Title

Analysis of the ubiquitin-modified proteome identifies novel host determinants of Kaposi's sarcoma herpesvirus lytic reactivation

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Abstract

Kaposi's Sarcoma Herpesvirus (KSHV) is the causative agent of Kaposi's Sarcoma (KS) and is associated with primary effusion lymphoma (PEL), multicentric Castleman's disease (MCD) and two inflammatory diseases. KSHV-associated cancers are primarily associated with genes expressed during latency, while other pathologies are associated with lytic gene expression. The major lytic switch of the virus, RTA, interacts with cellular machinery to co-opt the host ubiquitin proteasome system to evade the immune response as well as activate the program of lytic replication. Through SILAC labeling, ubiquitin remnant enrichment and mass spectrometry, we have analyzed the RTA dependent ubiquitin-modified proteome. We identified RTA dependent changes in the populations of polyubiquitin chains, as well as changes in ubiquitinated proteins in both cells expressing RTA and naturally infected cells following lytic reactivation. We observed an enrichment of proteins that are also reported to be SUMOylated, suggesting that RTA, a SUMO targeting ubiquitin ligase, may function to alleviate a SUMO dependent block to lytic reactivation. RTA targeted substrates directly through a ubiquitin ligase domain dependent mechanism as well as indirectly through cellular ubiquitin ligases, including RAUL. Our ubiquitome analysis revealed an RTA dependent mechanism of immune evasion. We provide evidence of inhibition of TAP dependent peptide transport, resulting in decreased HLA complex stability. The results of this analysis increase our understanding of mechanisms governing the latent to lytic transition in addition to the identification of a novel RTA dependent mechanism of immune evasion.

Introduction

Kaposi's Sarcoma Herpesvirus (KSHV), also known as HHV-8, is the causative agent of Kaposi's Sarcoma (KS) and is associated with primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD)(1, 2). KSHV is also linked to the inflammatory diseases, KSHV inflammatory cytokine syndrome and immune reconstitution syndrome-KS (3–5). KSHV is classified as a Group 1 carcinogen by the International Agency for Research on Cancer and the National Toxicology Program 14th Report on Carcinogens (6).

KSHV, a gamma herpesvirus, has a two-phase lifecycle consisting of latency and lytic replication. During latency, few genes are expressed, and genome replication occurs with the purpose of episome distribution to daughter cells during cell division. Lytic replication is initiated via expression of the viral Regulator of Transcription Activation (RTA) protein. This results in rapid expression of lytic genes, replication of viral DNA, assembly of virus particles and egress from the cell (7).

KSHV is primarily found in cells as a latent infection. Initiation of KSHV-associated cancers is, in part, associated with the genes expressed during latency, which maintain an environment that allows for the survival of cells harboring latent virus. To maintain a latent infection, several checkpoints and mechanisms of regulation are bypassed to progress through the cell cycle without triggering apoptosis, autophagy, or an antiviral response. Dysregulation of such critical processes as cell cycle control, apoptosis and immune response-related signaling pathways are believed to be associated with development of an oncogenic environment. In addition to virus propagation and proliferation, lytic gene expression is also thought to be required for tumorigenesis in KS and MCD. In PEL, most cells express latent genes, with 1-5% of cells exhibiting spontaneous reactivation and lytic gene expression(7).

The mechanisms responsible for lytic reactivation in natural infection are unclear, however chemical inducers of lytic reactivation such as phorbol esters and histone deacetylase inhibitors have been utilized to study the lytic program of gene expression and more recently cell lines harboring latent virus and doxycycline inducible RTA provide a more specific model for the study of reactivation (8, 9). Aside from being the major switch for reactivation of KSHV from latency, RTA has intrinsic ubiquitin ligase activity and has been shown to interact with cellular ubiquitination machinery, ubiquitin ligases and deubiquitinases(10–12). RTA has also been designated a SUMO targeting ubiquitin ligase, specifically targeting SUMO2/3 modified proteins for proteasomal degradation(13, 14). RTA targets IRF7 for proteasomal degradation as a mechanism to abrogate the interferon α/β response to viral infection(11). In addition, RTA is reported to degrade a number of known RTA repressors such as ID2, MyD88, Hey1, LANA and NFκB (p65)(15–20). This E3 ubiquitin ligase activity has been mapped to a Cys/His- rich domain between amino acids 118 and 207 of RTA(11). RTA was also shown to recruit and stabilize a cellular ubiquitin ligase, RAUL, via recruitment of the deubiquitinating enzyme HAUSP(10). We have previously reported RTA induced degradation of vFLIP through the cellular E3 ligase Itch (12, 21).

