCCGCCAGAAGTTGA
GCTGCTGCCCCAGCTGCAGGGGCGCCTGACGCCAGCATCAGGAACCTGGCTATGGAGAAGGTGGCCT
CGGCAGTCTGTTTCCCTGTAAGTATGCCACCACGGGCTGTTCCCTGACCCTGCACCATACGGAGAAACC
AGAACATGAAGACATATGTGAATACCGTCCCTACTCCTGCCATGTCTGGTGCTTCCTGCAAGTGGCAG
GGGTCCCTGGAAGCTGTGATGTCCATCTCATGCACGCCACAAGAGCATTACCACCCTTCAGGGAGAAG
ACATCGTCTTTCTAGCTACAGACATTAAC TTGCCAGGGGCTGTCGACTGGGTGATGATGCAGTCATGTTTT
GGCCATCACTTCATGCTGGTGCTGGAGAAACAAGAGAAGTACGAAGGCCACCAGCAGTTTTTTGCCATC
GTCCTGCTCATTGGCACCCGCAAGCAAGCCGAGAAC TTGCTACAGACTGGAGTTGAATGGGAACCGG
CGGAGATTGACCTGGGAGGCCACGCCCCGTTGCATTGATGACGGTGTGGCTGCGGCCATCATGAACAGC
GACTGCCTTGTTTTGCACACAGCCATAGCACATCTTTTGAGATAATGGGAACCTTGAATCAATGTTAC
TATTTCTACATGTTGTCCATGA CTCGA

GGA: *Bam*HI restriction site

CTCGAG: *Xho*I restriction site

CA/TG: in Wt CA/TG, in Rm GC

SUPPLEMENTARY MATERIAL

6.1.4 CHCHD4 plasmids

6.1.4.1 pFLAG-CMV-2 construct

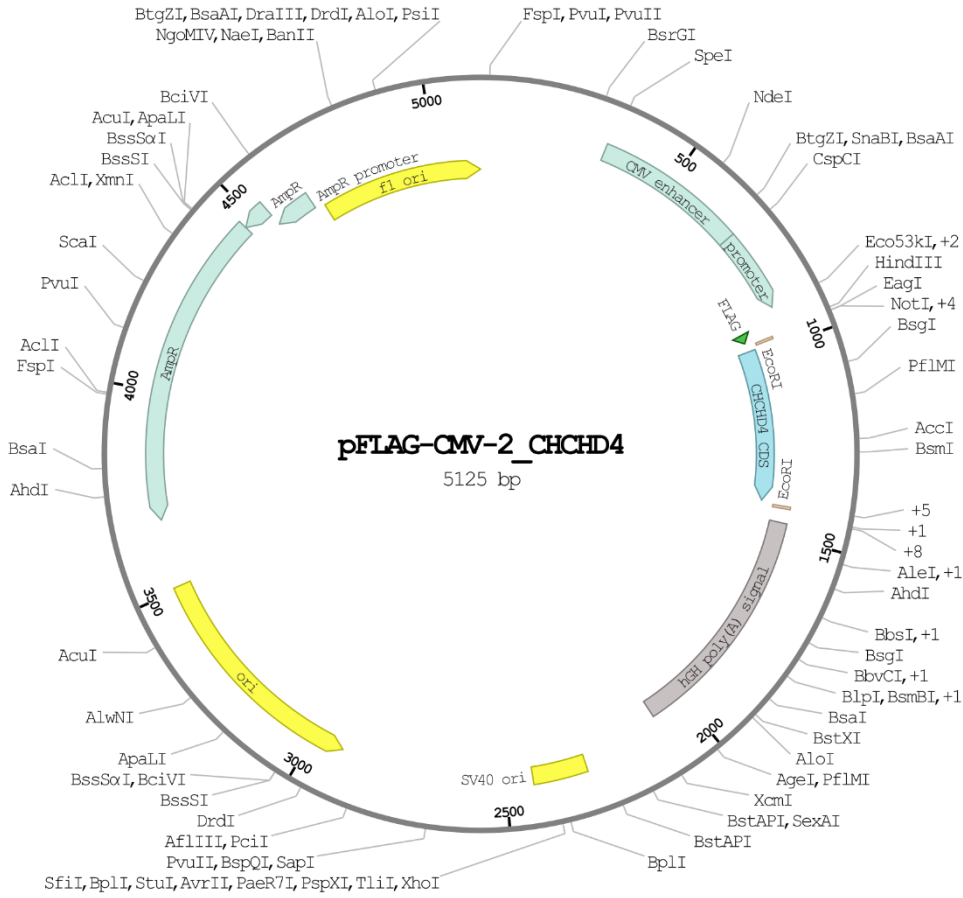


Fig. S12: pFLAG-CMV-2 vector containing CHCHD4.

Insert sequence CHCHD4 (cloned via *EcoRI* – *EcoRI*)

GAATTCGATTGGATCCATGTCCTATTGCCGGCAGGAAGGGAAGGATCGAATCATATTTGTAACCAAAGA
 AGATCATGAACTCCAAGCAGTGCAGAATTGGTGGCTGATGACCCCAACGATCCATACGAGGAGCATGG
 ATTGATACTGCCAAATGGAAACATTAAGTGAAGTGGCCATGCCTTGGGGGAATGGCCAGCGGTCCCTGT
 GGAGAACAGTTAAGTCAGCCTTTCTGCTTCCACTATAGCACGGAGGAGATCAAGGGGTCAGACTGT
 GTAGACCAGTTCGGGGCCATGCAGGAATGCATGCAGAAATACCCAGACCTCTATCCCAAGAGGATGAG
 GATGAGGAAGAGGAAAGAGAGAAGAAGCCAGCAGAACAGCAGAAGAAACAGCTCCATTGAGGCCA
 CTGCAACCAAAGAAGAGGAGGGATCAAGTTAA**TGAATTC**

GAATTC: *EcoRI* restriction site

SUPPLEMENTARY MATERIAL

6.1.4.2 pEGFP-N3 construct

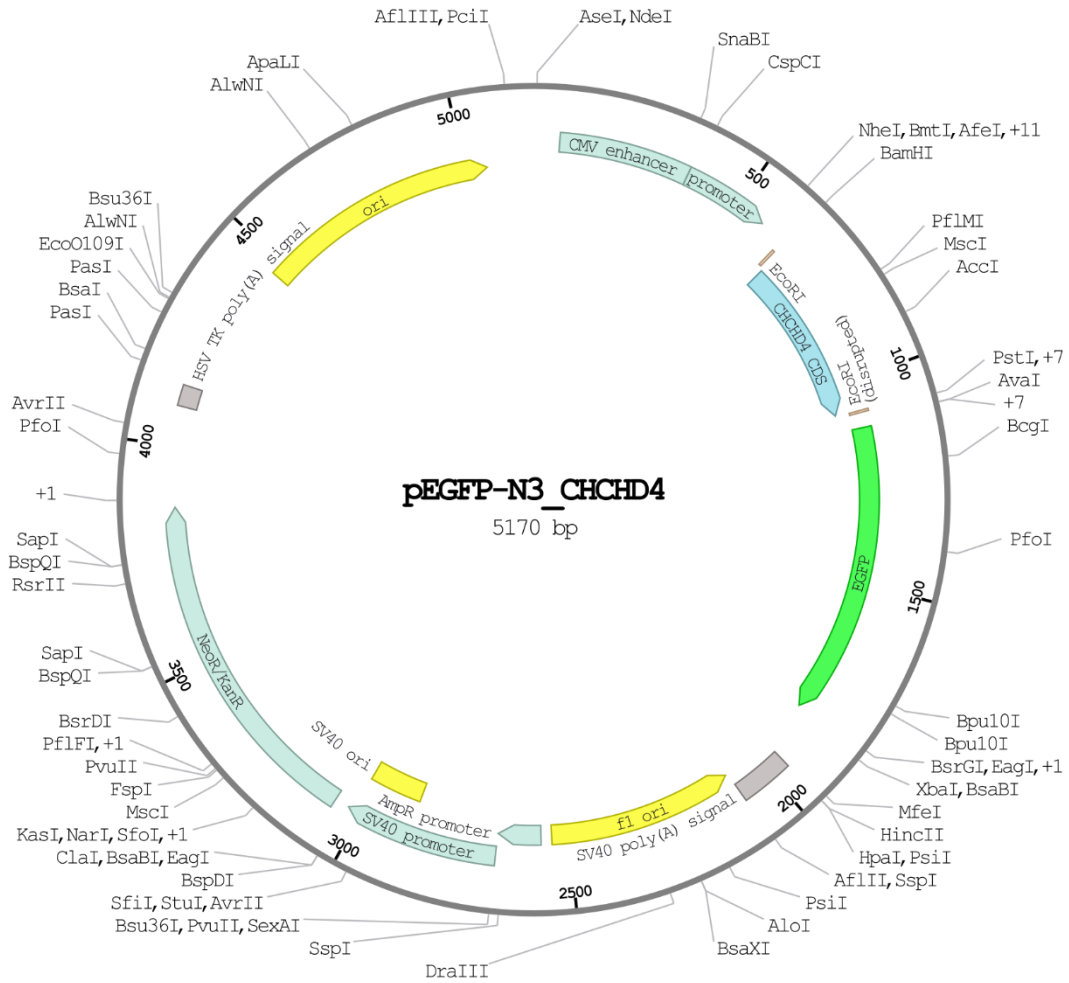


Fig. S13: pEGFP-N3 vector containing CHCHD4.

