H2AK119ub DUB screen post-heat shock

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Abstract

Polycomb repressive complex (PRC) 1 and 2 histone modifications H2AK119ub and H3K27me3 together mediate genetic silencing. This silencing is critically important for healthy stem cell biology. Heat shock leads to destabilization of these modifications at different kinetic rates, suggesting enzymatic removal of H2AK119ub. Here we sought to investigate the existence of deubiquitinating enzymes or a potential recruiter ZRF1 via shRNA knockdown screen. Furthermore, we investigated the effect of ZRF1 on Polycomb subunit CBX2 and cell survival. We observed no genes screened here to be responsible, individually or together, for H2AK119ub deubiquitination during heat shock, nor does ZRF1 appear to recruit enzymes involved in this. ZRF1 also does not appear to influence CBX2 behaviour. Further we show that K562 cells are reliant on ZRF1 expression to survive and even more reliant during stress conditions. Our findings show a continued gap-in-knowledge regarding the deubiquitination of H2AK119ub during heat shock, calling for further exploration. We eliminated some potential associations ZRF1 may have had with PRC1. Lastly, we show a reliance on ZRF1 for survival and proliferation, particular during stress.

Tags: Polycomb Repressive Complex, PRC1/2, Polycomb group protein family, PcG, H2AK119ub, H2A, H2B, H3, Deubiquitination, Ubiquitin, DUB, Heat Shock, BAP1, USP, MYSM1, ZRF1

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Screening for DUBs involved in post-heat shock H2AK119ub depletion

Tight regulation of self-renewal and lineage commitment is critical to maintaining lasting stem cell populations. Epigenetic modifications are key for dynamic and selective activation or silencing of genes. One epigenetic regulatory pathway specifically related to self-renewal and lineage commitment is the Polycomb group (PcG) protein family, first identified in *Drosophila Melanogaster* controlling *Hox* genes (Lewis 1978). The PcG family influences signalling through post-translational modifications on histone tails (Zhu et al., 2005, Di Croce & Helin 2013). Within the PcG family Polycomb Repressive Complexes 1 and 2 (PRC1/2) mediate important distinct epigenetic modifications on Polycomb target genes (figure 1). PRC2 trimethylates Histone 3 at lysine (K) residue 27 through subunits EZH1/2 to form H3K27me3 (Cao et al., 2002, Czermin et al., 2002 Kuzmichev et al., 2002). Canonical PRC1 recognizes H3K27me3 via its CBX subunit, through E3 ubiquitin ligases RING1A/B then mediates ubiquitination of Histone 2A at lysine residue 119 to form H2AK119ub (de Napoles et al., 2004, Wang et al., 2004, Buchwald et al., 2006, Tamburri et al., 2020). H2A ubiquitination has also been found to occur independently from PRC2 (Cooper et al., 2014, Blackledge et al., 2014). Specifically, non-canonical PRC1.1 can ubiquitinate H2AK119 independently of H3K27me3 (Tavares et al., 2012, Morey et al., 2013). This PRC2 independent pathway relies on the recognition CpG islands via subunit KDM2B to ubiquitinate H2AK119 via RING1A/B (van den Boom et al., 2016). Co-occupancy of Polycomb target genes by PRC1 and PRC2 histone modifications causes transcriptional repression (Bracken et al., 2006, Wang et al., 2006, Lee et al., 2007, Nakagawa et al., 2008, Blackledge et al., 2020).

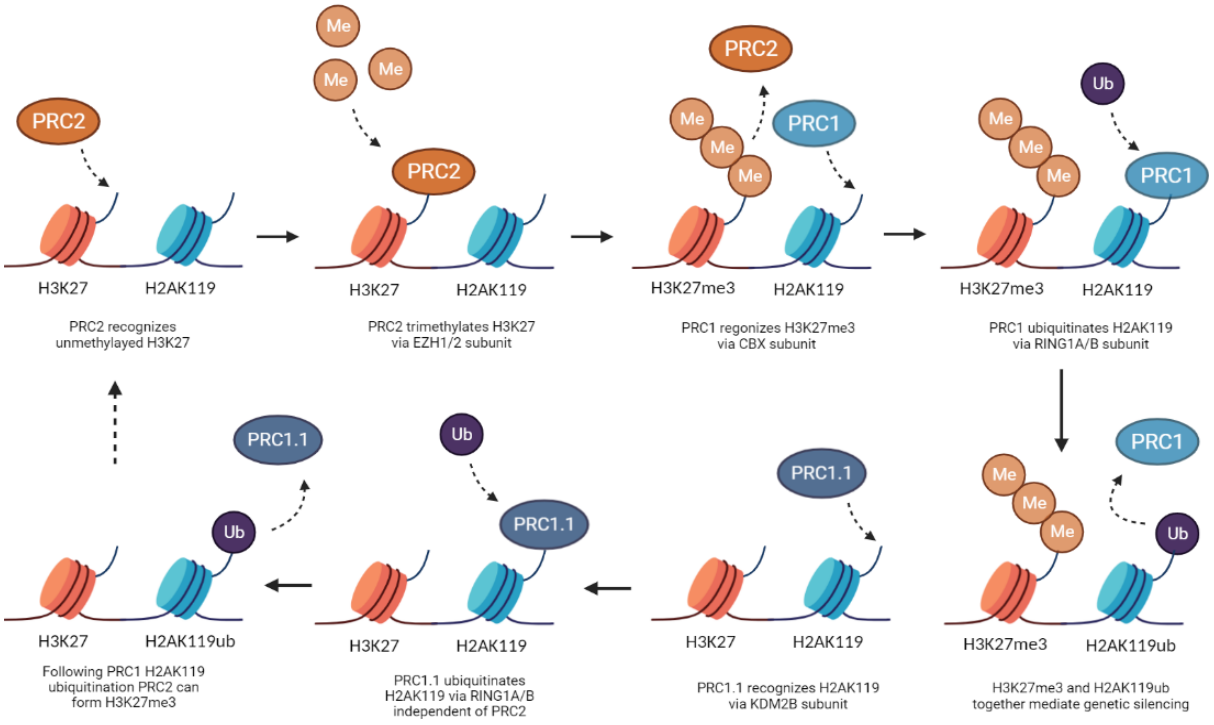

Figure 1. Simplified depiction of Polycomb Repressive Complex interactions with histones. PRC2 mediates trimethylation of Histone 3 at lysine residue 27 via its EZH1/2 subunits forming H3K27me3. Canonical PRC1 can recognize H3K27me3 via its CBX subunit and ubiquitinate Histone 2 at lysine residue 119 forming H2AK119ub. Alternatively non-canonical PRC1 can ubiquitinate H2A independently from H3K27me3 via subunit KDM2B. Co-occupancy of H3K27me3 and H2AK119ub leads to genetic silencing of the Polycomb target gene.

Disruption of PRC1 or 2, and thus disruption of epigenetic modifications, may give rise to various diseases. Loss of PRC1/PRC2 and variant PRC1 impairs self-renewing ability in mouse embryonic stem cells (Zepeda-Martinez et al., 2020). In humans haematopoietic stem cells (HSCs) exemplify a reliance on the PRC for self-renewal, survival and symmetrical division. A double knock-out of *BMI1*, which encodes the critical PRC subunit PCGF4, results in depletion of progenitor cells and increased differentiation of HSCs as symmetrical division is hampered (Lessard and Sauvageau 2003, Iwama et al., 2004, Rizo et al., 2008, Rizo et al., 2009, Schuringa & Vellenga 2010). CBX subunit variants haven been shown to play an important role in preserving balance between self-renewal and differentiation in HSCs. CBX7 promotes repression of progenitor-specific genes, when overexpressed in HSCs may lead to enhanced self-renewal causing leukaemia. Whereas overexpression of CBX2/4/8 brings about exhaustion of said HSCs (Klauke et al., 2013). These examples emphasize the role of and reliance on PRC for healthy stem cell biology.

Recently, Azkanaz et al., showed that Heat Shock (HS) destabilizes PRC1/2 from chromatin leading to epigenetic instability. Through LC-MS/MS analysis of the nuclear proteome before and after HS they observed that various PRC1 and PRC2 subunits, epigenetic regulators, HS proteins and proteasome components, were enriched in the nucleolus post-HS. Based on these and other data it is likely that nucleoli serve as a location for protein quality control (PQC) (Azkanaz et al., 2019). PQC removes erroneously folded proteins from cells through refolding or proteasomal degradation (Mimnaugh et al., 1997, Frottin et al., 2019). For this PQC is partly reliant on ubiquitin to mark proteins for degradation. In a model by Groothuis et al., it is proposed that freely accessible ubiquitin, under physiological conditions, is limitedly available in cells. When stress, such as HS, occurs ubiquitin demand sharply rises as unfolded and misfolded proteins accumulate quickly. This coincides with a rapid depletion of free ubiquitin within the cell and chromatin bound ubiquitin such as H2AK119ub (Carlson & Rechsteiner 1987, Mimnaugh et al., 1997, Dantuma et al., 2006).

HS also influences Polycomb epigenetic marks asides from PRC1/2 subunits, HS proteins, and proteasome components. Strikingly, H2AK119ub levels were nearly completely abolished after HS from Polycomb target genes, ALOX15, TCF21 and PAX7, whilst H3K27me3 diminished to a lesser extent. Upon closer observation Azkanaz et al., found that the removal rate of H2AK119ub was far higher than that of H3K27me3. Asides from H2AK119ub H2BK120ub is also deubiquitinated during HS (unpublished data). Based on this observed kinetic difference in removal we hypothesize that H2AK119ub deubiquitination is mediated by deubiquitinating enzymes (DUBs) during. DUBs for

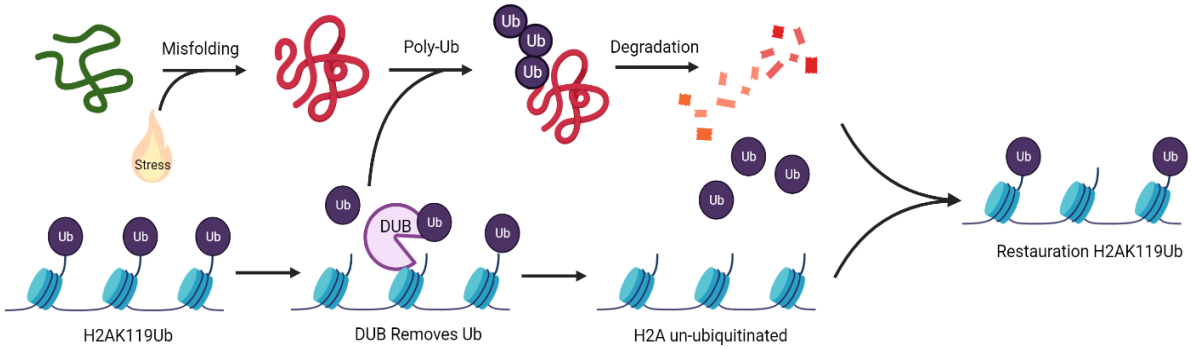

Figure 2. Ubiquitin flow during HS model. Under physiological conditions there is a limited supply of ubiquitin within cells. Upon HS mediated stress chromatin bound ubiquitin is rapidly made available via Deubiquitinating Enzymes (DUBs). Additional ubiquitin is used in Protein Quality Control to deal with misfolded proteins by means of proteasomal degradation. When normal conditions are restored, free ubiquitin is returned to chromatin.

specific genes are known to exist, such as BAP1 deubiquitinating *SLC7A11* (Zhang et al., 2019) thus DUBs may also act during HS. Taking inspiration from the model by Groothuis et al., and adapting it include ubiquitin behaviour during HS that includes DUBs we would imagine figure 2. Within this project we will try to answer the question: which DUB is responsible for the removal of H2K119ub under HS conditions? Based on previous research we selected candidate DUBs that have been found to be involved with or facilitate the deubiquitination of H2AK119ub (table 1).