We hypothesized that identification of additional substrates targeted for ubiquitination by RTA would provide insight into novel mechanisms of regulation of the latent-lytic transition. To this end, we carried out a comparative proteomics analysis in both RTA transfected 293T cells and TREX BCBL-1 RTA SILAC labeled cells. We identified 66 ubiquitination sites in 40 proteins shared between both data sets, representing proteins with RTA induced ubiquitination alterations in BCBL-1 cells. We validated a subset of the proteins for RTA dependent degradation in the context of a CURE (course-based undergraduate research experience) with 62 undergraduate students over three semesters. Undergraduate research experience is an important factor in persistence in STEM education and careers for students from diverse backgrounds(22).

HLA-C, CDK1, MCM7, VDAC1 and SUMO2/3 were among the proteins identified as targets of RTA induced ubiquitination and degradation. Our dataset was enriched with proteins that are also known to be SUMOylated, with more than a third of the proteins in the matched

dataset also reported to be SUMOylated. Inhibition of SUMOylation resulted in increased production of infectious virus, supporting a role for RTA induced degradation of SUMO in the transition to lytic replication. The ubiquitin ligase domains in RTA and RAUL are partially required for degradation of some of the substrates, suggesting that additional cellular factors are at play. We observed an RTA dependent decrease in the abundance of proteins associated with MHC class I antigen presentation and provide evidence supporting a novel mechanism of immune evasion associated with decreased TAP dependent peptide transport and intracellular HLA levels. This RTA dependent TAP2 inhibition represents a novel mechanism of attack on antigen presentation by KSHV early in lytic reactivation. In addition to identifying a novel mechanism of immune evasion, our study has yielded novel targets of RTA downmodulation will enhance our understanding of the latent to lytic transition and provided 62 undergraduate students with authentic research experience.

Methods

Cell Lines, Plasmids, Transfection, and Antibodies

HEK 293T, iSLK, and BAC16 iSLK were cultured in DMEM medium supplemented with 10% BS and TREx BCBL1 cells were maintained in RPMI supplemented with 20% FBS. Cells were grown at 37°C with 5% CO_2. The following plasmids were used in this study: FLAG tagged ORFs in pcDNA3.1+/C-(K)DYK were purchased from Genscript: CDK1 (NM_001786.5), MCM7 (NM_005916.5), VDAC1(NM_003374.3). HLAC (NM_002117.5) was obtained from Genecopoeia in a Gateway PLUS Shuttle clone and cloned into pcDNA-DEST40 (ThermoScientific). Flag tagged SUMO 2/3, RAUL (WT and C1051A) and RTA (WT and H145L) were provided by Diane and Gary Hayward. Cells were transfected using PEI (Polyethylenimine, Linear, MW 25000, Transfection Grade, Polysciences) at a ratio of 3ul PEL/1ug plasmid DNA. The following antibodies were used in this study: BACTIN (sc-69879V5), V5 (sc-271944) from Santa Cruz Biotech, FLAG M2 from Sigma Aldrich, FITC labeled HLA-ABC Antibody (11-9983-42 Invitrogen), PE Mouse Anti-Human CD184 (Clone 12G5, BD), Cdc2 p34 Sc-54 (Santa Cruz), HLA Class I ABC 15240-1-AP (Proteintech), MCM7 Sc-9966 (Santa Cruz), SUMO 2/3 AB374 (Abcam), VDAC1 Sc-390996 (Santa Cruz), RTA antibody was provided by Gary Hayward. Cells were transfected at 60-70% confluence using 1µg/ml polyethyleneimine (PEI) linear, MW~25,000 at a ratio of 1µg plasmid DNA:3µl PEI. After 15 min of incubation at room temperature the mixture was added to the cells.