Insert sequence CHCHD4 (cloned via *EcoRI* – *EcoRI*)

GAATTCGATTGGATCCATGTCCTATTGCCGGCAGGAAGGGAAGGATCGAATCATATTTGTAACCAAAGA
 AGATCATGAAACTCCAAGCAGTGCAGAATTGGTGGCTGATGACCCCAACGATCCATACGAGGAGCATGG
 ATTGATACTGCCAAATGGAAACATTAAGTGAAGTGGCCATGCCTTGGGGGAATGGCCAGCGGTCCCTGT
 GGAGAACAGTTTAAGTCAGCCTTTTCTGCTTCCACTATAGCACGGAGGAGATCAAGGGGTCAGACTGT
 GTAGACCAGTTCGGGGCCATGCAGGAATGCATGCAGAAATACCCAGACCTCTATCCCCAAGAGGATGAG
 GATGAGGAAGAGGAAAGAGAGAAGAAGCCAGCAGAACAAAGCAGAAGAAACAGCTCCATTGAGGCCA
 CTGCAACCAAAGAAGAGGAGGGATCAAGT**AATTC**

GAATTC: *EcoRI* restriction site

AATTC: disrupted *EcoRI* restriction site (to get CHCHD4 in frame with EGFP)

SUPPLEMENTARY MATERIAL

6.1.5 STUB1 plasmids

6.1.5.1 pCMV-Tag1 construct

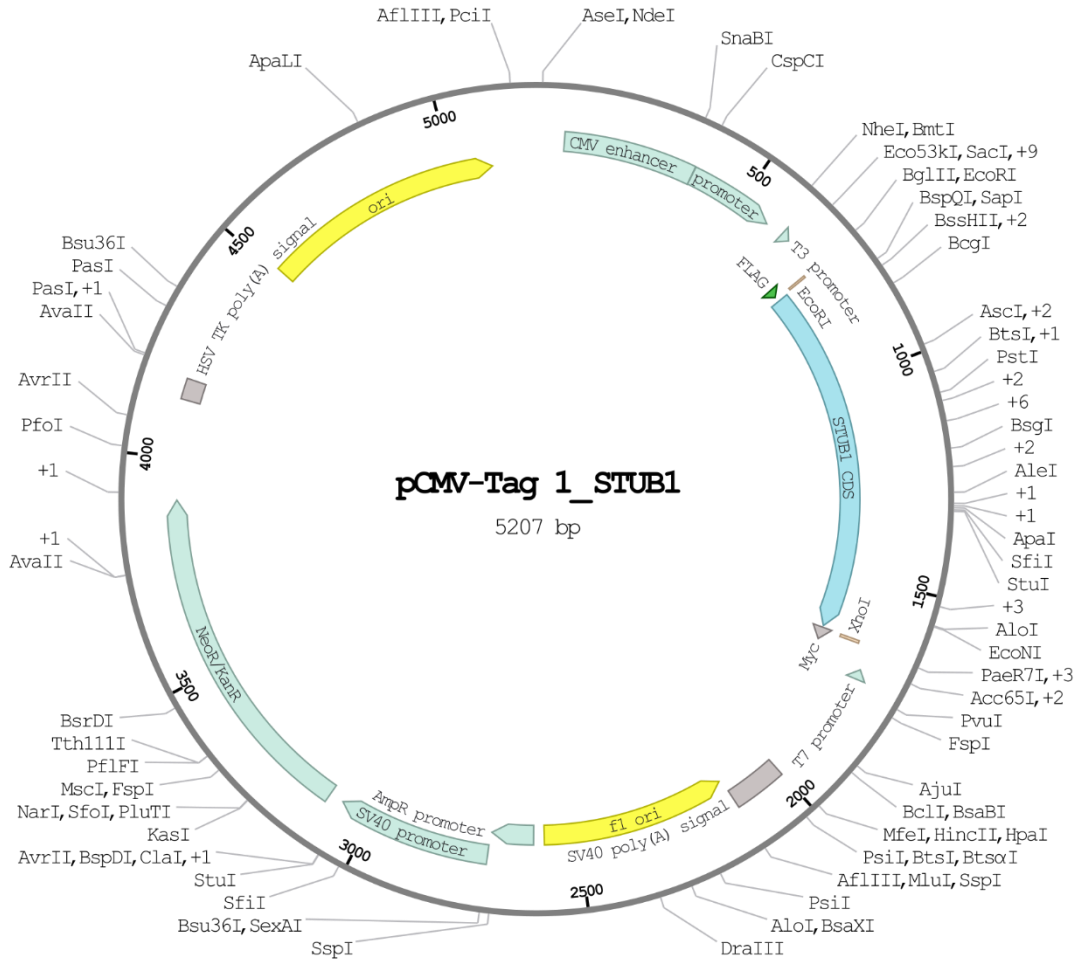


Fig. S14: pCMV-Tag 1 vector (mod. by A. Richter) containing STUB1. Cloned by N. Schlaudraff.

Insert sequence STUB1 (cloned via *EcoRI* – *XhoI*, cloned by N. Schlaudraff)

GAATTCATGAAGGGCAAGGAGGAGAAGGAGGGCGGCGCACGGCTGGGCGCTGGCGGCGGAAGCCCCG
AGAAGAGCCCGAGCGCGCAGGAGCTCAAGGAGCAGGGCAATCGTCTGTTCGTGGGCCGAAAGTACCCG
GAGGCGGCGGCCTGCTACGGCCGCGCGATCACCCGGAACCCGCTGGTGGCCGTGTATTACACCAACCGG
GCCTTGTGCTACCTGAAGATGCAGCAGCAGCAGCAGGCCCTGGCCGACTGCCGGCGCGCCCTGGAGCTG
GACGGGCAGTCTGTGAAGGCGCACTTCTTCTGGGGCAGTGCCAGCTGGAGATGGAGAGCTATGATGA
GGCCATCGCCAATCTGCAGCGAGCTTACAGCCTGGCCAAGGAGCAGCGGCTGAACTTCGGGGACGACAT
CCCCAGCGCTCTTCGAATCGCGAAGAAGAAGCGCTGGAACAGCATTGAGGAGCGGCGCATCCACCAGG
AGAGCGAGCTGCACTCCTACCTCTCCAGGCTCATTGCCGCGGAGCGTGAGAGGGAGCTGGAAGAGTGCC
AGCGAAACCACGAGGGTGATGAGGACGACAGCCACGTCCGGGCCAGCAGGCCTGCATTGAGGCCAAG
CACGACAAGTACATGGCGGACATGGACGAGCTTTTTTCTCAGGTGGATGAGAAGAGGAAGAAGCGAGA
CATCCCCGACTACCTGTGTGGCAAGATCAGCTTTGAGCTGATGCGGGAGCCGTGCATCACGCCAGTGGC
ATCACCTACGACCGCAAGGACATCGAGGAGCACCTGCAGCGTGTGGGTCAATTTGACCCCGTGACCCGG
AGCCCCCTGACCCAGGAACAGCTCATCCCCAAGTGGCTATGAAGGAGGTTATTGACGCATTATCTCTG
AGAATGGCTGGGTGGAGGACTACTGACTCGAG

GAATTC: *EcoRI* restriction site

CTCGAG: *XhoI* restriction site

6.1.6.1 pEGFP-N3 construct



AGATCTATGGCGGCCTCGGCAGCGGTGTTCTCGCGCTTGCGAAGCGGGCTCCGGCTCGGCTCGCGGGGA
CTGTGCACGAGGTTGGCGACGCCGCCCGGGCCCCAGATCAGGCCGAGAGATCGGGAGCCGCGG
GAGCACTAAGGCGCAAGGGCCACAGCAGCAGCCGGGCTCAGAGGGTCCAGCTATGCCAAAAAAGTTG
CGCTCTGGCTTGCTGGGCTGCTTGAGCTGGTGGGACTGTGAGCGTCGTCTATATCTTTGGAAACAACC
GGTGGACGAAAATGGTGCCAAGATTCTTGATGAGTTCGACAATGATCCAATTCTGGTACAGCAGTTGCG
CCGGACATACAAATATTTCAAAGATTATAGACAGATGATCATCGAGCCCACCAGCCCTTGCCTTCTCCCAG
ACCCTCTGCAGGAACCGTACTACCAGCCACCCTACACGCTCGTTTTGGAGCTCACCGGCGTCCTCTTGCAT
CCTGAGTGGTCGCTGGCCACTGGCTGGAGGTTTAAGAAGCGCCAGGCATCGAGACCTTGTTCCAGCAG
CTTGCCCCTTTATATGAAATTGTCATCTTTACGTCAGAGACTGGCATGACTGCGTTTCCACTCATTGATAGT
GTGGACCCCCATGGCTTCATCTCTACCGCTATTCCGGGACGCCACAAGATACATGGATGGACACCATG
TAAAGGATATTTATGTCTGAATCGGGACCCAGCTCGAGTAGTAGTTGTGGACTGCAAGAAGGAAGCCT
TCCGCTGCAGCCCTATAACGGCGTTGCCCTGCGGCCCTGGGACGGCAACTCTGATGACCGGGTCTTGTT
GGATCTGTCTGCCTTCTCAAGACCATTGCACTGAATGGTGTGGAGGACGTGCGAACCCTGCTGGAGCA
CTATGCCCTGGAGGATGACCCGCTGGCGGCTTTCAAACAGCGGCCAAAGCCGGCTAGAGCAGGAGGAGC
AGCAGCGCCTGGCCGAGCTCTCCAAGTCCAACAAGCAGAACCTCTTCCTTGCTCCCTACCAGCCGCTTG
TGGCCTCGCTCCAAACAGCCC

GTCGAC: *Sa*I restriction site

SUPPLEMENTARY MATERIAL

6.2 Mass spectrometry parameters

Tab. S1: LC-MSMS parameters. Adapted from data generated by the MS facility of Prof. Graumann at the MPI in Bad Nauheim, published in Deutschmeyer *et al.*, 2025.