Gene	Method of DUB	Author
BAP1	Associated with BRCA1, active DUB in the PR-DUB complex, BAP1 knockdown increases steady state H2AK119ub levels but does not prevent loss of H2Ak119ub after HS	Scheuermann et al., 2010, Nature
USP3	DUB activity in the context of S-phase progression and DNA Damage response	Nicassio et al., 2007, Curr Biol Lancini et al., 2014 J Exp Med
USP7	Potential role of DNMT1-associated deubiquitintylation, not in the context of HS-induced DUB activty since earlier experiments using USP7 inhibitor do not show effects of H2AK119ub loss after HS	Yamaguchi et al., 2017, Sci Rep
USP12	WDR48/WDR20 can independently and desynergistically activate USP12 activity, also DUB of PHLPP1 thereby supressing Akt dependent cell survival, developmental defects upon morpholino injection in Xenopus	Joo et al., 2011, J Biol Chem
USP16	H2AK119ub DUB at HoxD locus, counteracts Polycomb silencing, important for viability of hematopoietic mature and progenitor cell populations	Joo et al., 2007, Nature Cai et al., 1998, NAS Gu et al., 2016, PNAS
USP21	Expression levels correlate with H2AKub levels	Nakagawa et al., 2008, Genes Dev
USP22	Component of the SAGA co-activator complex and functional homolog of SAGA-associated Ubp8 in yeast. Mainly identified as H2B DUB but also has H2A DUB activity in vitro	Zhang et al., 2008 Mol Cell Zhao et al., 2008 Mol Cell
USP46	WDR48/WDR20 can independently and desynergistically activate USP12 activity, also DUB of PHLPP1 thereby supressing Akt dependent cell survival, developmental defects upon morpholino injection in Xenopus	Joo et al., 2011, J Biol Chem
MYSM1	Positive regulator AR-mediated txpn, unique domain structure (JAMM/MPN+ AND SANT, SWIRM domains) associated with but not recruited by p/CAF HAT. pCAF activity may affect 2a-DUB activity since preferred DUB acivity on hyperacetylated histones	Zhu et al., 2007, Mol Cell

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Table 1: DUB candidate genes and the explanation as to why these genes may mediate deubiquitnation.

Materials and Methods

Cell Transductions and Culture Conditions

HEK293T cells were cultured in DMEM 10% FCS 1% Pen-Strep at 37 °C 5% CO_2. K562 cells were cultured in RPMI 10% 1% Pen-Strep at 37 °C 5% CO_2. E. Coli bacteria were grown on LB agar plates containing 1:1000 ampicillin, or in LB broth with 1% ampicillin at 37 °C. For stress experiments three different culture conditions were used. HS: transduced K562 cells were plated and subjected to 44 °C for 20, 30 or 60 minutes. Samples were taken at desired timepoints and placed on ice before further experiments. For growth curves after HS cells were cultured at 37 °C 5% CO2. Irradiation: transduced cells were exposed to 5 Gray Cesium-137 radiation following exposure cells were cultured at 37 °C 5% CO_2. Hypoxia: transduced cells were cultured at 37 °C 1% O_2.

Hairpin Table

Below is the table containing the shRNAs used in this study their target gene and respective targeted area. Table 2 contains DUB candidate shRNA sequences, table 3 contains ZRF1 shRNA sequences.

Target Gene	shRNA Sequence	Targetting Area
BAP1-I	GCATGAAGGACTTCACCAAGG	ORF
USP3-I	CCTTGGGTCTGTTTGACTT	ORF
USP3-II	CCAAATCATCATTCACCAA	3'UTR
USP3-III	CACCCGCACTTCAGTTTAT	3'UTR
USP3-IV	GCAGCTGTTAAACAGTAAA	3'UTR
USP7-IV	GCAGAGAAAGGTGTGAAATTC	ORF
USP7-VI	GCGGTCACCGACCATGATATT	ORF
USP12-I	GCGTATAAGAGTCAACCTAGG	ORF
USP12-II	GCAAACAGGAAGCACACAAAC	ORF
USP12-III	GCCCATGATTCTGTCAGTAAA	3'UTR
USP16-I	GGAATGGAATATCTGCCAAGA	ORF
USP16-II	GCATGCCTTGAAGCACTATCT	ORF
USP16-III	GGTGTTACGTATGTGATAATG	ORF
USP21-I	GCTAGAAGAACCTGAGTTAAG	ORF
USP21-II	GCGAGAGGACAGCAAGATTGT	ORF
USP21-III	GCACCTGTGAAGCCCTTTAAA	3'UTR
USP22-II	GCATCATAGACCAGATCTTCA	ORF
USP22-III	GCACTTAAAGAATTCACATGG	3'UTR
USP46-I	GAGGGATCGCTGCAACCTTTA	5'UTR
USP46-III	GCCTCCATCTGTAATATGGGC	ORF
MYSM1-I	GGATAACACCATCAGTGAAGA	ORF
MYSM1-II	GGACGAGTTGTCTTCTCAAAC	ORF
MYSM1-III	GCTGTAGGCTGTGAAACTAAG	3'UTR

Table 2: DUB candidate shRNA sequences

Oligo annealing and ligation

Annealing buffer was mixed with oligos (48:2) for each respective hairpin. Heated to 95 °C for 4 minutes then actively cooled to 70 °C for 10 minutes before passively cooled to room temperature. Annealed oligos were mixed with gel-purified pLKO.1 mCherry plasmid cut open with EcoRI and AgeI, ligation buffer, and T4 DNA ligase, ligation reactions were incubated overnight at 16 °C.

Bacterial transformation

Ligated constructs were transformed into TOP10 E. Coli. Briefly, ligated constructs were mixed with competent E. coli, incubated on ice, briefly heat shocked for 1 minute at 42 °C. TOP10 were given 1ml of LB without antibiotics and kept at 37 °C for 45 minutes. TOP10 were subsequently plated onto agar plates with 1:1000 ampicillin and cultured at 37 °C overnight upside down.

Miniprep analysis

Single colonies of plated bacteria were transferred into 2.5 ml LB 1:1000 ampicillin and cultured overnight at 37 °C. Following day, 2 mL bacteria culture was spun down, 5000 rpm for 3 minutes, supernatant was discarded. Pellets were resuspended in 250 uL resuspension buffer by vortex. Next, 250 uL lysis buffer was added contents were mixed briefly and 350 uL neutralization buffer was added. Contents were spun down at maximum speed for 5 minutes. Supernatant was transferred onto spin column and spun at 14k rpm for 1 minute. Flow through was discarded after which 500 uL washing buffer was added to the spin column, spun for 1 minute at 14k rpm, this was repeated once, column was spun dry for 1 minute at maximum speed. A collection cup was placed underneath, 50 uL elution buffer was pipetted directly onto column membrane, spun for 2 minutes max speed. Isolated plasmid concentration was determined using Nanodrop. Plasmids were stored at -20 °C.

Sequence Validation and digest gel control

5 uL of Isolated plasmid DNA was diluted in FastDigest Green Buffer 10x and mixed with FastDigest XhoI cutting enzyme. Mix was stored at 37 °C for 10 minutes. Digested DNA fragments were loaded onto a 2% agarose gel with 1:10 000 SYBR Safe dye. Gel ran for 60 minutes at 120V and visualized using UV light. DNA bands in the gel were verified using a protein ladder and XhoI cut empty vector pLKO.1 for control. Plasmids not matching desired fragment size were discarded. 450 ng of correctly sized plasmid DNA along with 5 uL 5 pmol/ uL forward and reverse primers mix was sent to Eurofins for Sanger sequencing. This data was compared to the ordered hairpin oligo information to validate hairpin integrity in plasmids. Chromas analysis software was used to compare sequences with table 1 sequences for hairpins.

Rubidium chloride method for competent E. Coli.

Protocol used to generate TOP10 competent E. Coli. Psi broth (5 g/L of yeast extract, 10 g/L Tryptone, 5 g/L magnesium sulphate at pH 7.6 adjusted with potassium hydroxide) inoculated culture was kept at 37 °C for 3 hours, and constantly shaken. Bacteria were transferred into cups on ice, spun down and supernatant was discarded. Bacteria were resuspended in Tfbl (30mM Potassium Acetate, 100mM Rubidium Chloride, 10mM Calcium Chloride, 50mM Manganese Chloride, 15% v/v Glycerol at pH 5.8 adjusted with acetic acid) buffer for 15 minutes on ice. Bacteria were spun down at 0 °C 3500 rpm. Supernatant was removed and bacteria were resuspended in TfbII (10mM MOPS, 75mM Calcium Chloride, 10mM Rubidium Chloride, 15% v/v Glycerol at pH 6.5 adjusted with Natrium Hydroxide). Bacteria were filled out in 1.5 ml cup, flash frozen with liquid nitrogen and stored at -80 °C.

Lentivirus production

HEK293T cells were cultured in DMEM 10% FCS 1% Pen-Strep at 37 °C 5% CO_2. Packaging construct pCMV Δ8.91/PAX2, Glycoprotein envelope plasmid VSV-G and a plasmid containing hairpin of choice

were mixed with FugeneHD. The plasmid mixture was added dropwise to HEK293T cells and left overnight. Cells were checked for mCherry fluorescence to assess initial transfection success. DMEM was replaced with RPMI 10% FCS 1% Pen-Strep and left overnight. Lentivirus containing medium was harvested, filtered, and stored at -80 °C in labelled cryotubes. pLKO.1 vector containing non-targetting scrambled sequences were used for negative control, transduction with these would only lead to the expression of mCherry in transduced cells.

Lentiviral Transduction

Single Gene – USP and ZRF1 Hairpins

500.000 K562 cells in 1.5 mL were plated onto a 12 wells plate. 500 uL of one shRNA containing lentivirus was added to culture along with 1:1000 8ug/mL polybrene. Cells were left at 37 °C 5% CO_2 overnight. Lentivirus containing medium was washed away using 8 mL PBS 5-10% FCS thrice. Cells were resuspended in RPMI 10% FCS 1% Pen-Strep. To confirm transduction efficacy samples of cell populations were subjected to LSR flow cytometry scanning mCherry positive colouration to indicate success. Transductions aimed at the MYSM1 gene had low success rate, as measured by low mCherry positivity percentage of the total cell population. To assure reliable data MYSM1 transduced K562s were sorted using a 70 µm nozzle tip on day 3 post transduction.