Ubiquitin-modified proteome analysis

Proteomics experiments were conducted in collaboration with the Smoler Proteomics Center at the Technion-Israel Institute of Technology. Experiments were carried out using doxycycline inducible TREx BCBL1 RTA cells and 293T cells transfected with RTA or pcDNA control vector. Cells were maintained as described above. Prior to ubiquitin remnant motif K-ε-Gly-Gly (KGG) purification, cells were acclimated in SILAC media (lysine and arginine deficient media supplemented with stable isotope containing lysine and arginine ("heavy" media contained $^{13}C_6$ $^{15}N_4$ Arg and $^{13}C_6{}^{15}N_2$ Lys)) for 7 days and evaluated for efficient SILAC labeling (23). RTA expression was induced in TREx BCBL1 RTA cells via addition of doxycycline (1μg/ml) to cells for 24 and/or 48h as indicated. 293T cells were transfected with RTA or empty vector pcDNA 3.1 and harvested following 48h incubation. Cells were treated with 10μM MG132 6 h prior to harvesting. Cell pellets were washed with PBS and flash frozen in liquid nitrogen. Cells were lysed in 8M urea 400mM ammonium bicarbonate buffer pH 8, sonicated and cleared of cell debris by centrifugation. 2mg protein was processed for peptide purification by reducing with DTT (30min at 60°C, 3mM final concentration), cysteines were alkylated with 10mM idoacetamide, followed by trypsin digest (0.1μg/5ug protein) overnight. Following desalting by reverse phase

chromatography on SepPac disposable cartridges (Waters), peptides containing the ubiquitin remnant motif – K-ε-Gly-Gly were enriched via incubation with anti-K-ε-Gly-Gly antibody-bound agarose beads. Peptides were eluted with 0.2% TFA. Eluted peptides were desalted using C18 stage tips and dried prior to analysis via LC-MS/MS.
The peptides were resolved by reverse-phase chromatography on 0.075 X 180-mm fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr Maisch GmbH, Germany). The peptides were eluted with linear 180 minutes gradient of 5 to 28% 15 minutes gradient of 28 to 95% and 25 minutes at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.15 µl/min. Mass spectrometry was performed by Q Executive HFX mass spectrometer (Thermo) in a positive mode using repetitively full MS scan followed by collision induces dissociation (HCD) of the 30 most dominant ions selected from the first MS scan.
MS raw data was processed using MaxQuant software followed by identification of proteins using the Andromeda peptide search engine to search against the HHV8 and human UniProt databases (Uniprot-proteome 27.7.18 (73101 entries), Uniprot-proteome HHV8) (24, 25). Data merging and statistical analysis was done using the Perseus(26). Functional enrichment analysis was performed using DAVID (https://david.ncifcrf.gov/) and STRING (https://string-db.org/). Final data set was curated by matching the data from the TREx BCBL1 RTA dataset with the data from the 293T cell dataset to identify the RTA dependent ubiquitin-modified proteome. For time course proteomics experiments cells were cultured in the absence of MG132, lysates were separated on a 12% polyacrylamide gel and peptides extracted from Coomassie stained gel slices using in-gel digest and processed for LC-MS/MS without KGG purification. Experiments were performed in triplicate with a reverse SILAC labeling control, replicates with the highest Pearson's correlation coefficient were used for the final data analysis.

SDS-PAGE and Western Blot Analysis
Cells lysates were prepared by direct addition of 2X Laemmli buffer. Lysates were separated using a 4%-20% ExpressPlus PAGE Gel (Genescript) and MOPS running buffer. Proteins were transferred to a PVDF membrane using Trans-Blot Turbo using the Bio-Rad defined program for 1.5 mm gels. Membranes were blocked in 5% non-fat dry milk in PBS for 1 h followed by incubation with primary antibody overnight at 4°C and secondary antibody for 1 h at room temperature. Proteins were visualized by addition of ECL substrate and detection of chemiluminescence using a Licor C-DiGit or Azure 300.

TAP Peptide Transport Assay
iSLK cells were treated with 1ug/ml doxycycline at 90% confluence to induce RTA expression. 48 hrs. post doxycycline induction, cells were harvested, partially permeabilized and incubated with a peptide pool. Peptide pool contained the following cysteine AF 488 modified peptides at a concentration of 10nM: NST-F (RRYQNST[C]L), C4-F (RRY[C]KSTEL), and R9 (EPGY[C]NSTD). Cells were incubated with 10mM ATP or ADP for 30 minutes at either 37°C or on ice. Cells were washed 3x with PBS before being fixed with 2% paraformaldehyde and analyzed using a Guava EasyCyte Flow cytometer.

Surface and Intracellular Flow Cytometry
Cells were dissociated with 0.25% trypsin (Life Technologies) and resuspended in FACS wash buffer. Cells were washed twice, permeabilized (for intracellular staining only) and then incubated or directly incubated (surface staining only) with FITC labeled MHC ABC antibody (Invitrogen) or PE labled CXCR4 (BD Biosciences) at 4°C for 30 minutes. Cells were washed 3 times with FACS buffer at 4°C and analyzed using a Guava EasyCyte Flow cytometer.