Project	"richter.2018" --- SIAH3 Interactomics	"richter.2020" --- SIAH3 Induction Analysis (whole proteome)
Term	Value	
High Performance Liquid Chromatography (HPLC) Instrument	Thermo EASY-nLC	Thermo EASY-nLC
HPLC Vendor	Thermo Scientific	Thermo Scientific
Injected Sample Volume	4.00	4.00
Mass Spectrometer (MS)	Q Exactive - Orbitrap MS	Q Exactive HF - Orbitrap MS
MS Vendor	Thermo Scientific	Thermo Scientific
MS Model	QExactive	QExactive HF
MS Instrument Software	Xcalibur	Xcalibur
MS Instrument Software Version	2.9-290033/2.9.0.2926	2.11-211200/2.11.0.3007
MS Ionization Type	Electrospray Ionization (ESI)	Electrospray Ionization (ESI)
MS Electrospray Voltage	1500	2000
MS Heated Capillary Temperature	310	350
MS Analyzer Precursor Ion Spectrum (MS1)	orbitrap	orbitrap
MS Data Type Precursor Ion Spectrum (MS1)	Profile	Profile
MS Micro Spectra Precursor Ion Spectrum (MS1)	1	1
MS Resolution Precursor Ion Spectrum (MS1)	70,000	60,000
MS Resolution m/z Precursor Ion Spectrum (MS1)	200 m/z	200 m/z
MS Mass Range Precursor Ion Spectrum (MS1)	300 to 1650 m/z	300 to 1650 m/z
MS Ion Target Value Precursor Ion Spectrum (MS1)	3e6	3e6
MS Max. Injection Time Precursor Ion Spectrum (MS1)	20 ms	15 ms
MS Analyzer Fragment Ion Spectrum (MS2)	orbitrap	orbitrap
MS Data Type Fragment Ion Spectrum (MS2)	Profile	Profile
MS Micro Spectra Fragment Ion Spectrum (MS2)	1	1
MS Fixed First m/z Fragment Ion Spectrum (MS2)	-	-
MS Isolation Window Width Fragment Ion Spectrum (MS2)	1.8 m/z	1.2 m/z
MS Isolation Window Offset Fragment Ion Spectrum (MS2)	0.0 m/z	0.0 m/z
MS Fragmentation Mode (MS2)	HCD	HCD
MS Normalized Collision Energy Fragment Ion Spectrum (MS2)	NA	nce: 28
MS Resolution Fragment Ion Spectrum (MS2)	35,000	15,000

SUPPLEMENTARY MATERIAL

MS Resolution m/z Fragment Ion Spectrum (MS2)	200 m/z	200 m/z
MS Ion Target Value Fragment Ion Spectrum (MS2)	5e5	5e5
MS Max. Injection Time Fragment Ion Spectrum (MS2)	120 ms	15 ms
MS Loop Count (Top n) Fragment Ion Spectrum (MS2)	10	20
MS Charge States Excluded from Fragmentation	unassigned, 1	unassigned, 1, >8
MS Minimum Peak Intensity for Fragmentation	4.2e3	1.0e5
MS Fragmentation Trigger on Peak Apex	-	-
MS Dynamic Exclusion Length	20.0 s	20.0 s
MS Isotopologue Exclusion	on	on
MS Peptide Model for Excluded Isotope Clusters	on	preferred

Tab. S2: LC Gradient. Adapted from data generated by the MS facility of Prof. Graumann at the MPI in Bad Nauheim, published in Deutschmeyer *et al.*, 2025).

Time [mm:ss]	Duration [mm:ss]	Flow [nl/min]	Mixture [%B]
"richter.2018" --- SIAH3 Interactomics			
00:00	00:00	250	10
180:00	180:00	250	38
202:00	22:00	250	60
216:00	14:00	250	95
230:00	14:00	250	95
235:00	05:00	250	5
240:00	05:00	250	5
"richter.2020" --- SIAH3 Induction Analysis (whole proteome)			
00:00	00:00	400	5
05:00	05:00	400	5
125:00	120:00	400	38
132:00	07:00	400	60
138:00	06:00	400	95
143:00	05:00	400	95
148:00	05:00	400	5
150:00	02:00	400	5

SUPPLEMENTARY MATERIAL

Tab. S3: MaxQuant parameters. Adapted from data generated by the MS facility of Prof. Graumann at the MPI in Bad Nauheim, published in Deutschmeyer *et al.*, 2025.

Project	“richter.2018” --- SIAH3 Interactomics	“richter.20202 --- SIAH3 Induction Analysis
Parameter	value	
Version	1.6.2.6	1.6.10.43
User name	sjerats	jgrauma
Machine name	KI-S0114	KI-S0115
Date of writing	01/18/2019 23:53:20	07/05/2020 03:00:50
Include contaminants	True	True
PSM FDR	0.01	0.01
PSM FDR Crosslink	0.01	0.01
Protein FDR	0.01	0.01
Site FDR	0.01	0.01
Use Normalized Ratios For Occupancy	True	True
Min. peptide Length	7	7
Min. score for unmodified peptides	0	0
Min. score for modified peptides	40	40
Min. delta score for unmodified peptides	0	0
Min. delta score for modified peptides	6	6
Min. unique peptides	0	0
Min. razor peptides	1	1
Min. peptides	1	1
Use only unmodified peptides and	True	True
Modifications included in protein quantification	Oxidation (M);Acetyl (Protein N-term)	Oxidation (M);Acetyl (Protein N-term)
Peptides used for protein quantification	Razor	Razor
Discard unmodified counterpart peptides	True	True
Label min. ratio count	2	1
Use delta score	False	False
iBAQ	False	False
iBAQ log fit	False	False
Match between runs	True	False
Matching time window [min]	0.7	NA
Alignment time window [min]	20	NA
Find dependent peptides	False	False
Fasta file	C:\Users\sjerats\Desktop\Uniprot 20180716\Human_9606_canonical+isoforms_20180717.fasta	D:\20200703_richter.2020_relaxedparams\HUMAN_9606_canonical+isoforms_20200205_1888349.fasta
Decoy mode	revert	revert
Include contaminants	True	True
Advanced ratios	True	True
Fixed andromeda index folder		
Temporary folder		

SUPPLEMENTARY MATERIAL

Combined folder location		
Second peptides	True	True
Stabilize large LFQ ratios	True	True
Separate LFQ in parameter groups	False	False
Require MS/MS for LFQ comparisons	False	True
Calculate peak properties	False	False
Main search max. combinations	200	200
Advanced site intensities	True	True
Write msScans table	True	False
Write msmsScans table	True	True
Write ms3Scans table	True	True
Write allPeptides table	True	True
Write mzRange table	True	True
Write pasefMsmsScans table	True	True
Write accumulatedPasefMsmsScans table	True	True
Max. peptide mass [Da]	4600	4600
Min. peptide length for unspecific search	8	8
Max. peptide length for unspecific search	25	25
Razor protein FDR	True	True
Disable MD5	False	False
Max mods in site table	3	3
Match unidentified features	False	False
Epsilon score for mutations		
Evaluate variant peptides separately	True	True
Variation mode	None	None
MS/MS tol. (FTMS)	20 ppm	20 ppm
Top MS/MS peaks per Da interval. (FTMS)	12	12
Da interval. (FTMS)	100	100
MS/MS deisotoping (FTMS)	True	True
MS/MS deisotoping tolerance (FTMS)	7	7
MS/MS deisotoping tolerance unit (FTMS)	ppm	ppm
MS/MS higher charges (FTMS)	True	True
MS/MS water loss (FTMS)	True	True
MS/MS ammonia loss (FTMS)	True	True
MS/MS dependent losses (FTMS)	True	True
MS/MS recalibration (FTMS)	False	False
MS/MS tol. (ITMS)	0.5 Da	0.5 Da
Top MS/MS peaks per Da interval. (ITMS)	8	8
Da interval. (ITMS)	100	100
MS/MS deisotoping (ITMS)	False	False
MS/MS deisotoping tolerance (ITMS)	0.15	0.15
MS/MS deisotoping tolerance unit (ITMS)	Da	Da
MS/MS higher charges (ITMS)	True	True
MS/MS water loss (ITMS)	True	True
MS/MS ammonia loss (ITMS)	True	True
MS/MS dependent losses (ITMS)	True	True

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MS/MS recalibration (ITMS)	False	False
MS/MS tol. (TOF)	40 ppm	40 ppm
Top MS/MS peaks per Da interval. (TOF)	10	10
Da interval. (TOF)	100	100
MS/MS deisotoping (TOF)	True	True
MS/MS deisotoping tolerance (TOF)	0.01	0.01
MS/MS deisotoping tolerance unit (TOF)	Da	Da
MS/MS higher charges (TOF)	True	True
MS/MS water loss (TOF)	True	True
MS/MS ammonia loss (TOF)	True	True
MS/MS dependent losses (TOF)	True	True
MS/MS recalibration (TOF)	False	False
MS/MS tol. (Unknown)	0.5 Da	20 ppm
Top MS/MS peaks per Da interval. (Unknown)	8	12
Da interval. (Unknown)	100	100
MS/MS deisotoping (Unknown)	False	True
MS/MS deisotoping tolerance (Unknown)	0.15	7
MS/MS deisotoping tolerance unit (Unknown)	Da	ppm
MS/MS higher charges (Unknown)	True	True
MS/MS water loss (Unknown)	True	True
MS/MS ammonia loss (Unknown)	True	True
MS/MS dependent losses (Unknown)	True	True
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Site tables	Oxidation (M)Sites.txt	Oxidation (M)Sites.txt

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6.3 Additional results

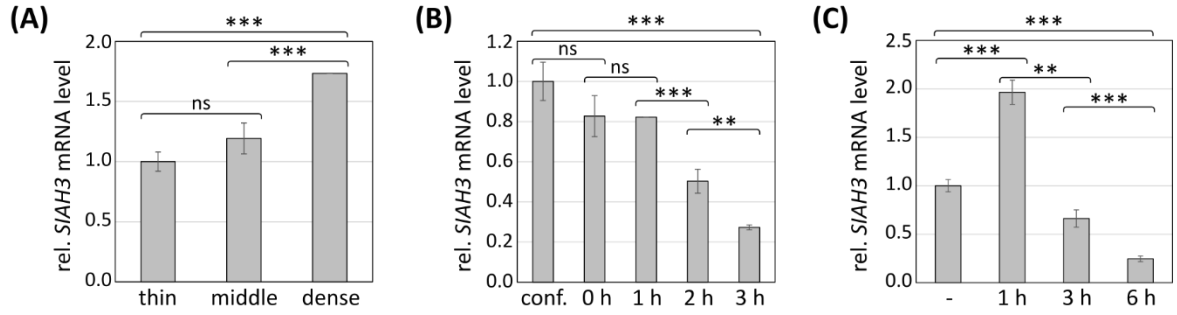


Fig. S17: *SIAH3* expression depends on cell density (replicates).

(A) Density dependence of *SIAH3* expression. Same numbers of C918 cells were cultured on dishes of 3 different sizes until the smallest was confluent. RNA was isolated and mRNA level was determined by RT-qPCR. *SIAH3* expression was normalised to *ACTB*. (B) Separation of cells led to a decrease in *SIAH3* expression. C918 cells were cultivated until being confluent, then separated by trypsin and kept isolated by rotating for 1 to 3 h at 37 °C. RNA was isolated at the indicated time points and *SIAH3* expression was determined by RT-qPCR. *SIAH3* expression was normalised to *ACTB*. (C) Stability of *SIAH3* mRNA. C918 cells were treated with α -amanitin (10 μ M) and RNA was isolated at the indicated time points. *SIAH3* expression was determined by RT-qPCR. *SIAH3* expression was normalised to *28S*-RNA.