Multiple Genes – USP Hairpins

To target multiple genes identical procedures were taken except instead of 1 shRNA containing lentivirus lentiviruses aimed at all potential DUB genes were added. These were selected based on knockdown success found with single gene transductions. Selected were USP 3-1 USP 12-1, USP 16-2, USP 21-3, USP 22-2, and USP 46-3. Either 100 uL of each lentivirus aimed at potential DUBs for a total of 700 uL or 400 uL of each was added for a total of 2.8 mL, both conditions had corresponding concentrations of polybrene added, 1:1000 8ug/ml.

KD validation

On day 4 after transduction cells RNA was isolated using RNeasy Plus mini kit. Cells were lysed using RLT+ Buffer. Genomic DNA was eliminated using spin column. Then RNA was dissolved in RNase free water. Through RT-PCR 500ng RNA was turned into cDNA using iSCRIPT cDNA Synthesis Kit. cDNA was diluted in 380 uL H_2O and stored at -20 °C.

qPCR

5 uL cDNA for each cell population was loaded on to a 384 wells plate. 5 uL Sybersafe and 0.2 uL primer mix, containing forward and reverse primers, for the gene of interest were added. qPCR was then preformed for detailed knockdown success validation (n=3), RLP27 served as housekeeping throughout.

Western Blot

Western blot analysis utilized the following primary antibodies: Rabbit anti H2AK119ub (1:1000), Rabbit anti-H3 unmodified (1:1000), Rabbit anti-H2BK120ub (1:1000), Goat anti-β-Actin (1:1000). Primary antibodies are incubated overnight in cold room. Secondary antibodies: Rabbit anti-Goat (1:5000) Swine anti-Rabbit (1:5000). Secondary antibodies were incubated for 90 minutes at room temperature on rollers. Each lane was loaded with 10 uL sample containing approximately 50k cells.

Cytospin

Cells transduced with lentiviruses containing desired hairpin were cytospinned onto glass slides. Subsequently cells were fixated using 4% paraformaldehyde solution in PBS. Paraformaldehyde was washed away using PBS 0.1% Triton X-100 twice. Finally, glasses were then washed using PBS and

dried. Cells were stained using Vectashield with DAPI and sealed in. Fluorescence microscopy was used to visualize DAPI stained DNA, GFP-CBX2 and verify mCherry positivity from transduction.

Results

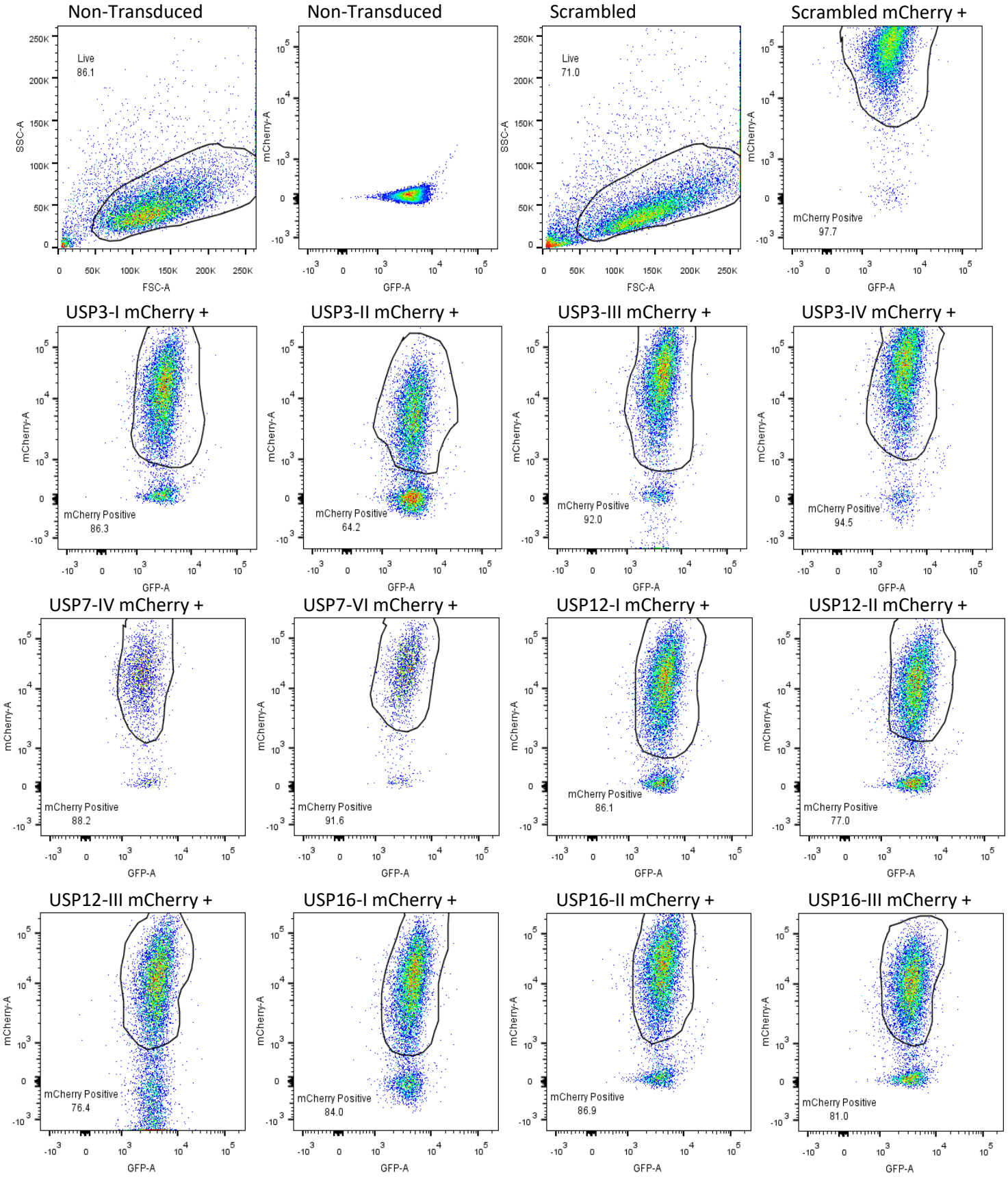
HS is a form of cellular stress leading to unfolding of proteins and is accompanied by rapid deubiquitination of chromatin (Frottin et al., 2019, Azkanaz et al., 2019). The kinetics of this process suggest that chromatin is actively deubiquitinated upon HS. This project focuses on the identification of the DUB(s) responsible for this rapid deubiquitination of H2AK119ub. For this, we used a shRNA approach, where we knocked down previously identified H2AK119ub DUBs (table 1). We generated lentiviral vectors targeting our candidate DUBs and transduced K562 cells. In this first set of experiments we validated the knockdown efficiencies of the various shRNAs and tested their effect of post-HS loss of H2AK119ub.

Validation of DUB shRNA lentiviral vectors

To confirm lentiviral transduction success K562 cells were subjected to flow cytometry four days after transduction before further experiments. Cell sample data was first gated for life cells (data not shown). Flow cytometry data showed clear mCherry positive signal indicating successful transduction of cells (figure 3). MYSM1 lentiviruses were found to have low success on initial transduction therefor newly transduced K562-MYSM1 cells were sorted for mCherry positivity on day three post transduction. MYSM1 transduced cells were then again validated on day four post transduction, for mCherry positivity (figure 3).

shRNAs are successful in knocking down DUBs

On day four post transduction, cell samples were taken followed by RNA isolation and subsequent cDNA synthesis. Next, qPCR analysis was performed to assess knockdown efficiencies. mRNA expression of designated DUB was compared to housekeeping gene RPL27. Overall, for each candidate DUB at least one shRNA sequence was found to cause a significant knockdown (figure 4). Of note, some hairpins did not induce a significant knockdown for their target gene (i.e., USP3-3; figure 4), while they were used in subsequently H2AK119ub level analyses after HS. The results related to this shRNA should therefore be disregarded in figure 5.


Non-Transduced
Live
86.1
SSC-A
FSC-A
Non-Transduced
mCherry-A
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USP3-II mCherry +
mCherry Positive
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USP3-III mCherry +
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94.5
USP7-IV mCherry +
mCherry Positive
88.2
USP7-VI mCherry +
mCherry Positive
91.6
USP12-I mCherry +
mCherry Positive
86.1
USP12-II mCherry +
mCherry Positive
77.0
USP12-III mCherry +
mCherry Positive
76.4
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mCherry Positive
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USP16-III mCherry +
mCherry Positive
81.0