SEAP Assay
Vero rKSHV.294 and 293T MSR tet-OFF cells were kindly provided by David Lucak(27). Day one:

Vero rKSHV.294 were plated in a six-well plate and treated with 2-D08, DMSO, or NaBr where indicated. Day two: 293T MSR tet-OFF cells were plated in a six-well plate. Day three: media from 293T MSR tet-OFF cells was replaced with virus containing media from the rKSHV.294 cells. Day five: 25 μl of media was harvested and heat inactivated at 65°C for 30 minutes. SEAP activity was quantified using the Great EscAPe SEAP assay (Clontech). Fluorescence was read on a Filtermax F5 plate reader (Molecular Devices).

Results

Characterization of the RTA dependent KSHV ubiquitin-modified proteome.

Given that RTA is the master lytic switch and has been shown to not only have intrinsic ubiquitin ligase and SUMO targeting ubiquitin ligase activity but is also reported to interact with cellular components of the ubiquitin proteasome system, we reasoned that identification of host proteins that displayed RTA dependent differential ubiquitination would increase our understanding of the latent to lytic transition. KSHV encodes two ubiquitin ligases (K3 and K5) that are expressed during lytic replication; however, our goal was to identify RTA specific targets.

To identify targets of RTA induced differential ubiquitination, we designed our workflow in two disparate cell lines, doxycycline inducible TREx BCBL1 RTA cells, which contain the entire viral genome, and RTA transfected 293T cells, which contain RTA only, with the rationale that the matched datasets would yield RTA specific targets. Cells were adapted to SILAC media as follows: light media contained “light” lysine and arginine and heavy media contained “heavy” lysine (13C6;15N2) and arginine (13C6;15N4) amino acids based on the protocol of Ong and Mann, 2007(23). Following confirmation of stable labeling, cells were either treated with doxycycline to induce RTA expression or transfected with RTA or vector control. Cells were either harvested for proteomics to evaluate lytic induction and protein abundance or treated with MG132 and incubated with anti-K-ε-Gly-Gly antibody-bound agarose to isolate peptides containing ubiquitin remnants. Ubiquitinated peptides were enriched with anti-K-ε-GG antibody, followed by processing for mass spectrometry on a Q-Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer. For proteomics, to evaluate protein abundance, cells were harvested at the indicated time points, and lysates processed for MS. Data was analyzed using MaxQuant and Perseus (Fig.1a).

Time-course of lytic induction.

A proteomics experiment was first conducted in TREx BCBL1 RTA cells to assess lytic gene expression over a 24h time-course. Peptides of viral origin displayed an increase in peptides of lytic origin as well as a sustained presence of other proteins such as LANA and vIL6 (Supplemental Figure 1a-b). In TREx BCBL-1 RTA cells, analysis of peptides of cellular origin revealed a decrease in the detection of proteins previously reported to be degraded following lytic induction (Supplemental Figure 1c). HLA-A/ HLA-B or MHC class I is known to be downregulated via the action of viral encoded ubiquitin ligases K3 and K5(28–30). We also observed a decrease in BST2/Tetherin, also reported to be downregulated by K5 via endocytosis and subsequent downregulation in the endosome(31). SPN/leukosialin was also significantly downregulated but has not been described previously (Supplemental Figure 1c). We also observed a modest but significant (q=0.012285714) increase in Integrin alpha-6 (Supplemental Figure 1d). This was interesting due to the involvement of integrins in cytomegalovirus entry and induction of angiogenesis (32, 33).

Ubiquitin-modified proteome analysis in RTA transfected and naturally infected cells.

Analysis of anti-K-ε-GG enriched ubiquitinated peptides resulted in the identification of 193 differentially ubiquitinated sites in 138 proteins in cells transfected with RTA, and 272 sites in 208 proteins in doxycycline treated TREx BCBL1 RTA cells. Sixty-six of these sites in 40 proteins were shared between both data sets, representing proteins with RTA induced ubiquitination

alterations in BCBL-1 cells; 29 displayed increased ubiquitination and 11 displayed decreased ubiquitination (Fig. 1b-c). Experiments were performed in triplicate and the experiments with the highest Pearson correlation coefficient (293T RTA r=0.97 and TRExBCBL-1 RTA r=0.68) were used for further analysis (Supplemental Fig. 2 a-b). While we observed greater variability within the TREx BCBL-1 RTA experiment, we did observe an overall similar trend in the behavior of many ubiquitination sites in both cell lines (Fig. 1d, Supplemental Fig. 2 c-d).