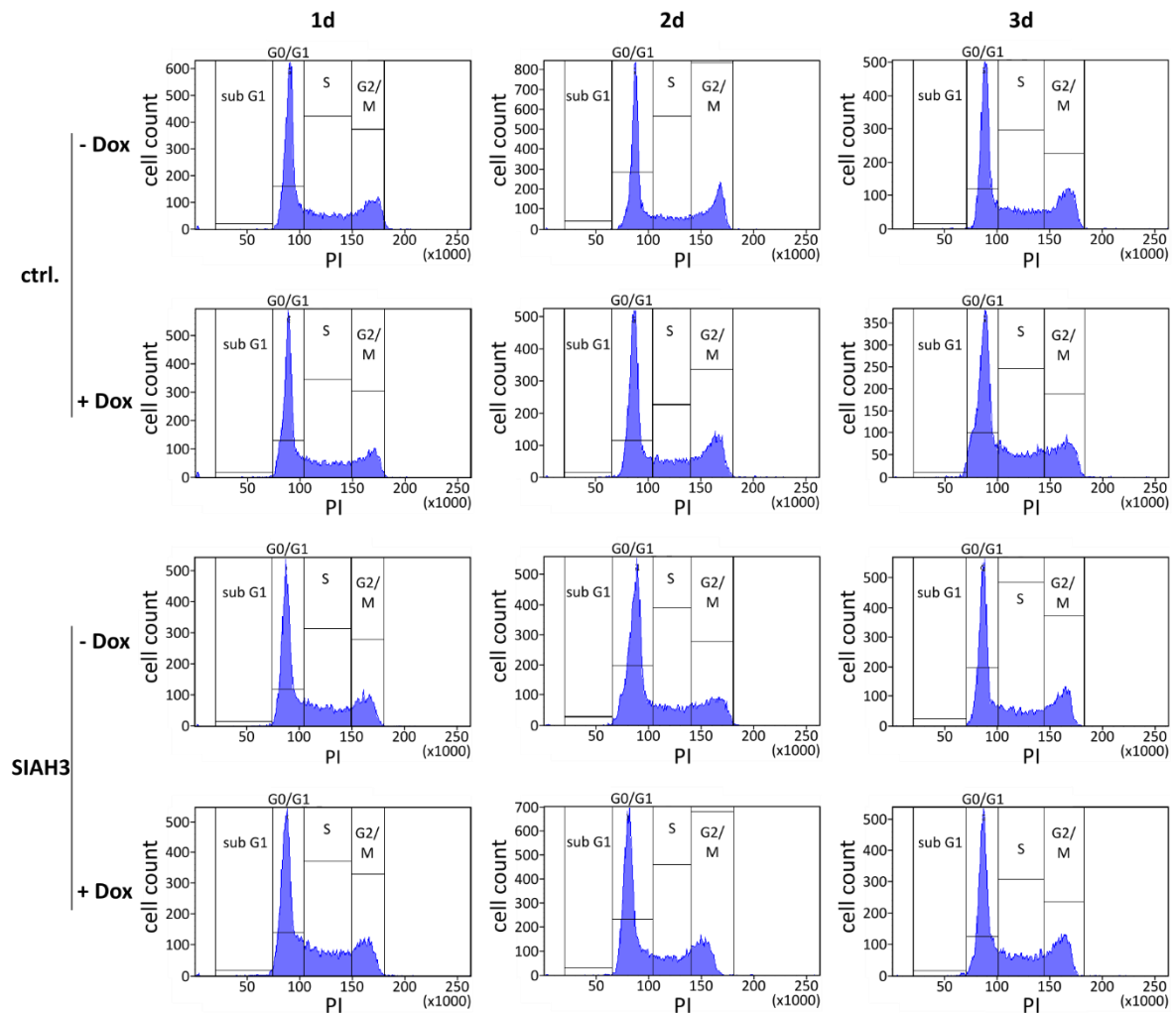


Fig. S18: *SIAH3* has no effect on cell cycle progression.

Cell cycle distribution of T-REx *SIAH3* cells was analysed by flow cytometry. Graphs depict the cell count per DNA-content, represented by the propidium iodide (PI) signal, gated to sub G1, G0/G1, S, and G2/M phases.

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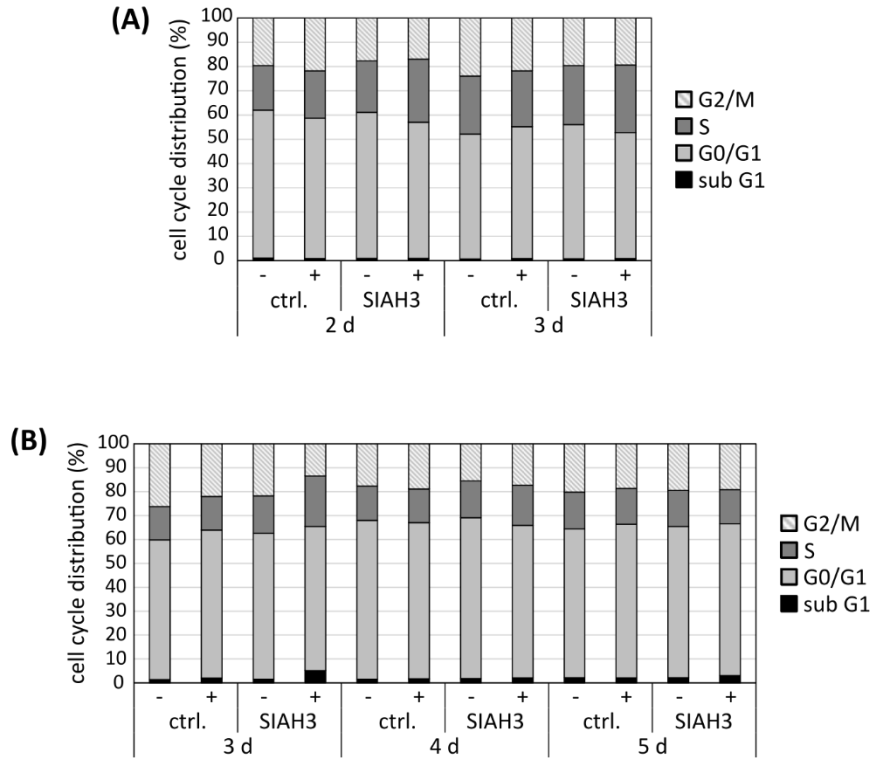


Fig. S19: Further experiments showing that *SIAH3* has no effect on cell cycle progression.

Cell cycle distribution of T-REx *SIAH3* cells (pool of 3 clones), compared to T-REx control cells, was analysed by flow cytometry and propidium iodide staining. **(A)** Analysis after 2 and 3 days of *SIAH3* induction. **(B)** Analysis after 3 to 5 days of *SIAH3* induction.

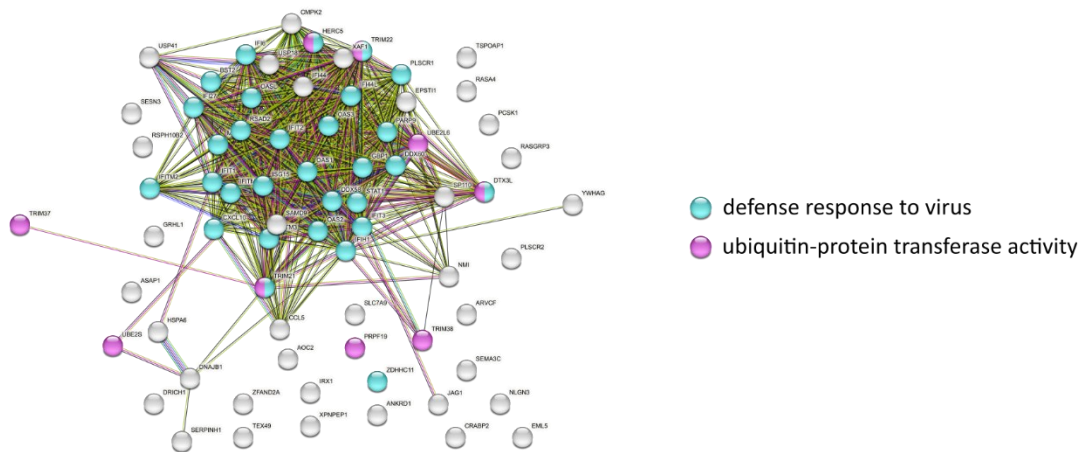


Fig. S20: Microarray analysis of another gene, that was analysed in the same microarray as *SIAH3*.

Functional enrichments within the protein-protein interaction network presenting the genes that are upregulated ≥ 2 or downregulated ≤ 0.5 (EGFP-IRX1 vs. EGFP-empty overexpression). PPI enrichment value: $< 1 \times 10^{-16}$. The genes are coloured according to the described enrichment groups: “Defense response to virus” is from the GO-term “Biological Process” (FDR = 6.68×10^{-33}), “ubiquitin-protein transferase activity” is from the GO-term “Molecular Function” (FDR = 0.0289). Analysis was performed with *STRING*.