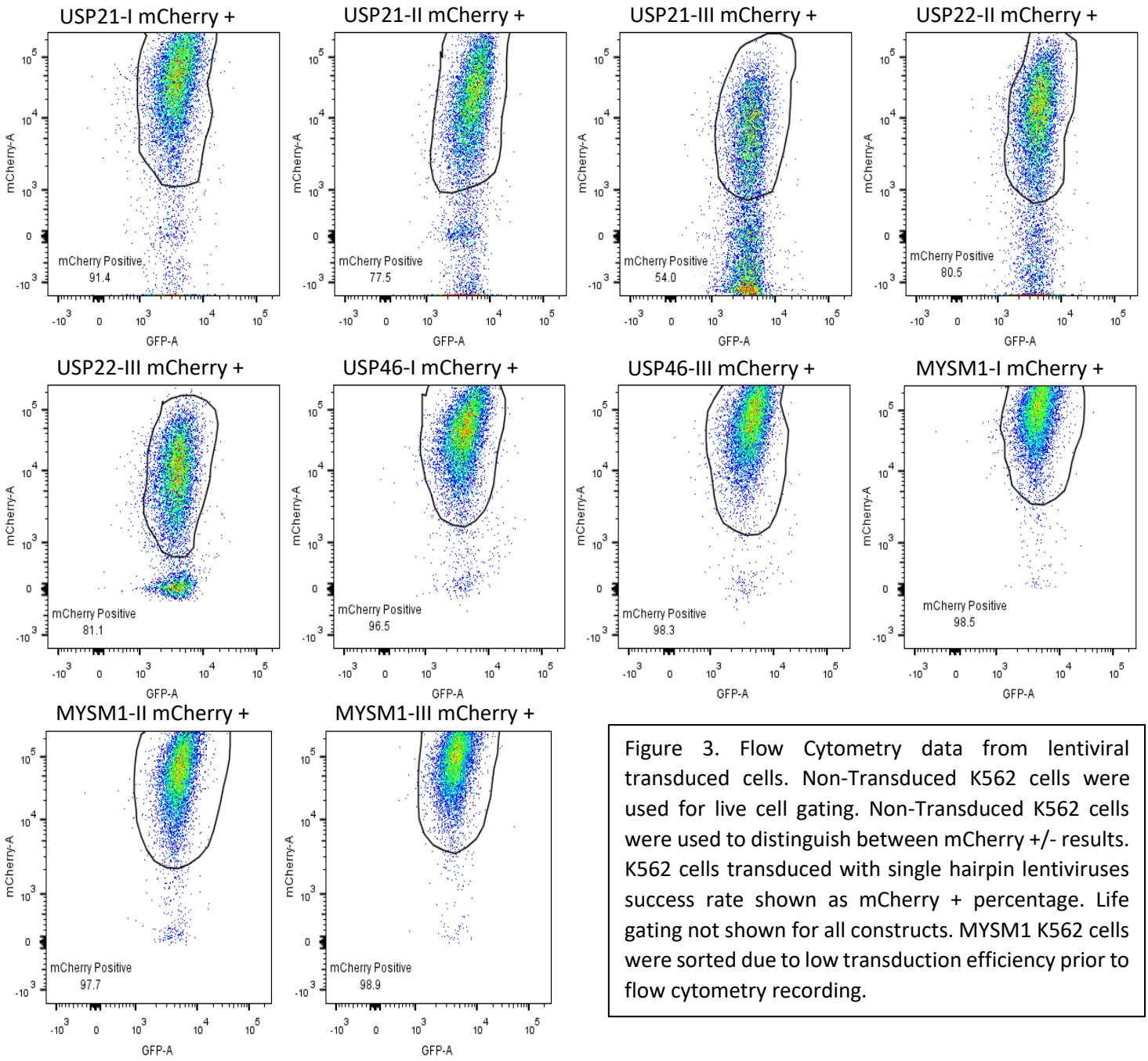


Figure 3. Flow Cytometry data from lentiviral transduced cells. Non-Transduced K562 cells were used for live cell gating. Non-Transduced K562 cells were used to distinguish between mCherry +/- results. K562 cells transduced with single hairpin lentiviruses success rate shown as mCherry + percentage. Life gating not shown for all constructs. MYSM1 K562 cells were sorted due to low transduction efficiency prior to flow cytometry recording.

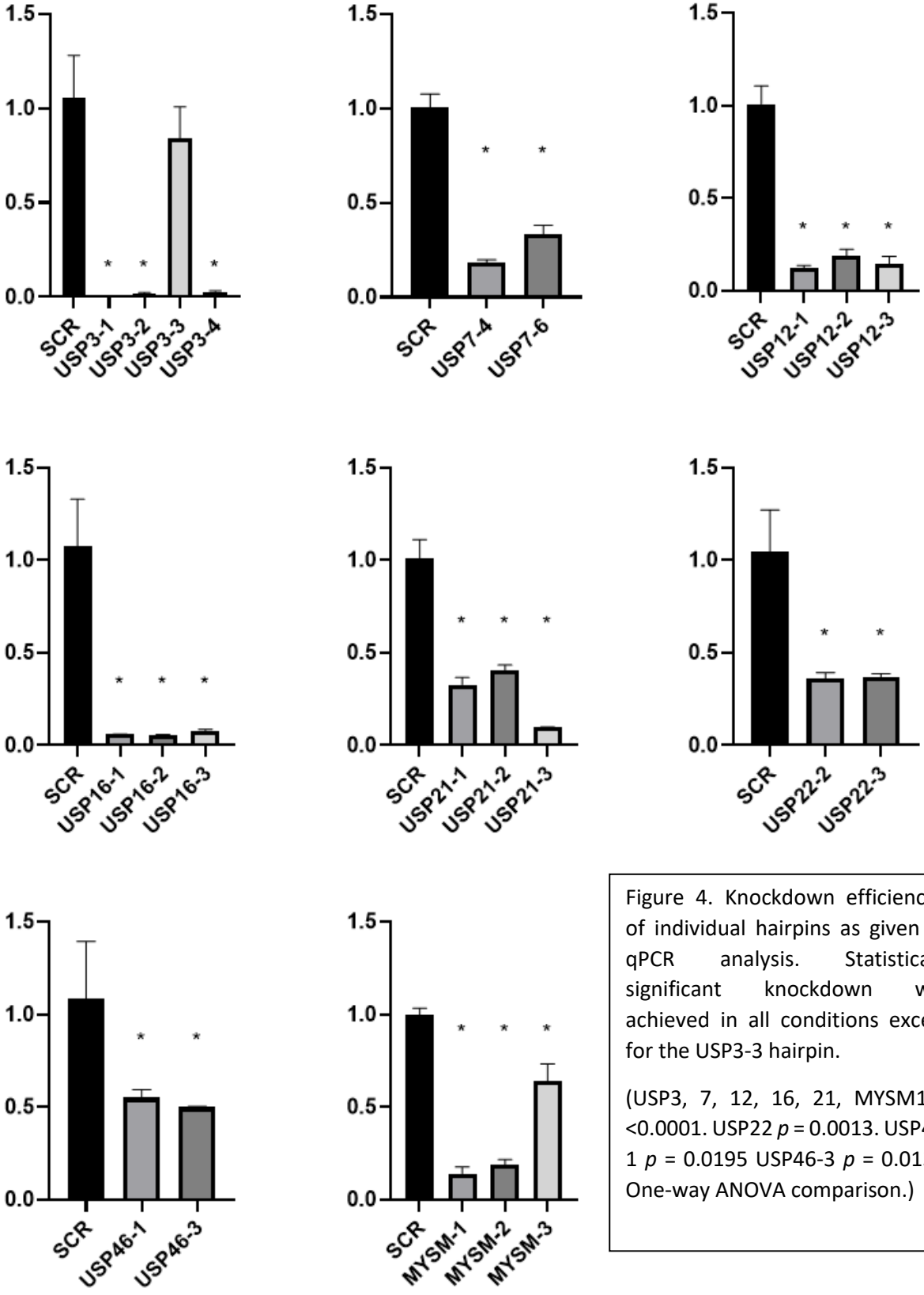


Figure 4. Knockdown efficiencies of individual hairpins as given by qPCR analysis. Statistically significant knockdown was achieved in all conditions except for the USP3-3 hairpin.

(USP3, 7, 12, 16, 21, MYSM1 p <0.0001. USP22 p = 0.0013. USP46-1 p = 0.0195 USP46-3 p = 0.0130. One-way ANOVA comparison.)

Individual DUB knockdowns do not prevent H2AK119ub deubiquitination after HS

Next, we analysed the effect DUB knockdown on H2AK119ub levels, before and directly after HS. Transduced K562 cells were subjected to a 30 min 44 °C HS to induce H2AK119ub deubiquitination. In these western blot analyses we also included H2BK120, a non-Polycomb histone mark (Wojcik et al., 2018), that has also been found to be diminished post-HS (Van den Boom et al., personal communication). Cells expressing SCR hairpins functioned as negative control and actin served as housekeeping gene. As expected, control cells showed a strong decrease in H2AK119ub upon HS (figure 5). Upon knockdown of a DUB involved in post-HS H2AK119ub deubiquitination one would expect an impaired loss of H2AK119ub after HS. However, for the DUBs screened there was still a reduction in H2AK119ub observed in post-HS samples. Reductions were still observed in H2B120ub compared to pre-HS samples (figure 5). Suggesting that none of the investigated genes, on their own, mediate DUB activity in HS conditions. There still is the possibility that redundancy exists between DUBs, meaning that multiple genes may need to be knocked down before changes in post-HS levels of H2AK119ub are visible.

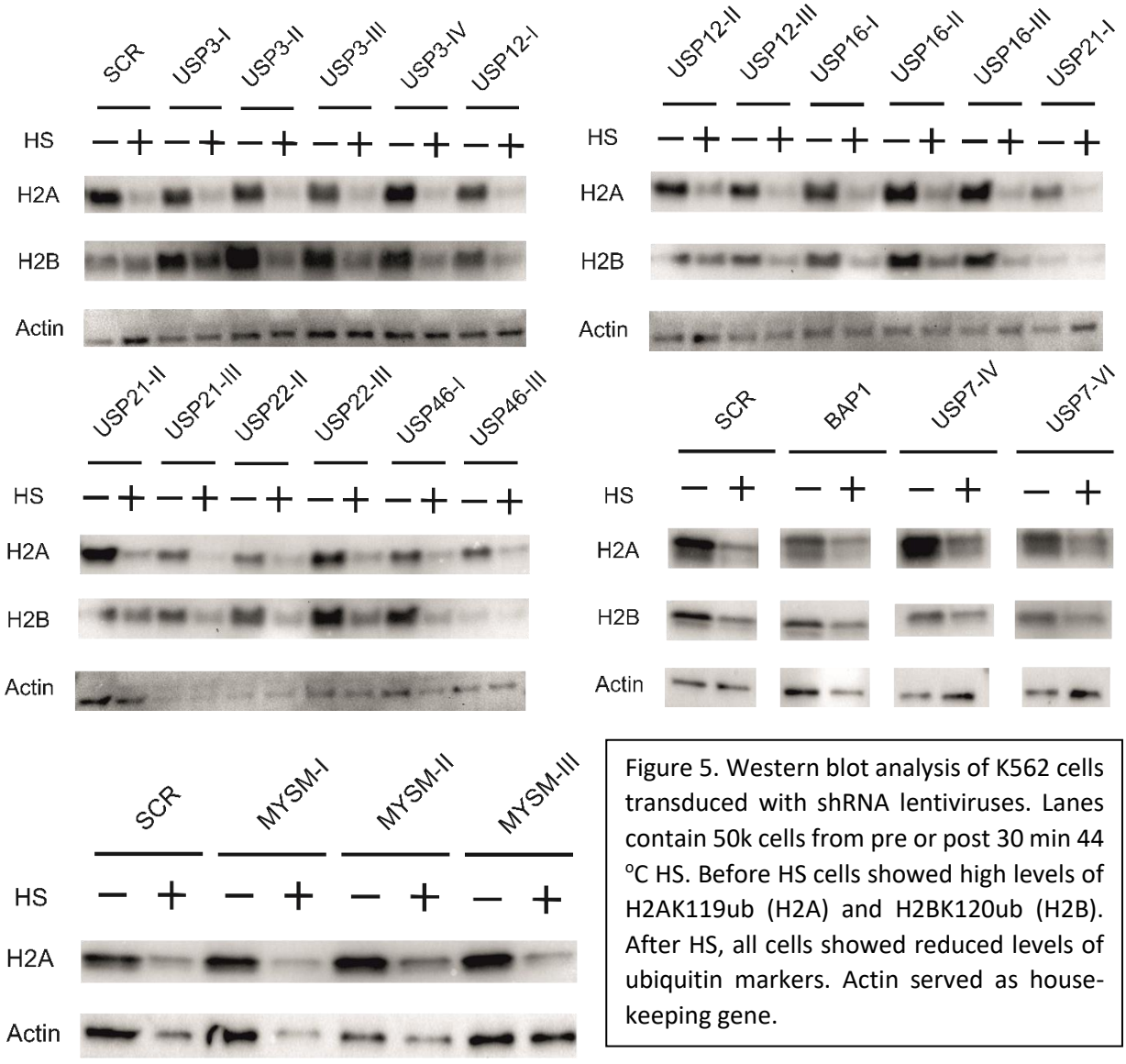


Figure 5. Western blot analysis of K562 cells transduced with shRNA lentiviruses. Lanes contain 50k cells from pre or post 30 min 44 °C HS. Before HS cells showed high levels of H2AK119ub (H2A) and H2BK120ub (H2B). After HS, all cells showed reduced levels of ubiquitin markers. Actin served as house-keeping gene.