STRING analysis of proteins displaying an RTA dependent decrease in ubiquitination revealed two functional groups of proteins (Fig. 1e). One cluster is involved in DNA damage repair and the other is associated with ubiquitin binding. Analysis of proteins with increased ubiquitination in RTA expressing cells relative to control indicated functional clusters of proteins involved in diverse cellular processes including antigen presentation, DNA replication, DNA damage repair, cell cycle, SUMOylation, translation, transcription, and the proteasome (Fig 1f)(34). Gene-annotation enrichment analysis using DAVID resulted in clustering into functional groups (35, 36). The groups with the lowest Benjamini values are included in Supplemental Fig. 2e. We also observed an increase in K48 and K63 linked polyubiquitin chains in transfected 293T cells as well as a significant increase in ubiquitination of SUMO 2/3 in the matched dataset (Supplemental Fig. 2f and Figure 3a). These observations are consistent with the reported functions of RTA as a ubiquitin ligase, manipulator of the ubiquitin proteasome system and SUMO targeting ubiquitin ligase (10–13).

Evaluation of the stability of selected cellular proteins in the presence of RTA.
To validate the results of our proteomics experiment, in collaboration undergraduate students participating in a CURE (course-based undergraduate research experience), we selected proteins based on student generated hypotheses to evaluate protein stability in the presence of RTA expression. Flag tagged ORFs were expressed in 293T cells and evaluated for stability in the presence of RTA or pcDNA vector control. The following proteins that displayed increased ubiquitination in the ubiquitin-modified proteome screen, exhibited decreased stability in the presence of RTA: HLA-C, CDK1, MCM7, VDAC1 and SUMO2/3 (Fig 2 a). Decreased abundance of endogenous proteins was also observed in TREx BCBL-1 cells induced with doxycycline for 24 or 48hr (Fig. 2b.) Transcript levels in both transfected 293T cells (Fig. 2c) and doxycycline induced BCBL-1 cells (Fig. 2d) did not correlate with the observed protein levels, suggesting that the observed decrease in protein abundance was occurring post transcriptionally. The degradation of these proteins occurred rapidly as observed by cycloheximide chase and was dose dependent, as delivery of increasing amounts of RTA resulted in decreasing abundance of the substrate (Supplemental figure 3a-b).

Characterizing the mechanism of RTA induced degradation of selected substrates.
RTA has ubiquitin ligase activity and interacts with cellular ubiquitin ligases. To further explore the mechanism responsible for the observed RTA dependent decrease in protein stability, we utilized two different mutants. RTA ubiquitin ligase activity has been mapped to an N-terminal RING-like domain(11). Substitution of leucine for histidine at position 145 has been shown to abolish ubiquitin ligase activity and stabilize substrates of RTA (11, 20, 37, 38). To determine whether RTA is destabilizing CDK1, HLA-C, MCM7, SUMO2/3, and VDAC1, in the context of a CURE, 18 undergraduate students transfected 293T cells with epitope tagged substrate and wild-type or mutant H145L RTA and observed substrate specific effects on stability. CDK1 was not stabilized in the presence of RTA H145L, however HLA-C, MCM7, SUMO2/3 and VDAC1 displayed varying levels of stability. VDAC1 and HLA-C displayed the most evident stabilization, suggesting that RTA ubiquitin ligase activity is required for degradation (Fig. 4a). RTA is known to recruit and stabilize the cellular ubiquitin ligase RAUL (UBE3C) (10). To evaluate whether RAUL ubiquitin ligase activity is required for degradation of CDK1, HLA-C, MCM7, SUMO2/3, and VDAC1, 293T cells were transfected with RTA and/or wild-type or mutant RAUL C1051A and the

indicated substrate. Wild-type and mutant RAUL expression had a stabilizing effect on MCM7 only (Fig. 4b). We have previously observed overexpression of one protein in a multiprotein ubiquitin ligase complex to have a dominant negative affect due to the disruption of the stoichiometry of the complex. We observed this effect with MCM7. RTA expression decreased MCM7 abundance, while co-transfection with both wild-type or mutant RAUL had a stabilizing effect, suggesting that both RTA and MCM7 function in the degradation of MCM7.

RTA targets SUMO2/3 for ubiquitination and degradation.