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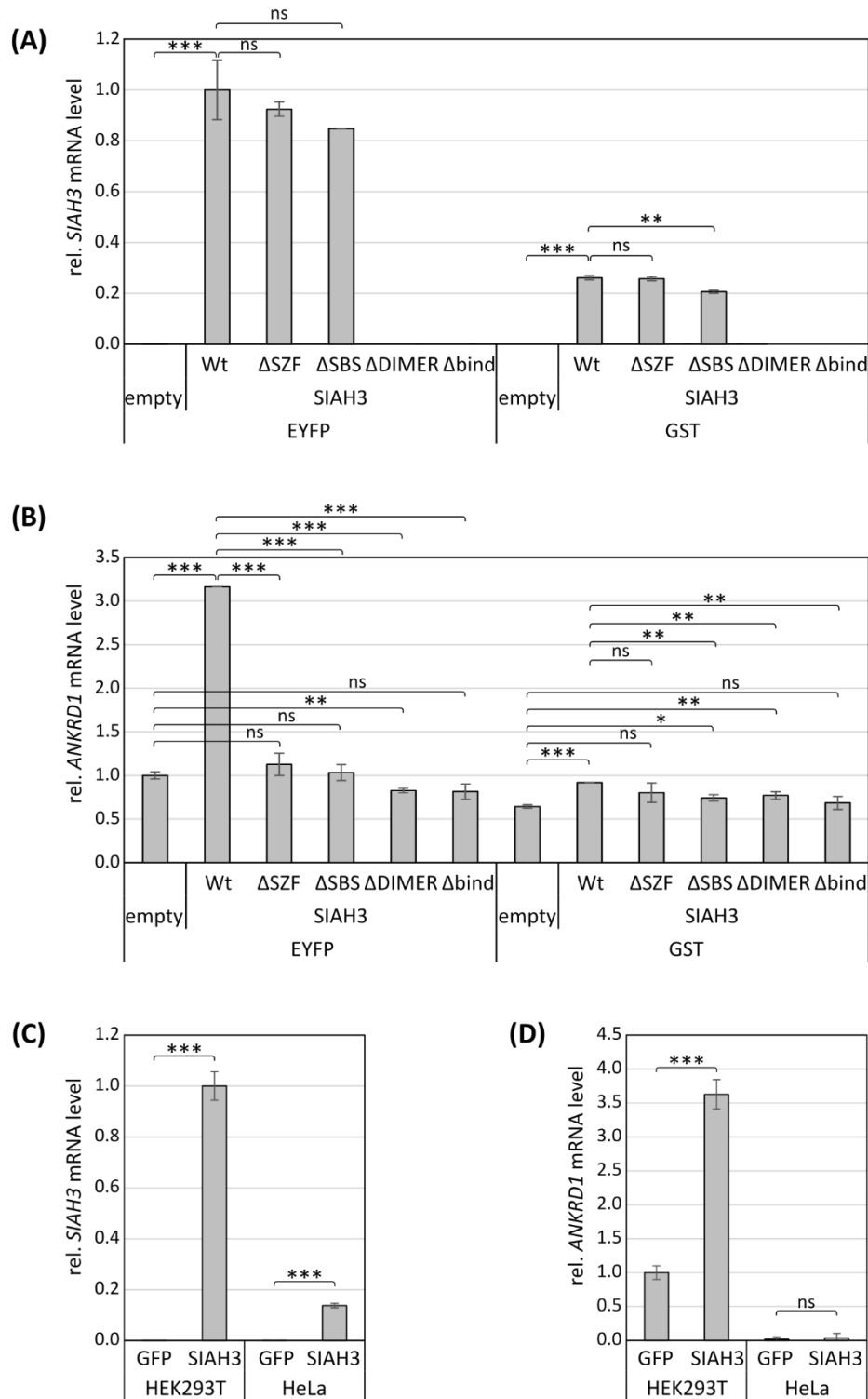


Fig. S21: *ANKRD1* is upregulated by *SIAH3* in HEK293T cells.

(A-B) EYFP-*SIAH3* (Wt/domain deletion mutants), GST-*SIAH3* (Wt/domain deletion mutants) or the corresponding empty controls were overexpressed in HEK293T cells for 48 h. RNA was isolated and *SIAH3* (A) and *ANKRD1* (B) mRNA level were determined by RT-qPCR. *SIAH3* and *ANKRD1* expression were normalised to *ACTB*. *SIAH3* expression of the Δ DIMER and Δ bind mutants are missing because primers bind within this region and therefore no amplification was possible. **(C-D)** EGFP-*SIAH3* or EGFP-empty were overexpressed in HEK293T or HeLa cells for 24 h. RNA was isolated and *SIAH3* (C) and *ANKRD1* (D) mRNA level were determined by RT-qPCR. *SIAH3* and *ANKRD1* expression were normalised to *ACTB*.

*/**/** indicate the significance levels based on Student's t-test.

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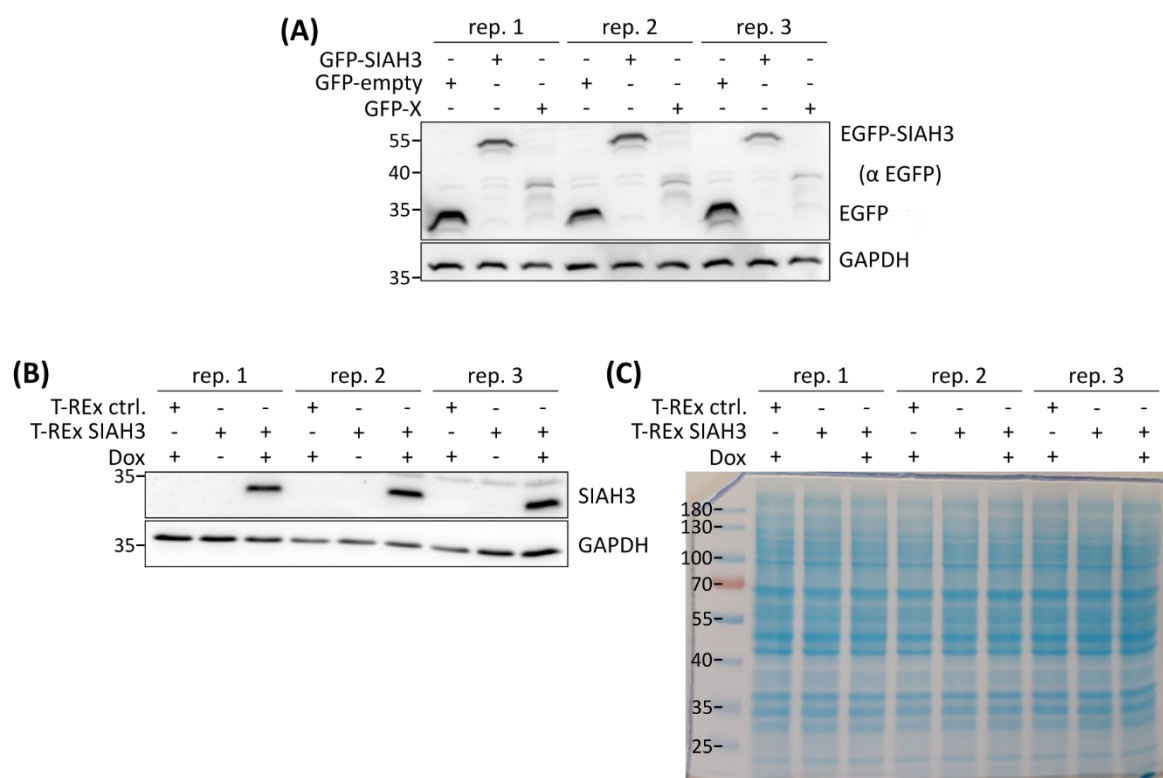


Fig. S22: WBs and Coomassie-staining belonging to the MS experiments.

(A) Successful overexpression of SIAH3. EGFP-SIAH3 or EGFP-empty were overexpressed in HEK293T cells for 24 h. Three independent experiments were performed. Proteins were isolated and an aliquot of the lysate was used to proof SIAH3 overexpression by WB. EGFP and SIAH3 were detected via an AB against the GFP-tag. GAPDH was detected using a protein-specific AB. GFP-IP was carried out using the remaining lysates, which were then sent for MS analysis. GFP-X: Another GFP-tagged protein of interest from a colleague that was analysed alongside SIAH3. The experiment was performed by Dr. A. Richter. **(B)** Successful induction of SIAH3. T-REx control cells or T-REx SIAH3 cells (pool of 3 clones) were uninduced or induced with Dox for 24 h. Three independent experiments were performed. Proteins were isolated and sent for MS analysis. In advance, an aliquot of the lysate was analysed by WB. SIAH3 and GAPDH were detected using protein-specific ABs. **(C)** Coomassie-staining of the corresponding SDS gel.

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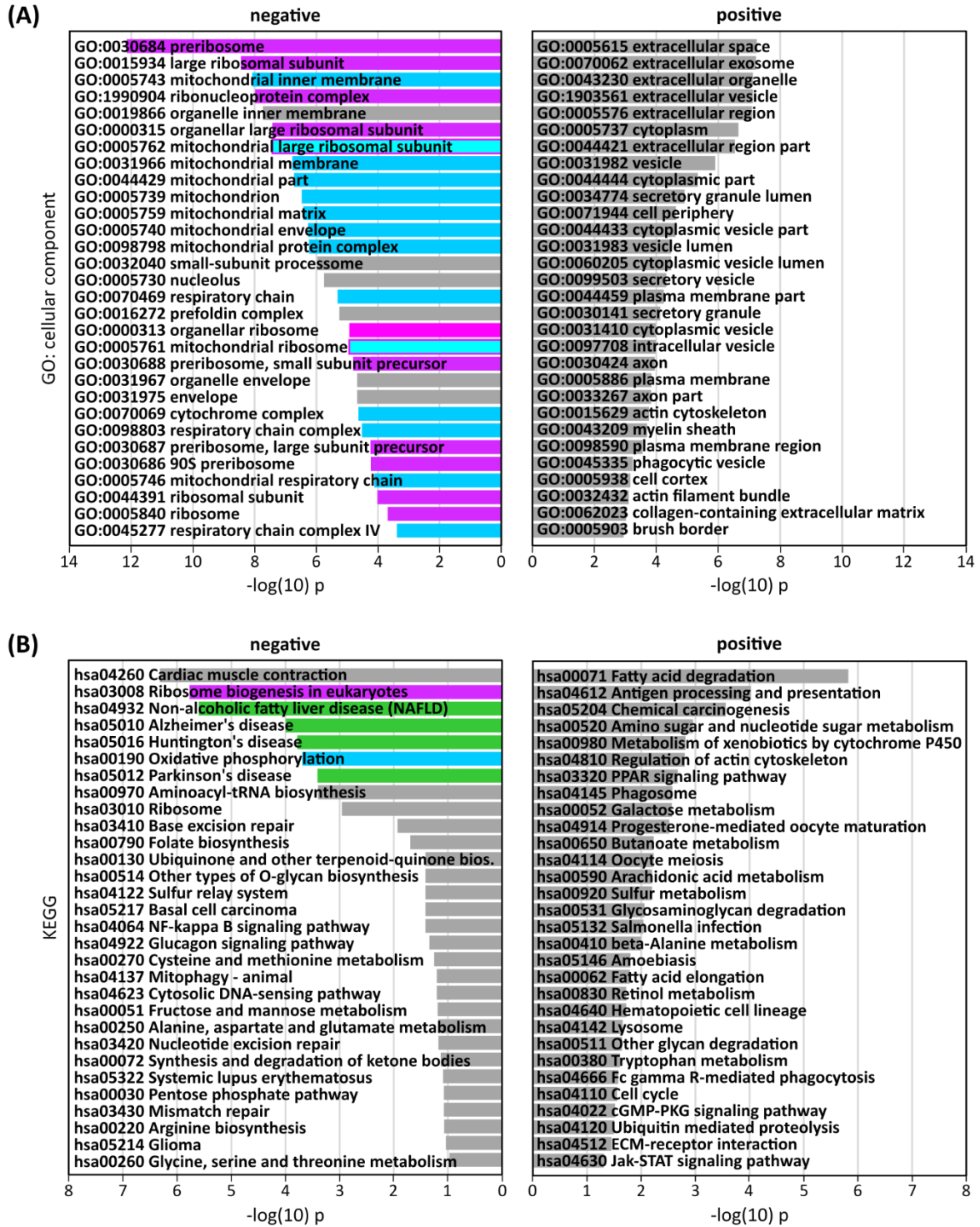


Fig. S23: SIAH3 expression is not correlated with the level of proteins associated with (pre-)ribosomes.