Multiple shRNA transduction leads to significant simultaneous knockdown of DUBs

Since we observed that no single gene knockdown prevented H2AK119ub deubiquitination upon HS we set out to explore a possible mechanism of redundancy. For this we transduced K562 cells with multiple shRNA lentiviruses directed against various DUBs. We tried two different approaches, one condition with 100 uL lentivirus per shRNA and a second with 400 uL lentivirus per shRNA. The hairpin sequences chosen were from the best preforming knockdowns found in figure 4. Flow cytometry showed both approaches resulted in high levels of mCherry positive live cell populations (figure 6). qPCR analysis of alive cells showed that 100 uL lentivirus per gene was insufficient for significant knockdown of DUBs (figure 6) whilst 400 uL per gene did result in significant knockdowns of all target DUBs (figure 6).

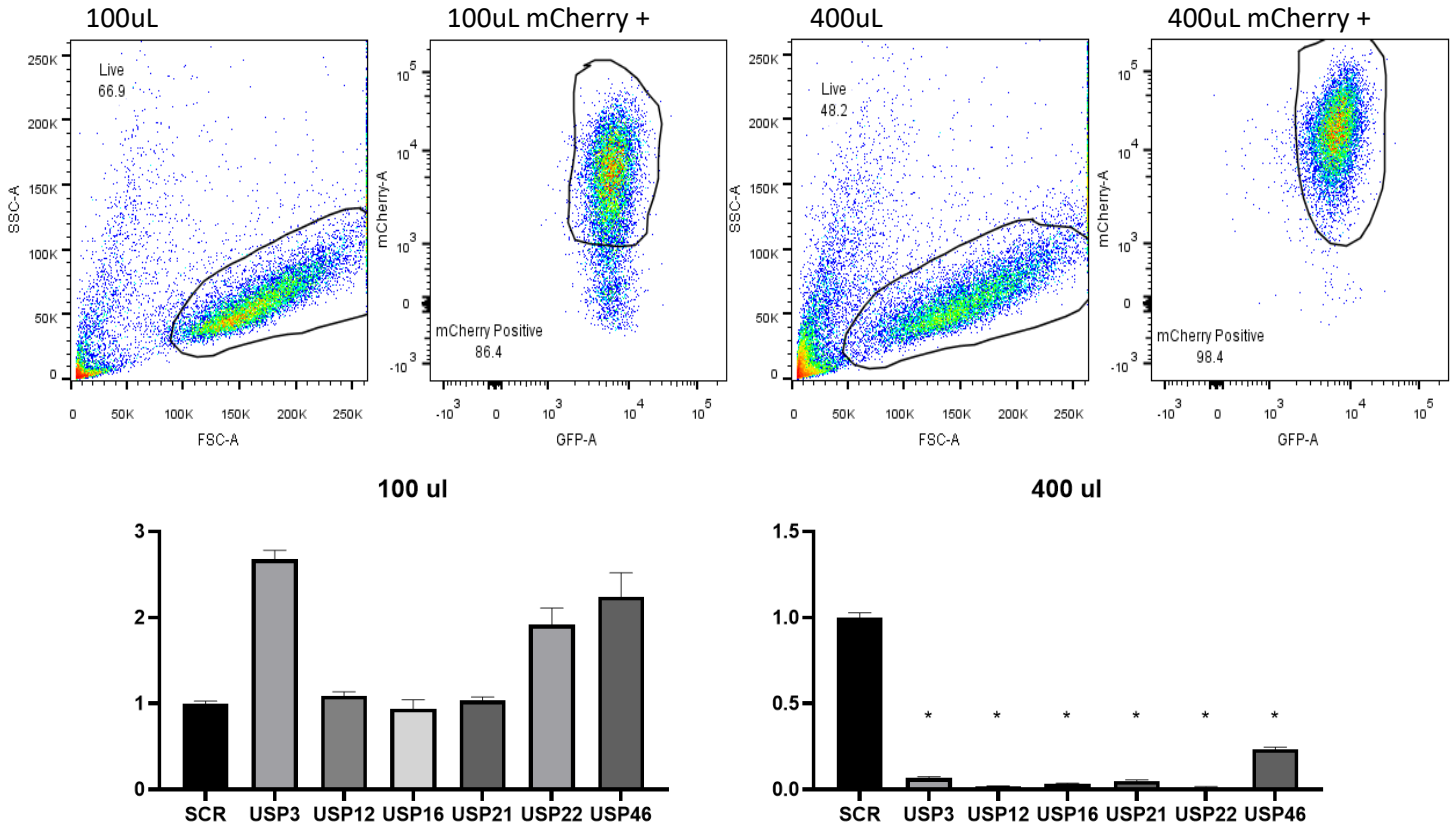


Figure 6. Flow cytometry analysis of multiple virus transduced K562s. Cells transduced with either 100 ul or 400 ul of multiple hairpins showed high levels of mCherry positivity. Knockdown efficiencies of multiple virus treated K562 as found by qPCR analysis. No statistical significance was achieved for cells transduced with 100 ul. Cells transduced with 400 ul were all significantly knocked down for all genes observed (p <0.0001).

No redundancy appears to exist among screened DUBS in post-HS H2AK119ub deubiquitination

With the knowledge that cells treated with 400 ul of multiple lentiviruses displayed significant knockdown we subjected the transduced cells to 30 min 44 °C HS. This to see if redundancy amongst DUBs could explain the deubiquitination of H2Ak119. As expected, all transduced cells, including SCR controls, displayed of H2AK119ub pre-HS (figure 7). In post-HS conditions, unsurprisingly, SCR cells showed clear H2AK119ub deubiquitination (figure 7). Cells transduced with 100 uL showed no reduced H2AK119ub deubiquitination post-HS (figure 7) this was to be expected as no significant knockdowns were achieved by this method of transduction. Notably cells transduced with 400 uL virus also showed reduced levels of H2AK119ub post-HS, on four and seven days after transduction (figure 7). Unmodified H3 was used as a housekeeping gene. Taken together, these data suggest that there is no mechanism of redundancy for among the screened DUBs for H2AK119ub deubiquitination after HS. Based on these data we can conclude that either the specific post-HA H2AK119ub DUB is not included in our screen, or that the loss of H2AK119ub after HS is perhaps not enzymatically driven.

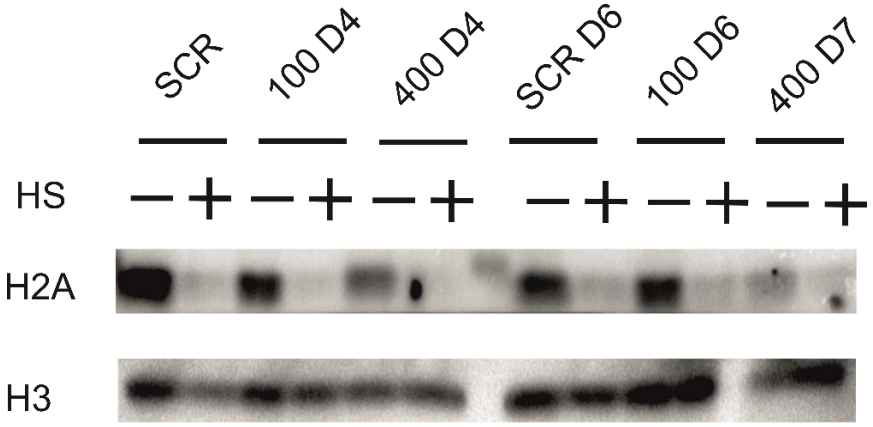
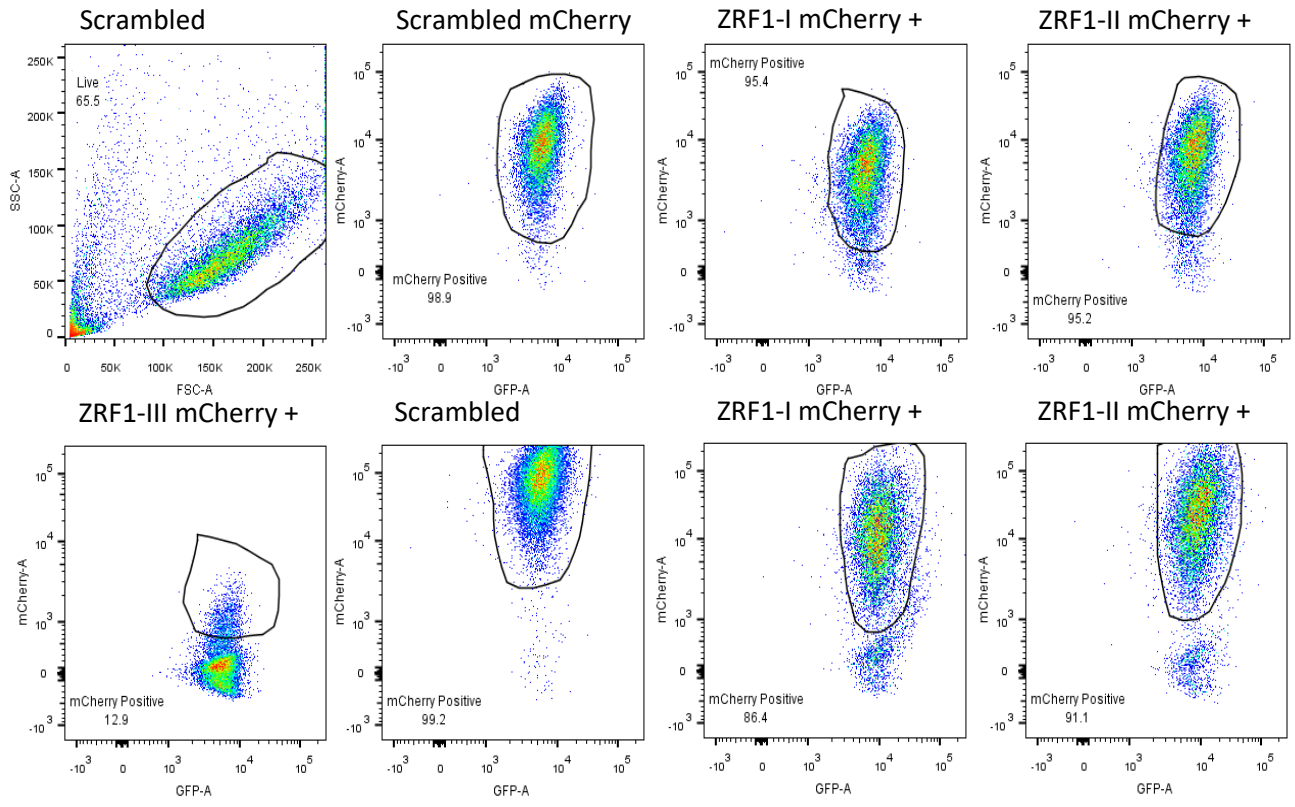