RTA is a well characterized SUMO targeting ubiquitin ligase (STUbL) (13), therefore it was not surprising when SUMO 2/3 was identified as highly ubiquitinated at K11 and K45 (range 1.3-2.9 H/L ratio) in RTA expressing 293T and doxycycline induced TREx BCBL-1 RTA cells (Fig 3a). Further analysis of the ubiquitin-modified proteome dataset suggests that the RTA induced, or lytic, ubiquitin-modified proteome is enriched for proteins that are SUMOylated. The human proteome contains approximately 12,434 proteins that are ubiquitinated, of those proteins, 2,434 or 19.5% are also reported to be SUMOylated, however within the RTA dependent ubiquitin-modified proteome 30.7% (TREx BCBL-1), 38.3% (293T), and 34.4% (matched matrix) of the proteins are also SUMOylated (Fig 3b). In fact, 61.5% of the proteins from the matched matrix that localize to the nucleus are reported to be SUMOylated (Fig 3b). This observation suggests that the STUbL activity of RTA may be partly responsible for the observed increase in ubiquitinated proteins, specifically in the nuclear compartment. RTA is known to have intrinsic ubiquitin ligase activity as well as interact with cellular ubiquitin ligases and these, in addition to indirect mechanisms, may account for the remaining ubiquitination observed.

The RTA dependent degradation of SUMO 2/3 is dose dependent (Supplemental Fig 3a) and occurs rapidly (Fig 3d and Supplemental Fig 3b). SUMOylation appears to play a role in maintaining latency, as inhibition of SUMOylation resulted in increased production of infectious virus as quantified by secreted alkaline phosphatase assay (Fig. 3e). We utilized Vero rKSHV.294 cells that contain a reporter gene that expresses secreted placental alkaline phosphatase (SeAP) from a tetracycline responsive promoter. SeAP is secreted into the media only after successful infection of 293 MSR Tet OFF cells. Vero rKSHV.294 cells were treated with increasing concentrations of 2-D08. After 48h incubation, virus containing media was collected and added to 293 MSR Tet OFF cells. Media was assayed for SeAP activity following 72h incubation. We observed a significant dose dependent increase ($p<0.0001$) in SeAP activity at concentrations of 30 μM and higher. These data suggest that RTA targets SUMO 2/3 for degradation and that SUMOylation is important for maintaining latency in Vero rKSHV.294 cells.

RTA targets antigen presentation through diverse mechanisms.

Analysis of the ubiquitin-modified proteome yielded two proteins that are associated with antigen presentation: HLA (A, B and C) and TAP2. These proteins play a role in antigen processing and presentation (Fig 5a). We initially observed a decrease in HLA A and B abundance in our time course experiment (Supplemental Figure 1c). While KSHV K3 and K5 are known to downregulate MHC class I via endocytosis(39), ubiquitination was observed in RTA transfected 293T cells in addition to doxycycline treated TREx BCBL-1 cells, suggesting that there may be an additional RTA dependent mechanism for HLA downregulation (Fig 2 a-b). TAP2 is an ABC transporter that, in complex with TAP1, participates in the transport of proteasome generated peptides from the cytosol to the endoplasmic reticulum for loading onto nascent MHC class I (HLA) molecules. The TAP1-TAP2 peptide transport complex is targeted by multiple viruses, serving as an effective mechanism of immune evasion. TAP2 is reported to interact with Epstein Barr virus BLNF2a, herpes simplex virus US12/ICP47, and adenovirus E3-19K(40–43). Through mass spectrometry,

we observed TAP2 ubiquitination at K356 which is located within the peptide binding loop. We observed RTA dependent degradation of HLA following 24h RTA expression (Fig 2a and b). This degradation was dose dependent with RTA expression reducing HLA half-life to 2h post cycloheximide addition compared to 6h in empty vector transfected cells (Fig. 5b). Similarly, we observed a decrease in TAP2 abundance in both 293T and TREx BCBL-1 cells (Supplemental figure 4b and c, respectively).

Based on these observations, we hypothesized that peptide transport was being inhibited through inhibition of TAP2. To evaluate this hypothesis, we employed a peptide transport assay based on the method described by Fishbach et al. (44). This assay utilized a peptide pool containing FITC labeled peptides. Doxycycline inducible iSLK RTA cells lacking BAC16 were subjected to mild permeabilization and incubated with peptide pools under the indicated conditions. Following 30 min incubation, cells were washed, and peptide translocation was quantified by flow cytometry detection of FITC. We observed translocation of peptides into the ER in iSLK cells incubated at 37°C in the presence of ATP, however cells with doxycycline induced RTA expression displayed decreased peptide translocation, suggesting that RTA is interfering with TAP dependent transport of peptides into the ER (Fig 5c). Proteins that are not properly folded are targeted for degradation by ERAD. Specifically, HLA molecules that fail to bind β-2-microglobulin or peptide are retained, retrotranslocated, ubiquitinated and degraded (45). Mass spectrometry analysis of HLA ubiquitination induced by β-2-microglobulin depletion identified two luminal lysines, K200 and K267(46). We observed RTA induced ubiquitination of HLA-B and C on K200 in our mass spectrometry data. If inhibition of peptide transport and subsequent degradation of HLA via ERAD were to occur, we would expect to see decreased intracellular staining of HLA. iSLK cells lacking BAC16 were treated with doxycycline or media control followed by staining for endogenous intracellular HLA A, B, and C or CXCR5 as a control. RTA expressing cells displayed significantly decreased (9-fold average decrease in mean fluorescence intensity) intracellular HLA staining compared to untreated controls. There was no impact on the unrelated receptor CXCR5 (Fig 5d-e). Taken together these data supports the model that KSHV RTA targets antigen presentation through inhibition of peptide import into the ER resulting in a decrease in HLA biosynthesis.