MS analysis of the whole proteome in the T-Rex SIAH3 cells (pool of 3 clones) compared with the proteome of T-Rex control cells (both induced with Dox for 24 h). **(A)** ORA of SIAH3 cells induced versus induced control cells regarding the GO database “cellular component”. The 30 most significantly over-represented terms (positively and negatively regulated) are depicted in p-values (-log10). **(B)** ORA utilizing the Kyoto Encyclopedia Genes and Genomes (KEGG) database. The 30 most significantly over-represented groups (positively and negatively regulated) are depicted in p-values (-log10).

Terms associated with the mitochondrial respiratory chain are coloured in blue, terms of diseases in which mitochondrial dysfunction plays an important role are coloured in green and terms related to the (pre-)ribosome are marked purple.

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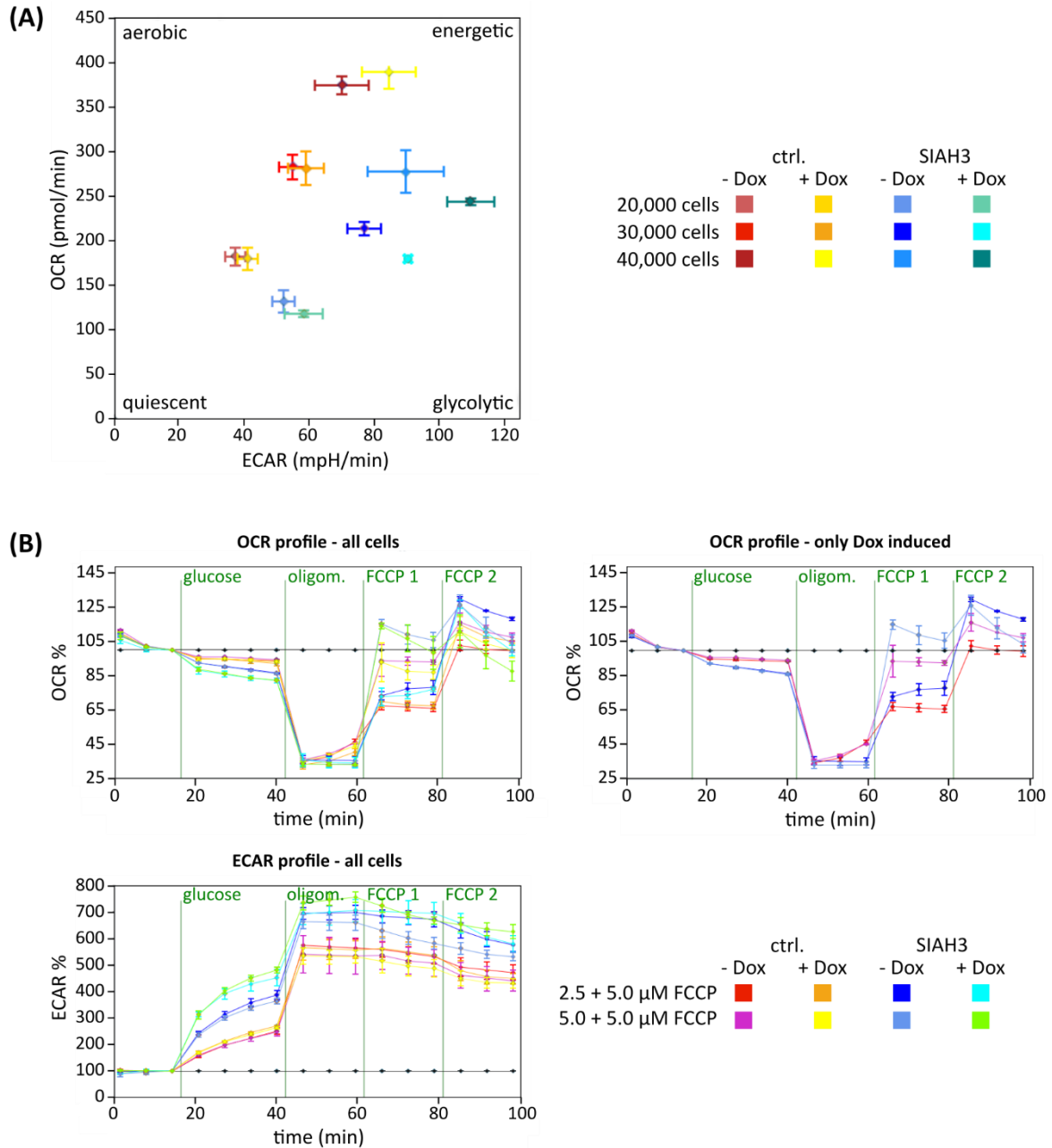


Fig. S24: Determination of the optimal cell count and FCCP concentration for real-time cell metabolic analyses.

20,000, 30,000 and 40,000 T-REx cells/well (T-REx-SIAH3 (pool of 3 clones) and control cells; induced or uninduced with Dox) were plated and after 24 h a modified mitochondrial stress test profile (with acute glucose injection, without Antimycin A/Rotenone addition, but with 4 different FCCP concentrations) was created. **(A)** Optimal cell count is 30,000 cells per well. Energy map showing OCR versus ECAR relation in the four different cell types and 3 different cell counts at measurement point 7 (induced level before oligomycin injection) from the modified mitochondrial stress test. **(B)** Optimal FCCP concentration ranges from 0.75 to 1 μ M. OCR and ECAR profiles of the modified mitochondrial stress test (30,000 cells/well). In a first injection step, 2.5 or 5.0 μ M FCCP (10x concentrated) were added, followed by a second injection with 5 μ M FCCP (10x concentrated). Thus, the resulting effect of 0.25, 0.5, 0.75 and 1.0 μ M FCCP was evaluated. The black line represents the baseline and the dots the different measurement points. Error bars represent the standard deviation of each measurement point.

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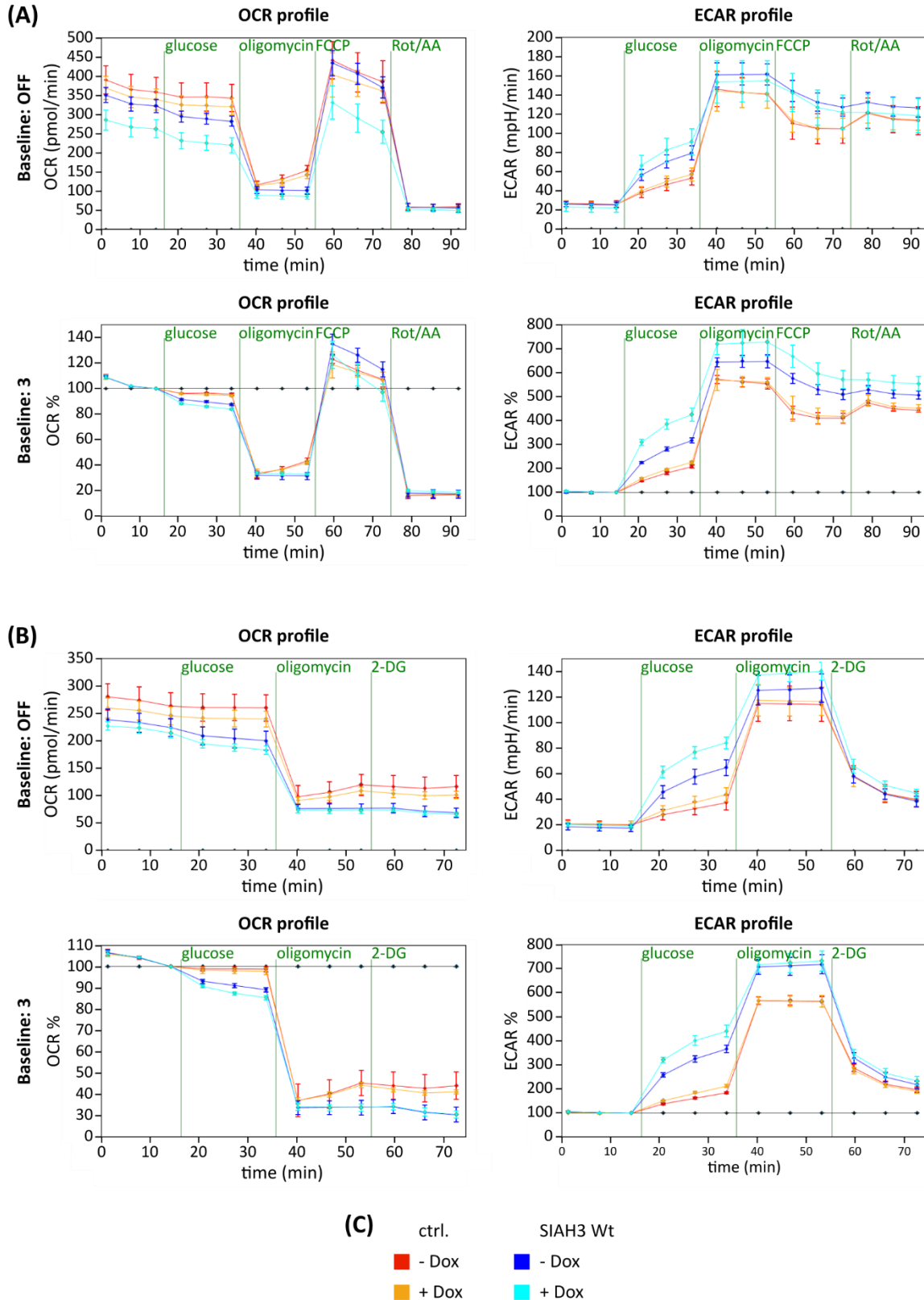


Fig. S25: OCR and ECAR profiles of the mitochondrial and glycolysis stress tests of SIAH3 Wt cells.