Figure 7. Western blot analysis of K562 cells transduced with multiple shRNA lentiviruses. Samples were obtained either pre or post 30 min 44 °C HS, with 50k cells per lane. Samples were obtained from multiple days after transduction. 100 indicates that 100 uL of each virus was used for transduction. 400 indicates that 400 uL of each virus was used for transduction. 100 uL transduced cells were determined to be non-informative due to no significant knockdown having been found in qPRC. 400 uL treated cells showed comparable results to the SCR cell line. Pre-HS high levels of H2AK119ub (H2A) was found and post-HS H2AK119ub levels

Zuotin-related factor 1 knockdown in K562 cells

Based on the experiments performed so far aimed falling short at uncovering the DUB active during HS we decided to turn attention on potential DUB recruiters in hopes of more promising results. A candidate recruiter we decided to explore further is DNAJ Heat Shock Protein Family Member C2 (HSP40), also referred to as Zuotin Related Factor 1 or ZRF1 (Qui et al., 2006, Richly et al., 2011, Aloia et al., 2015). First described in mice where its function was unclear (Hughes et al., 1995). This protein was later found to interact with H2AK119ub at Polycomb target genes (Richly et al., 2010). Upon binding ZRF1 displaces PRC1 and actively recruits USP21 for deubiquitination of H2AK119ub (Richly et al., 2010). This displacement promotes a transcriptionally active chromatin state. ZRF1 thus likely plays a role in switching repressed Polycomb target genes to an active state. Therefore, we chose to knockdown ZRF1 and analyse whether loss of ZRF1 expression interferes with post-HS H2AK119ub deubiquitination. New lentiviruses containing shRNA sequences aimed at ZRF1 (table 2) were made and transduced into K562 cells. Flow cytometry was used to assess mCherry positivity after transduction, showing that transduction efficiency was variable between hairpins (figure 8). When considering the knockdown efficiencies given by qPCR analysis no notable differences appeared between the hairpins used (figure 8).



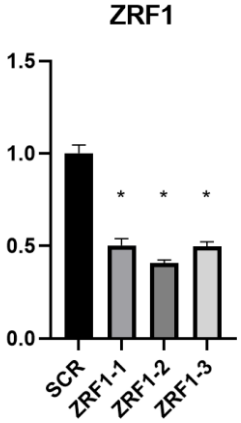


Figure 8. Flow cytometry analysis of K562 treated with lentivirus containing SCR control or ZRF1 shRNA, cells were checked for mCherry +. Cells used in initial hairpin knockdown efficiency assessment and subsequent HS western blotting. Second depiction of scrambled, ZRF1-I and ZRF1-II were used in later growth curve experiments. ZRF1 knockdown efficiencies as found by qPCR. All hairpins yielded statistically significant knockdown effects $p < 0.0001$ One-way ANOVA.

Target Gene	shRNA Sequence	Targetting Area
ZRF1-I	AGAAGATGATCTGCAATTATT	ORF
ZRF1-II	CAATGAGGCAGACCGTGTTAA	ORF
ZRF1-III	GCTTGAACTGGCAAGCTTACA	ORF

Table 3: ZRF1 shRNA Sequences

ZRF1 does not appear to recruit DUBs upon HS

We again sought to use western blot analysis of pre- and post-heat shocked cells to assess if deubiquitination was affected upon expression of ZRF1 hairpins. Again, we see that in pre-HS samples H2AK119ub levels were comparable between SCR and ZRF1 knockdown samples (figure 9). Post-HS samples of ZRF1 knockdown samples still showed reduced levels of H2AK119ub (figure 9). Thus, it appears that ZRF1 plays no role in the recruitment of DUBs or process H2AK119ub deubiquitination during HS.

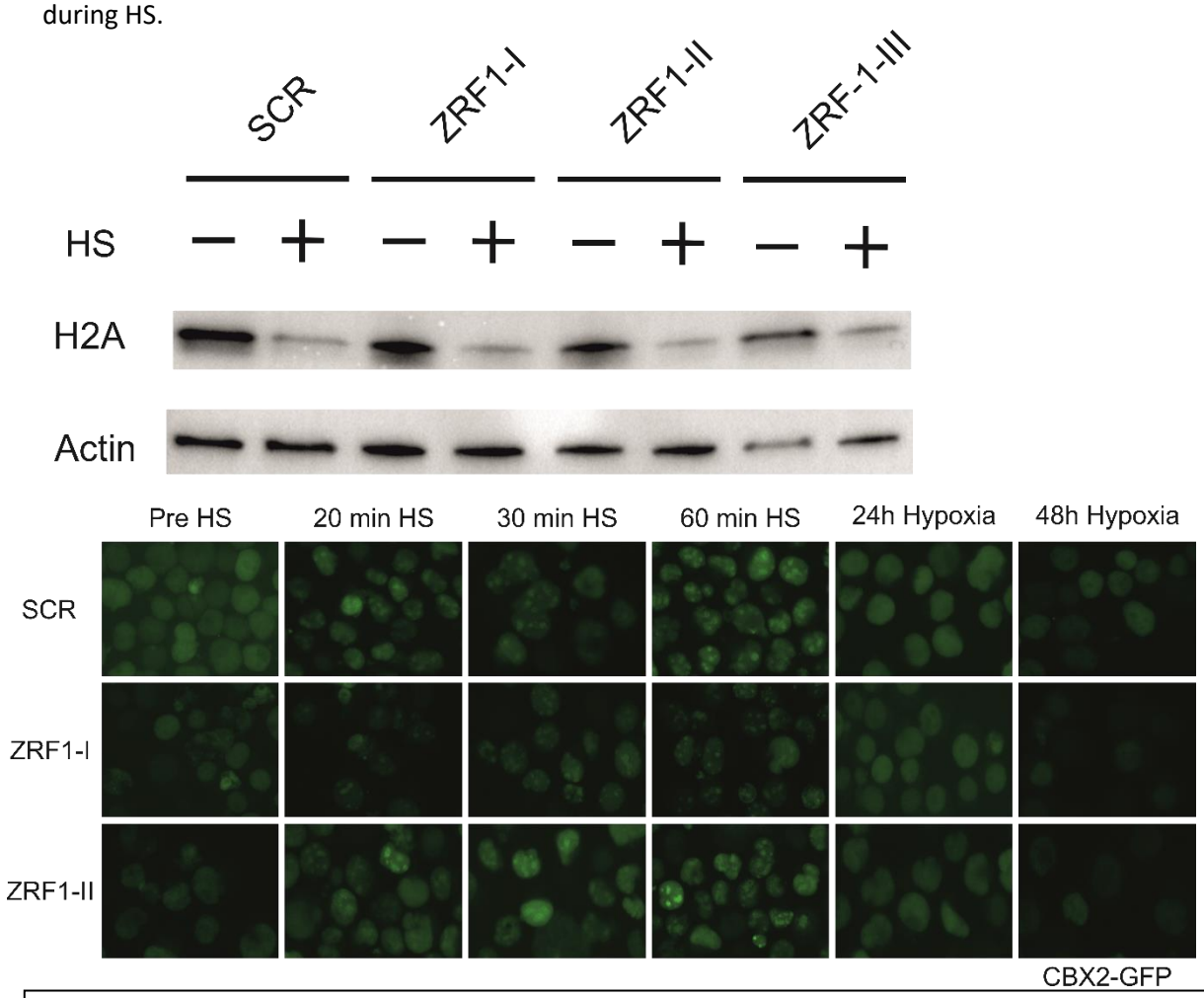


Figure 9. Western blot analysis of K562 cells transduced with ZRF1 shRNA containing lentivirus. Samples were taken pre or post 30 min 44 °C HS. Pre-HS cells showed levels of H2AK119ub comparable to that of SCR transduced cells. Post-HS cells showed reduced levels of H2AK119ub comparable to the SCR cells. Cytospin photos of cells transduced with either SCR control or ZRF1 shRNA. Visualized here is GFP-CBX2 conjugate protein. In pre-HS conditions CBX2 is spread throughout the nucleolus, during HS CBX2 accumulates in nucleolar. This accumulation is comparable between SCR cells and ZRF1 knockdown cells. Under hypoxia conditions cells show no accumulation of CBX2 in nucleolar.

ZRF1 knockdown does not affect CBX2 aggregation in nucleolar upon HS

ZRF1 was previously identified as a factor that competes with PRC1 for H2AK119ub binding (Richly et al., 2010) as PRC1 chromatin binding is lost upon HS, leading to nucleolar accumulation of PRC1 subunits we hypothesized that ZRF1 may be a causal factor in inducing PRC1 displacement after HS. Therefor we performed ZRF1 knockdown experiments to evaluate whether reduced ZRF1 expression may affect GFP-CBX2 nucleolar accumulation after HS. For this we used K562 cells expressing CBX2 proteins tagged with GFP which we then transduced with ZRF1 shRNA containing lentiviruses, using s scrambled sequence as control. We then exposed these transduced cells to different stress conditions and observed GFP-CBX2 behaviour using fluorescence microscopy. Looking at the fluorescence microscopy pictures we can see that during non-stress conditions CBX2 is spread throughout cells (figure 9). Upon exposure to HS, we observe that GFP-CBX2 concentrates in nucleolar (figure 9). In ZRF1 knockdown cells, GFP-CBX2 shows the same behaviour, during normal conditions GFP-CBX2 is spread out (figure 9) following HS it accumulates in nucleolar, and during hypoxia no accumulation appears to occur (figure 9). Thus, ZRF1 knockdown does not influence GFP-CBX2 behaviour during normal or stressed conditions.

ZRF1 knockdown causes impaired proliferation, and is exacerbated by stress conditions

During our experiments working with ZRF1 knockdown cells we noted that at a certain point there was a decline in the number of cells in our culture flasks. To confirm whether there was indeed a phenotype we set out an experiment in which ZRF1 knockdown cell growth was assessed during different conditions. In these growth experiments we compared regular K562 cells transduced with scrambled lentivirus as a control to K562 cells transduced with ZRF1 hairpins. Comparing regular growth conditions, at first ZRF1 shRNA expressing cells appear viable as they divide and multiply, albeit at a slower rate than scrambled cells (figure 10). At later time points, around day four, ZRF1 cells appear to show impaired proliferation whilst the scrambled cells continue to grow (figure 10). This same behaviour is found in heat shocked, irradiated and hypoxia exposed cells, where the SCR cells recover and eventually continue to multiply, whilst the ZRF1 appear to plateau and then decline quickly (figure 10). Looking more closely at the onset of cell decline it appears that ZRF1 knockdown cells struggle earlier in stressed conditions compared to non-stressed conditions. Decline onset in control cultured cells appears to occur after day three or four whilst decline onset appears to start on day two for stress conditions. Despite not affecting H2AK119ub deubiquitination during HS ZRF1 appears to play an important role in cell survival long term and cells with reduced ZRF1 expression appear more susceptible to stress conditions compared to non-transduced cells.