Discussion

RTA is the primary regulator of the latent to lytic transition in KSHV. RTA is not only the transcriptional transactivator responsible for the activation of lytic gene expression, but also has ubiquitin ligase activity and is known to both directly and indirectly target proteins of viral and cellular origin for ubiquitination. Many of the targets of RTA induced degradation represent repressors of lytic reactivation. Targets of RTA include SMC5/6, ID2, SUMO 2/3, vFLIP, IRF7, MyD88, Hey1, LANA and NFκB (p65) (10, 12, 13, 15, 17–19, 38, 47, 48) Using the rationale that RTA targets repressors of lytic reactivation we set out to identify novel regulators of the latent to lytic transition using a proteomics-based approach. We utilized SILAC labeling and ubiquitin remnant enrichment to identify proteins that display RTA dependent differential ubiquitination. We observed an RTA dependent increase in K-48 and K-63 linked polyubiquitin chains. We identified 40 proteins that displayed RTA dependent differential ubiquitination. These proteins function in cellular processes including DNA damage repair, transcriptional regulation, DNA replication, apoptosis, and immune evasion. Through the development of a course-based undergraduate research experience, designed to provide authentic research experiences to diverse populations of undergraduate students, we selected proteins to validate and further characterize. Students selected proteins, generated hypotheses, and validated degradation of HLA-C, CDK1, TAP2, MCM7, VDAC1, and SUMO2/3. In a subsequent semester, students asked whether RTA is directly ubiquitinating these substrates and utilized a ubiquitin ligase domain mutant of RTA to evaluate protein stability. Participation in this course over a three-year period exposed 62 undergraduate students to the process of research and inspired a few of them to consider biomedical research as a career path.

In this study we present the following observations: 1. RTA expression results in cell specific reprogramming of the ubiquitome 2. RTA targets the degradation of SUMOylated proteins to promote lytic replication and 3. RTA expression decreases TAP2 protein levels abrogating peptide transport into the ER resulting in decreased intracellular HLA.

RTA is a well characterized STUbL, so it was not surprising when we observed SUMO 2/3 to be among the most highly ubiquitinated proteins identified in our RTA dependent ubiquitin-modified proteome(13). In fact, our ubiquitome was enriched for ubiquitinated proteins that are reported as SUMOylated via Phosphosite Plus, often on the same lysine. When we analyzed the SUMOylation status and cellular localization of the proteins that displayed an increase in ubiquitination, we found that 61.5% of the of the proteins that were also SUMOylated are localized to the nucleus. RTA is primarily localized to the nucleus. Taken together, this observation supports a model where nuclear localized RTA targets SUMOylated proteins to promote lytic reactivation. To probe the functional role of SUMOylation in maintaining latency we utilized the infectivity assay developed by the Viera and Lucak labs(27, 49) When Vero rKSHV.294 cells, containing virus with the gene encoding secreted alkaline phosphatase, were treated with increasing doses of a small molecule inhibitor of SUMOylation, we observed a dose dependent increase in production of infectious virus. These data are consistent with reports that RTA STUbL activity is required for lytic reactivation and gene expression (13).

To explore the mechanism of RTA induced degradation of selected proteins, we asked whether the ubiquitin ligase domains of RTA and RAUL are required for degradation. The RTA ubiquitin ligase domain appears to be required for degradation of most of the proteins evaluated, however we did not observe 100% recovery to control levels, except for VDAC1. When we evaluated the role of RAUL ubiquitin ligase activity, we only observed stabilization with MCM7. These data suggest that RTA induced degradation occurs through diverse mechanisms that include direct ubiquitination by RTA and recruitment of cellular ubiquitin ligases like RAUL. This observation is in line with what has been observed by others (10, 12, 18, 19). Additional experiments to identify RTA binding partners could shed light on the mechanism used by RTA to target different proteins.