Uninduced (-Dox) and induced (+Dox) T-Rex SIAH3 (Wt) and T-Rex control cells were analysed using the Seahorse XF96 Analyzer. All used drugs are indicated. The profiles correspond to the experiments shown in main Fig. 43A-C and show the OCR or ECAR level in respect to the time (without baseline or with baseline (100%) set at measurement point 3). **(A)** OCR and ECAR profiles of the mitochondrial stress test. **(B)** OCR and ECAR profiles of the glycolysis stress test. **(C)** Legend for graphs in A and B. The black lines in the graphs represent the baseline and the dots the different measurement points. Error bars represent the standard deviation of each measurement point. OCR profiles of panel A adapted from Deutschmeyer *et al.*, 2025.

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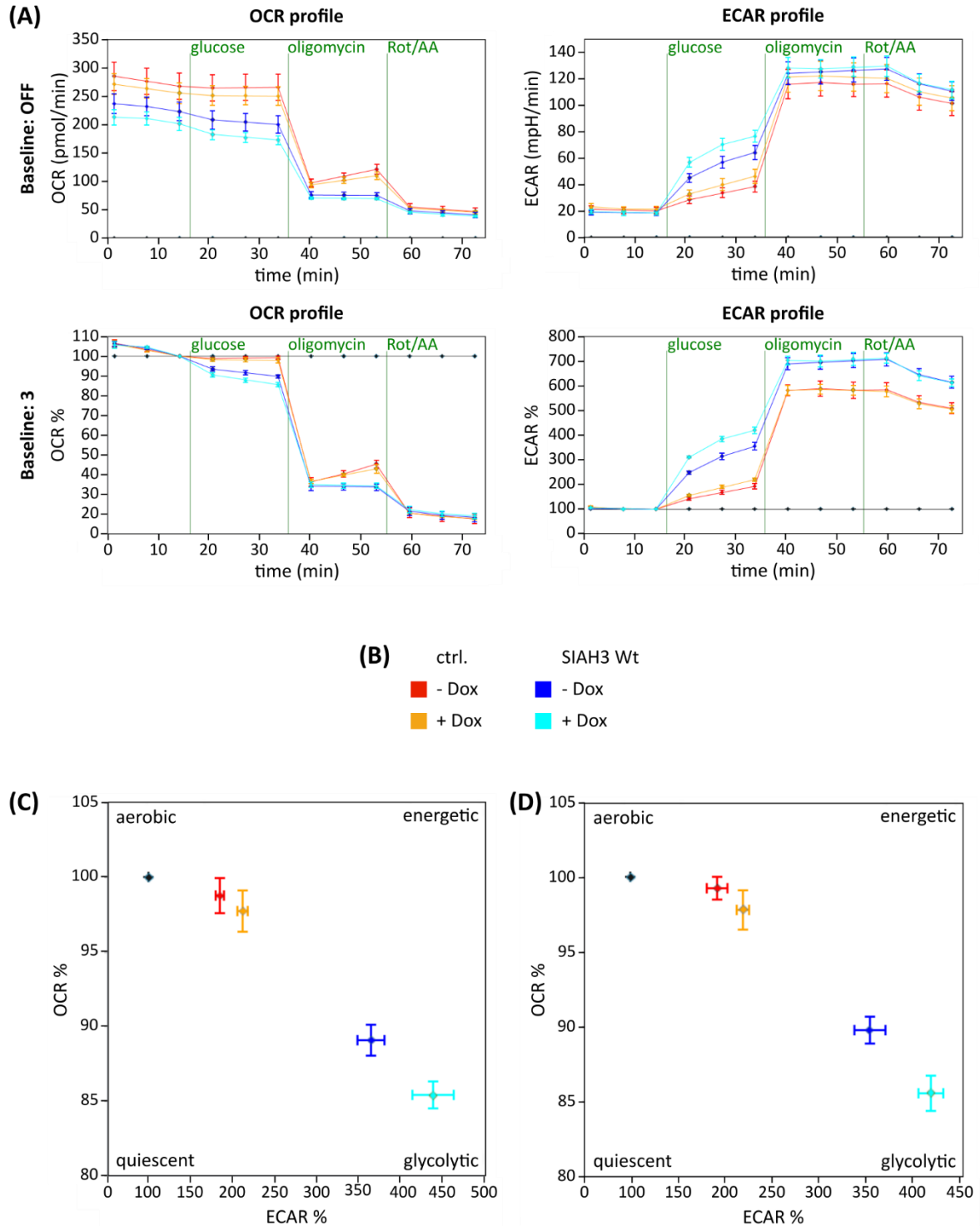


Fig. S26: OCR and ECAR profiles of the ATP rate assay and energy maps from the glycolysis stress test and the ATP rate assay (T-Rex-ctrl. and SIAH3 Wt).

Uninduced (-Dox) and induced (+Dox) T-Rex SIAH3 (Wt) and T-Rex control cells were analysed using the Seahorse XF96 Analyzer. All used drugs are indicated. **(A)** OCR and ECAR profiles corresponding to the ATP rate assay experiment shown in main Fig. 43D. The OCR or ECAR levels are depicted in respect to the time, without baseline (upper row) or with baseline (100%) set at measurement point 3 (lower row). The black line in the graph represents the baseline and the dots the different measurement points. **(B)** Legend for graphs in A, C and D. **(C)** Energy map derived from the glycolytic stress test (main Fig. 43B). **(D)** Energy map derived from the ATP rate assay (main Fig. 43D). The graphs show the OCR (induced level after glucose injection) versus ECAR relation in the four different cell types at measurement point 6 (after glucose addition), baseline set at measurement point 3 (before glucose addition).

Error bars represent the standard deviation of each measurement point.

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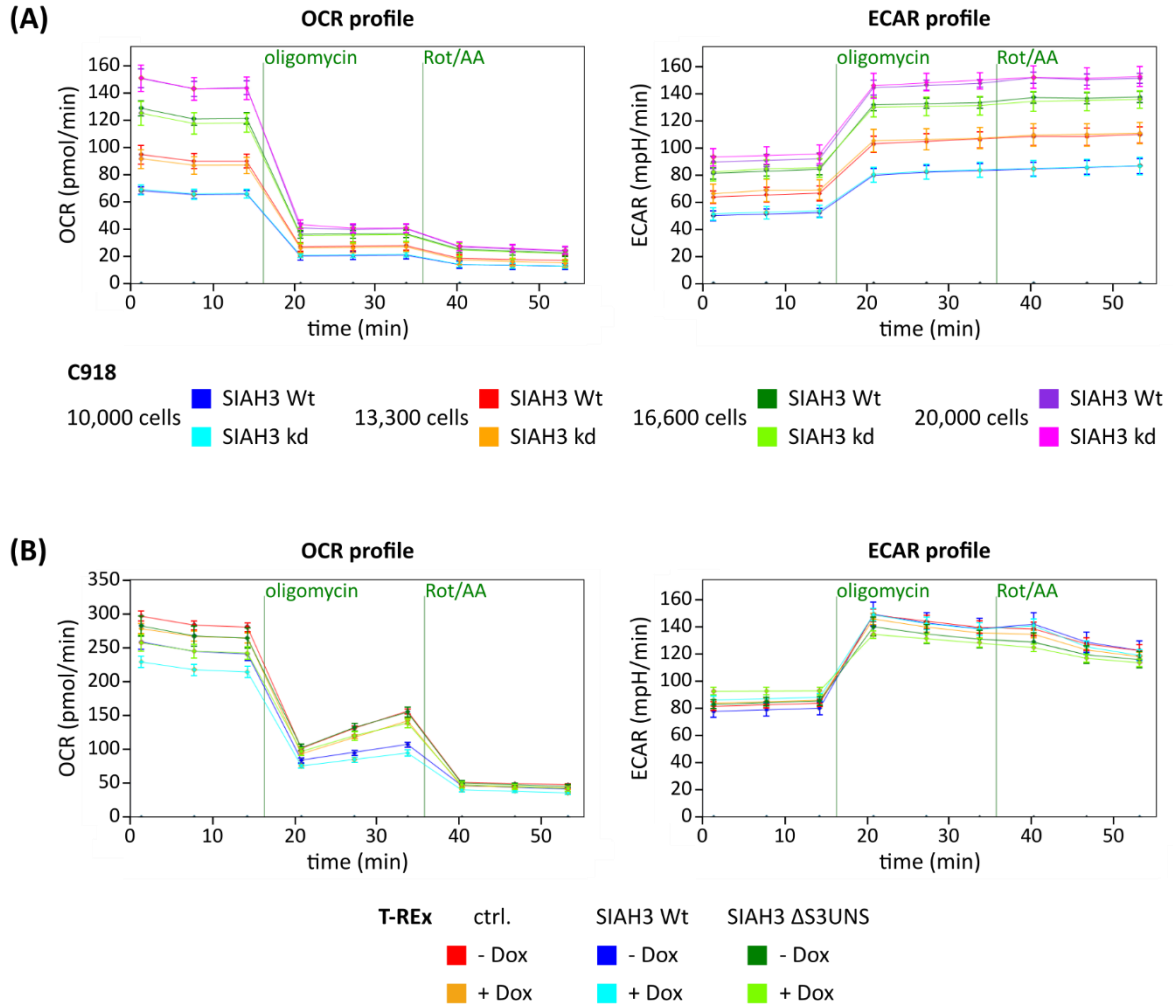


Fig. S27: OCR and ECAR profiles of the ATP rate assays of C918 and T-REx cells incl. SIAH3 ΔS3UNS

(A) C918 Wt and SIAH3 knockdown cells (kd) were analysed using the Seahorse XF96 Analyzer. All used drugs are indicated. OCR and ECAR profiles correspond to the ATP rate assay experiment shown in main Fig. 44. The OCR or ECAR levels are depicted in respect to the time (with no baseline set). **(B)** Uninduced (-Dox) and induced (+Dox) T-REx SIAH3 Wt, SIAH3 ΔS3UNS and control cells were analysed using the Seahorse XF96 Analyzer. All used drugs are indicated. OCR and ECAR profiles correspond to the ATP rate assay experiment shown in main Fig. 45. The OCR or ECAR levels are depicted in respect to the time (with no baseline set). The dots in the graph near the x-axis represent the different measurement points. Error bars represent the standard deviation of each measurement point. OCR profiles adapted from Deutschmeyer *et al.*, 2025.