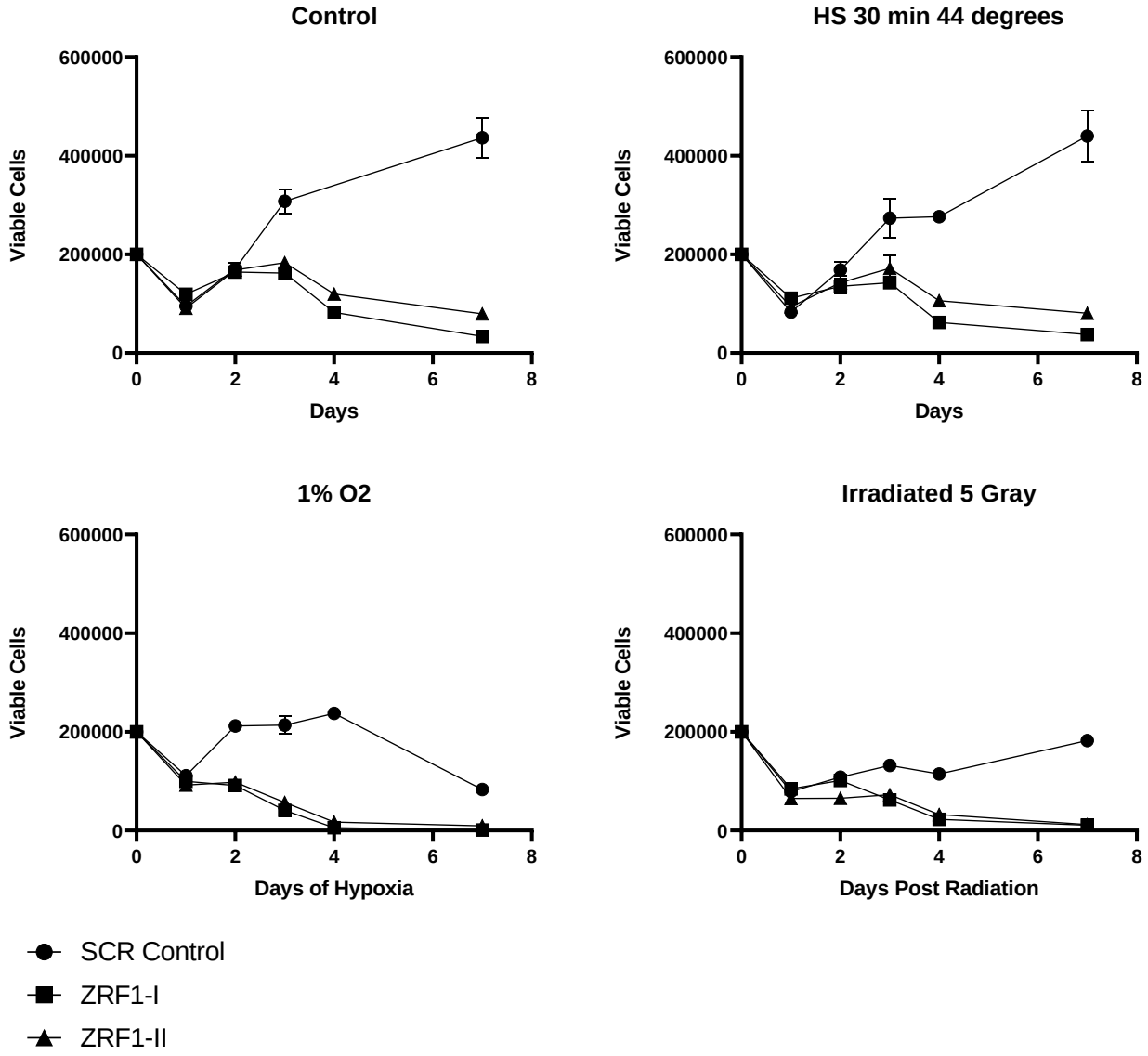


Figure 10. Growth curves observing cell proliferation and survival in different culture conditions following ZRF1 knockdown (N=3). SCR transduced cells serve as control. Cells were cultured at 37 °C following exposure to stress. Control cells and HS cells appear similar in survivability, SCR recover whilst ZRF1 knockdown cells expire. In hypoxia and irradiated conditions ZRF1 phenotype appears to occur earlier compared to control and HS conditions.

Conclusion and Discussion

In this study we explored our hypothesis that H2AK119ub deubiquitination upon HS is mediated by DUBs. We also investigated whether ZRF1 is involved in DUB recruitment for H2AK119ub deubiquitination. We found that the genes screened, USP (3, 12, 16, 21, 22, 46) MYSM1 and BAP1, (table 1) do not appear to be DUBs responsible for the enzymatic removal of H2AK119ub during HS. Whilst the generated hairpins were successful in knocking down target genes, they did not seem to influence H2AK119ub levels after HS, regardless of whether we targeted single or multiple genes. We also found that ZRF1 is not involved in H2AK119ub deubiquitination nor does ZRF1 knockdown influence CBX2 behaviour. ZRF1 does appear to strongly influence cell survival, when knocked down using shRNA we found reduced cell proliferation and eventual population decline, this was further exacerbated by stress conditions.

Despite this extensive screen the existence of a conclusive DUB or DUBs aimed at H2AK119ub during HS remain elusive. Considering the significance of knockdowns and the timeframe at which experiments were preformed we feel confident in stating that no single DUB in this screen is affecting H2AK119ub deubiquitination during HS on a western blot detectable level. However, the experiments preformed were limited in their ability to detect smaller changes potentially conceived by knockdowns. In future higher resolution experiments that allow for insights on individual genes should be included. Performing CHiP analyses of Polycomb genes in the presence of potential DUB knockdowns might reveal more nuanced results compared to broader insights given by western blots. Besides different experiments expanding the scope of DUBs studied is warranted as far from all DUB family members have been screened. The shRNA approach seems solid enough to be repeated given effective hairpins can be designed and fabricated.

Within this study the possibility of redundancy was accounted albeit in a heavy-handed way as no MIO assessment having taken place prior to transductions. Regardless, significant simultaneous knockdowns have been achieved, yet no changes to post-HS H2AK119ub were observed. We cannot guarantee that the entire cell population was affected evenly by the lentiviruses multiple with our method of transducing. However, qPRC analysis was preformed utilized a homogenized sample of transduced cells, meaning that DUB expression levels found should be representative of the entire cell population. Furthermore, the dosage used per gene was comparable to the amount of lentivirus used in single gene knockdowns which for the chosen hairpins was clearly sufficient (400 ul vs 500 ul). It is also worth pointing out that within the redundancy screen a HS was performed on both four- and seven-days post transduction, limiting the chance that remnants of target gene proteins persisted. Thus, the finding that between the studied genes no mechanism of redundancy exists seems reliable. The limitation touched on regarding western blot result resolution still applies, therefor preforming CHiP experiments with multiple knockdowns for Polycomb genes is to be considered. Also, if more single gene knockdowns for other DUB candidates are preformed there will also be a need to reaffirm redundancy potential. Alternatively, to the shRNA approach a full knockout experiment might be worth setting up utilising CRISPR-CAS9 this would eliminate the possibility that residual activity of knockdown genes is sufficient to allow for DUB activity. Assuming these experiments show comparable results to shRNA approach it would nearly solidify the idea that DUBs screened are not responsible for post-HS deubiquitination of H2AK119ub. Besides showing that DUBs central to this study do not influence H2AK119ub deubiquitination during HS we also show that they do not influence H2B120ub deubiquitination upon HS. Another experiment worth considering is to observe the effect of these DUB knockdowns on long term H2AK119ub under regular conditions.

Lastly it remains possible that no DUB is active during HS. This would go strongly against the observed kinetic difference between H3K27me3 and H2AK119ub by Azkanaz et al., and seems unlikely that this

extreme difference is not caused by an underlying process. Perhaps though there is a case to be made for a mix of active and passive removal of H2AK119ub during HS. Where some genes would be subject to DUB activity to lose the ubiquitin histone mark and part of the removal being caused by the protein unfolding that occurs during HS. As noted, earlier BAP1 is involved in the deubiquitination of *SLC7A11* perhaps during HS this is also the case whilst other genes may or may not have DUBs catering to them. This could explain why no definitive DUB has been found as a single or even multiple genes getting knocked down would not result in cells unable to undergo deubiquitination of H2AK119ub. Since then, limited regions would be affected by knockdowns, perhaps this could be further investigated by studying DUB-gene association before HS and then under influence of knockdowns if these genes are not deubiquitinated post-HS. This, asides from being pure speculation, would raise the question of how and why there would be a distinction between passive and active removal of bonds.

Whilst ZRF1 seems not play a role in the recruitment of DUBs during HS for H2AK119ub deubiquitination it cannot be stated as absolute certainty. ZRF1 knockdowns, whilst significant, did not reach the level of knockdown found in the DUB candidate shRNA knockdowns, or a level that could be considered near complete. The lack of a near full knockdown may be explained by the nature of the cell line used rather than the quality of the hairpin. Previous research has shown that leukemic cells may present overexpression of ZRF1, and depletion of ZRF1 may inhibit leukaemia progression (Demajo et al., 2014). Considering K562 cells are a leukemic cell line they may have an inherently elevated level of ZRF1 expression, and even be in part reliant on ZRF1 for survival. Whilst a comparison between K562 normal occurring levels of ZRF1 expression versus that of different cell types was not included it may be an interesting follow-up experiment. This could in future help decide whether K562 cells are the right cells to perform ZRF1 knockdown reliant experiments with. Furthermore, it might be worth including a western blot analysis to assess ZRF1 protein levels, both to compare between different cell types and to gain better insight into the effectiveness of knockdowns. Still with no noticeable effect on DUB activity post-HS despite significant knockdowns does suggest that ZRF1 may not be responsible for recruiting DUBs. ZRF1 also appears to not influence CBX2 behaviour upon HS in any noticeable form. Neither the rate nor the amount of accumulation occurring at any time following HS appeared to be changed following ZRF1 knockdown. This raises the question which specific aspects of PRC1 are affected by ZRF1 knockdown and is worthy of future studies as displacement of PRC1 by means of ZRF1 does occur (Richly et al., 2010, Aloia et al., 2015). Additional PRC1 subunits tagged with fluorescent proteins might be a viable approach here to allow for visualization of RING1A/B or KDM2B and how these proteins behave following ZRF1 knockdowns with or without the effect of HS. ZRF1 interactions with PRC1 might even be different depending on whether it is canonical, variant or non-canonical PRC1.

Whilst a full knockdown of ZRF1 might not have been achieved in this study the effects of a partial knockdown on cell proliferation and survival is pronounced. This is in line with other studies observing the role of ZRF1 in different cell types (Aloia et al., 2014, Demajo et al., 2014, Rath et al., 2016). ZRF1 knock out via CRISPR-CAS9 even leads to early post implantation lethality in mice embryos (Helary et al., 2019). Notably we also found that ZRF1 knockdown combined with stress conditions appears to further exacerbate population decline. These findings should still be validated further, with better controlled starting population sizes, less disparity between cell counting measures, better controlled culture conditions for hypoxic conditions, wider range of Gray irradiation dosages and different cell types. Still specific nature of the decline resulting from ZRF1 knockdown does remain a question to be answered. Speculating one potential explanation is that by interfering with ZRF1 nucleotide excision repair (NER) is impaired. Studies have found that ZRF1 interacts with H2AK119ub to decondense chromatin structures and recruit the DICER protein to mediate NER (Chital et al., 2017A/B Garcheva et al., 2016). Though this seems not to be the most logical or likely explanation for the observed

decline as NER is primarily utilized to repair damage mediated by UV radiation (Garcheva 2016) and not by cesium-137 as was used in this study. Perhaps an even better argument against impaired NER explaining the decline is that the need for DNA repair seems implausible when we consider that neither hypoxia nor HS leads to explicit DNA damage.

An alternative explanation to the observed cell decline might be found in a more intricate pathway. PRC1 and ZRF1 are known to compete for binding to Histone 2A (Richly et al., 2010). PRC1 mediates ubiquitination and ZRF1 by competing would limit this process. One interesting target for PRC1 in the context of cell death is *SLC7A11* a component of the cystine/glutamine exchanger, also known as xCT. BAP1 has been implicated as a specific DUB for this gene (Zhang et al., 2019). From this it could follow that when ZRF1 is knocked down PRC1 can ubiquitinate *SLC7A11* more easily thus, together with PRC2, leading to repression. Normally xCT regulates the influx of cystine into a cell and is downstream converted by Gpx4 into GSSG (Bersuker et al., 2020). This conversion allows Gpx4 to also transform lipid peroxides into lipid alcohols, by removing the peroxides Gpx4 inhibits ferroptosis mediated cell death (Yang et al., 2014). GSSH 's downstream metabolites also function as inhibitors of ferroptosis (Bersuker et al., 2020). Due to the knockdown of ZRF1 *SLC7A11* could remain repressed by PRC eventually resulting in increased amounts of lipid peroxides, stimulating ferroptosis, whilst also decreasing GSSH metabolism thereby limiting ferroptosis inhibition. The observed increase in cell decline following ZRF1 knockdown might thus be explained by increased activity of ferroptosis. Furthermore, stress is known to be able to trigger ferroptosis activation (Distéfano et al., 2017, Li et al., 2019), potentially explaining the increased cell death observed between stress and non-stress culture conditions (figure 11). Neither our preformed experiments nor studies observing ZRF1 directly confirm the process of ferroptosis taking place however it does seem a potential candidate. Direct observations into the level of *SLC7A11* transcription and xCT activity after ZRF1 knockdown would give more insight into whether ferroptosis is the cause of the decline. Another experiment that could be used to elucidate this phenotype is assessing lipid peroxidase levels and its biproducts MDA and 4-HNE. If ferroptosis is at play the expectation would be to find elevated levels in cells that have ZRF1 knocked down. Clarity on this would not only explain the phenotype found in this study but also could help explain the cause of increased cell death in other studies observing ZRF1 phenotypes.

PRC 1 and 2 together mediate epigenetic silencing through H2AK119ub and H3K27me3. HS leads to displacement of said histone markers. This displacement shows kinetic difference between H2A and H3 suggesting H2A deubiquitination might be caused by deubiquitinating enzymes. We screen potential DUBs in this study and found no evidence that they mediated enzymatic deubiquitination either on their own or together. Suggesting more extensive screens or nuanced experiments might be needed to find the explanation behind the observed differences. We also explored the potential role of ZRF1 in recruiting DUBs upon HS, which in our results appeared to not be the case, nor does ZRF1 affect CBX2 behaviour. We did observe increased cell population decline in cells with ZRF1 knocked down, which was further exacerbated by stress conditions. Whilst more experiments are needed to confirm it seems plausible that the observed cell death might be explained through increased ferroptosis. The interactions between epigenetic markers, PRC 1 and 2 and ZRF1 remain as intricate as at the start of this study, with only more future experiments waiting to be performed to shed light on results here.

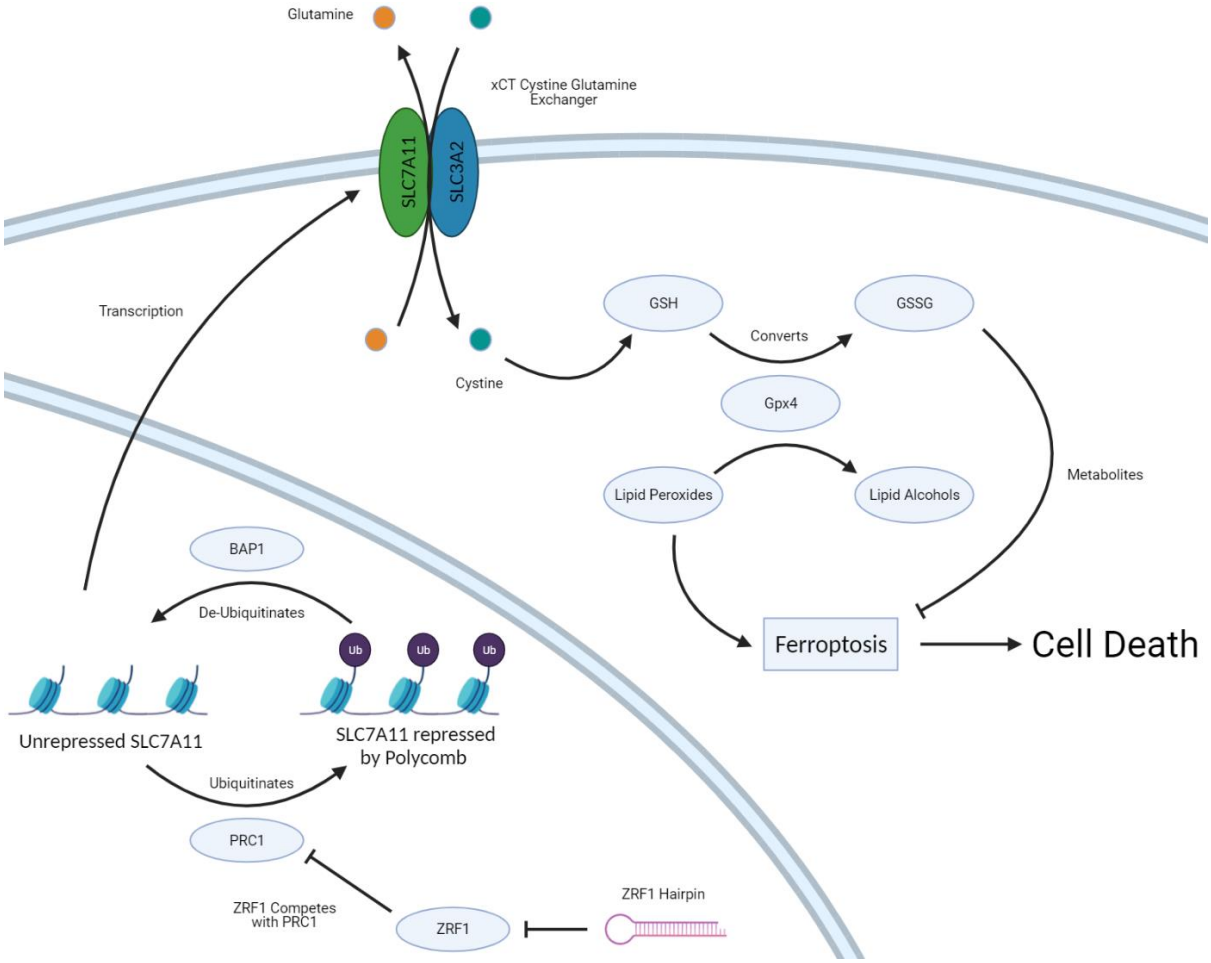
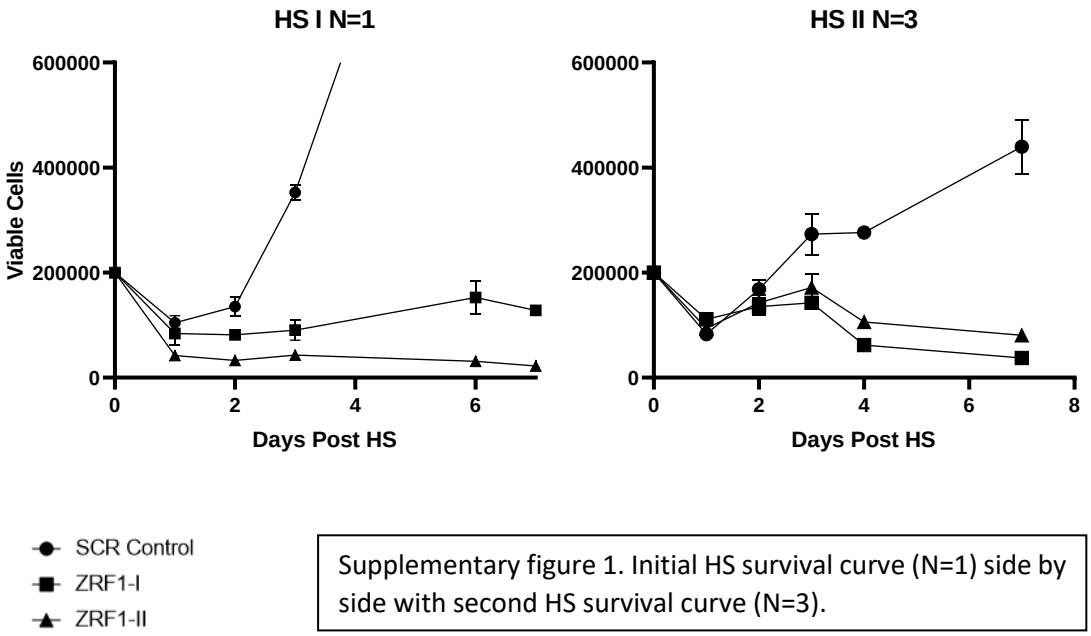


Figure 11. PRC1 and BAP1 under normal conditions ubiquitinate and deubiquitinate *SLC7A11*, a component of the xCT exchanger. ZRF1 competes with PRC1 for Histone 2 binding. By inhibiting ZRF1 transcription via hairpin PRC1 could perhaps ubiquitinate SLC7A11 more easily thereby repressing transcription. This repression then leads to reduced cystine influx, limiting the ability of Gpx4 to remove Lipid Peroxides leading to increases in ferroptosis. This reduction in cysteine also eventually causes, reduced downstream GSSG metabolites which normally inhibit ferroptosis. These interactions combined would lead to increased cell death via ferroptosis.

Supplementary figures

Initially we performed a small-scale growth experiment to validate further investigation of ZRF1 knockdown phenotypes. Here we observed that ZRF1 knockdown did appear to have a phenotype over time. With that we set out to do a better controlled experiment with a larger sample size.



Supplementary figure 1. Initial HS survival curve (N=1) side by side with second HS survival curve (N=3).

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