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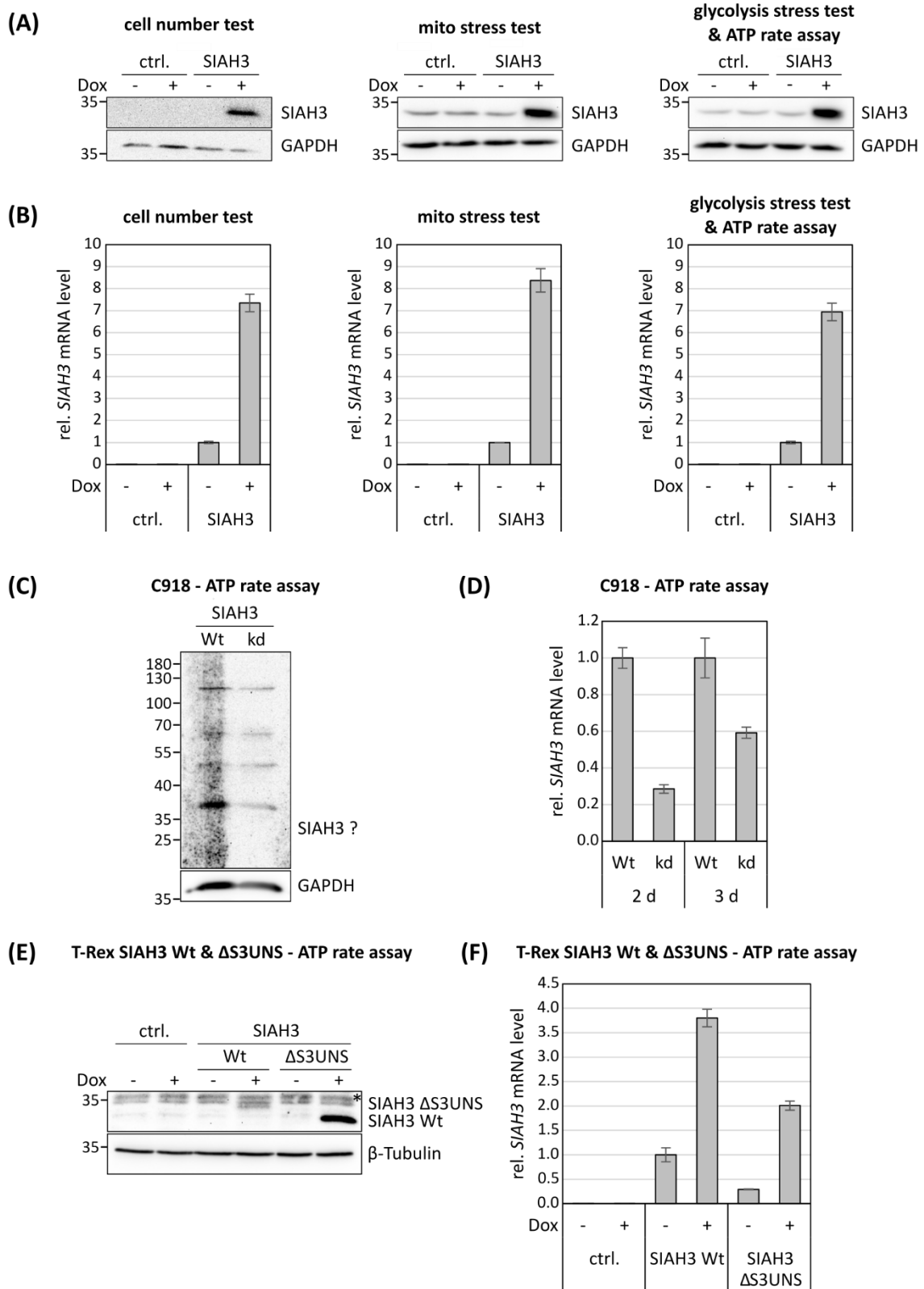


Fig. S28: Western blots and qPCRs prove the intended SIAH3 expression for the real-time cell metabolic analyses

(A) & (B) T-REx SIAH3 and control cells were induced with Dox for 24 h. On the day of cell metabolism analysis, protein and RNA were isolated, too. **(A)** Protein levels of SIAH3, corresponding to the three experiments shown in main Fig. 43 and supplementary Fig. S24, were analyzed by SDS-PAGE and WB. SIAH3 and GAPDH were detected using protein specific ABs. **(B)** The respective mRNA expression levels of *SIAH3* were determined by RT-qPCR, normalised to *ACTB*. **(C) & (D)** SIAH3 protein and mRNA corresponding to the ATP rate assay in C918 cells (see Fig. 44). **(C)** Proteins were isolated after two days of siRNA treatment, the day the cells were plated on the Seahorse cell plate (one day before measurement). No clear signal for SIAH3 was

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observed in the WB. SIAH3 and GAPDH were detected using protein specific ABs. **(D)** qPCR, however, proved a significant reduction of *SIAH3* mRNA after siRNA treatment. RNA was extracted both on the day of cell seeding and on the day of metabolic analysis, followed by RT-qPCR (*SIAH3* mRNA levels were normalised to *ACTB*). **(E) & (F)** SIAH3 protein and mRNA corresponding to the ATP rate assay in T-Rex control, SIAH3 Wt & SIAH3Δ S3UNS cells. (see Fig. 45). Protein and RNA were isolated at the day of measurement). **(E)** SIAH3 protein levels were analysed by SDS-PAGE and WB. SIAH3 and GAPDH were detected using protein specific ABs. * unspecific band. **(F)** The respective mRNA expression levels of *SIAH3* were determined by RTR-qPCR, normalised to *ACTB*.

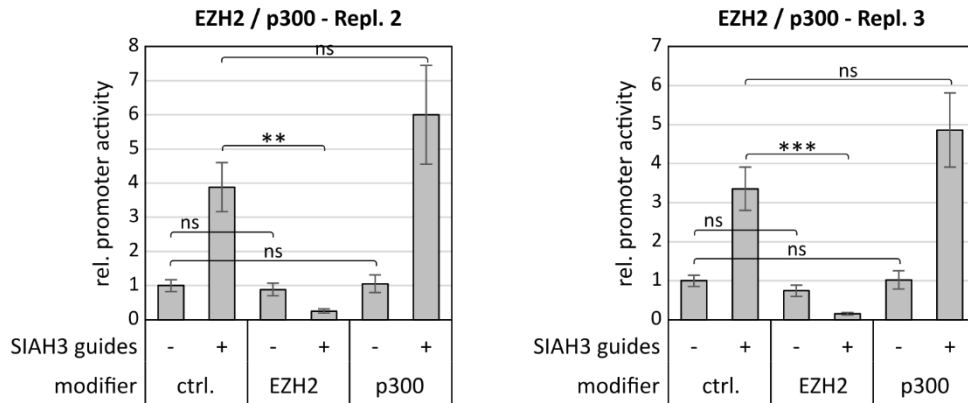


Fig. S29: Epigenetic regulation of an artificial *SIAH3* promoter construct by histone modifiers.

SIAH3 promoter activity was quantified using the Dual-Luciferase Reporter Assay. EZH2 and p300 (core), coupled to an inactive Cas9 (dCas9), were recruited by a mix of 3 sgRNAs, to the artificial *SIAH3* promoter-reporter-construct. Experiments were conducted in HEK293T. Histone methyltransferase EZH2 represses, while histone acetyltransferase p300 slightly activates the *SIAH3* promoter. Shown are replicates of the experiment depicted in Fig. 52A.

Acknowledgements

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To all my friends: Thank you for your encouragement, patience and support – thank you for these valuable friendships that helped me to get through this time!

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Eidesstattliche Erklärung

Eidesstattliche Erklärung

„Ich erkläre: Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Ich stimme einer evtl. Überprüfung meiner Dissertation durch eine Antiplagiat-Software zu. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

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 - ☒ Verstehen von Konzepten, Recherche von Fakten und Definitionen
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Folgende KI-Tools habe ich verwendet, damit aufgeführte Teile meines Textes von dem Tool wie folgt profitiert haben:

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- ChatGPT (OpenAI)
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Ich habe die KI-Tools (v.a. JLU kiChat und ChatGPT) hauptsächlich zum Verbessern der englischen Sprache einzelner Sätze verwendet. Dafür habe ich den Satz selbst formuliert und dann sprachlich korrigieren lassen. Bei jeder Veränderung habe ich dann selbst überprüft, ob sie den Sinn trifft, den ich ausdrücken möchte. Ebenfalls habe ich die KI dazu genutzt, die richtige Schreibweise von Wörtern wie z.B. von speziellen Chemikalien oder Gennamen herauszufinden. Weiterhin habe ich die KI-Tools zur Recherche von Fakten, Erklärungen und Hintergrundinformationen genutzt (v.a. Perplexity AI). Auch habe ich mit Hilfe der KI-Tools nach passender Literatur / Veröffentlichungen gesucht. Bei Literaturverweisen habe ich mir die Quelle genau angeschaut und kontrolliert, ob sie wirklich die besagte Information beinhaltet. Außerdem habe ich Lösungsvorschläge der KI bei Softwareproblemen genutzt (z.B. bei Schwierigkeiten mit Inkscape oder Layoutproblemen in Word). Insgesamt wurde jede Information von mir nach besten Möglichkeiten überprüft und hinterfragt.