KSHV encoded ubiquitin ligases K3 and K5 play an important role in immune evasion via ubiquitination and subsequent endocytosis of HLA(39). We observed increased ubiquitination and degradation of HLA in both doxycycline induced TREx BCBL-1 RTA and RTA transfected 293T cells in addition to decreased levels of peptides of HLA origin in TREx BCBL-1 cells as early as 12hrs post lytic induction. Ubiquitination and subsequent degradation of HLA-C was observed in RTA transfected 293T cells, suggesting an additional, K3/K5 independent, mechanism of HLA targeted immune evasion. DNA viruses have evolved diverse mechanisms to target antigen presentation, frequently targeting the TAP peptide transporter. Herpesviruses in particular frequently target TAP function. Cytomegalovirus US6 targets TAP by inhibiting ATP binding(42, 50). HSV-1 ICP47 inhibits peptide transport by blocking the peptide-binding site and EBV interferes with both peptide and ATP binding (40, 43). We observed TAP2 ubiquitination at K356 which is located within the peptide binding loop(51). Expression of RTA in iSLK cells resulted in reduced peptide transport. The exact mechanism of inhibition of peptide transport remains unclear. Potential mechanisms include RTA induced degradation of TAP2 or ubiquitination of TAP2, resulting in decreased peptide binding.

RTA is primarily localized to the nucleus. This begs the question of how RTA is targeting cytoplasmic proteins. 13/29 of the proteins that displayed increased ubiquitination are nuclear, however what about the 16 that are cytoplasmic? RTA is translated in the cytoplasm and imported into the nucleus. It is possible that some activity occurs prior to nuclear import or that a subset of RTA localizes to the cytoplasm. This model is supported by our observation that the RTA has the

largest impact on HLA stability 24h post transfection/doxycycline induction, with a decreased effect at 48h when RTA has presumably localized to the nucleus. Alternatively, RTA may be exerting its effects on cytoplasmic proteins indirectly, either through increasing expression of cellular ubiquitin ligases or destabilizing a deubiquitinase that is both nuclear and cytoplasmic. In the case of TAP2, this complex is localized to the ER membrane. The ER membrane is contiguous with the nuclear envelope, increasing the potential for interactions while the RTA is being imported into the nucleus. It is likely that aside from the SUMO targeting activity of RTA, one model will not explain how all proteins are targeted and this remains an area for future research.

Taken together, here we have identified 40 proteins that display RTA dependent differential ubiquitination. We validated 17.2% of the proteins that displayed increased ubiquitination for RTA dependent degradation. Our dataset was enriched with proteins that are also known to be SUMOylated. Inhibition of SUMOylation resulted in increased production of infectious virus, supporting a role for RTA induced degradation of SUMO and SUMOylated proteins in the transition from latency to lytic replication. We observed RTA dependent degradation of proteins associated with MHC class I antigen presentation and provide evidence supporting a novel mechanism of immune evasion associated with decreased TAP dependent peptide transport and increased intracellular HLA and proteasome subunit degradation. This report contributes to our understanding of KSHV biology by identifying new points of virus-host interaction and describing a novel mechanism of immune evasion by KSHV RTA during lytic replication that likely contributes to the success of the virus in establishing a lifelong infection. Future study of the RTA targets identified in this study have the potential to further our understanding of the latent to lytic transition and lead to the identification of new chemotherapeutics.

Figure 1. Analysis of the ubiquitin-modified proteome in RTA transfected and KSHV infected cells. (A) Schematic describing experimental design for comparative proteomics analysis. Experiments were carried out in both TREx BCBL1 RTA and RTA transfected 293T cells. Cells were grown in SILAC media containing heavy or light lysine and arginine, supplemented with dialyzed FBS. Cells were treated with MG132 6h prior to harvest. 25ug of protein was saved for proteomic analyses following preparation of tryptic peptides. Tryptic peptides were enriched for ubiquitin remnants with anti-K-ε-GG antibody, followed by processing for mass spectrometry. Data was analyzed using Maxquant and Perseus. Created with Biorender.com. (B) Venn diagram illustrating the number of proteins that displayed increased (top) and decreased (bottom) ubiquitination in each dataset and shared between datasets following filtering in Perseus. (C) Heat map displaying shared ubiquitination sites between RTA transfected and KSHV infected cells. Duplicate experiments displayed for each cell type. Red=H/L ratio >0.5, Green=H/L ratio <0.5. (D) Heat map illustrating shared ubiquitination sites that displayed increased ubiquitination in RTA expressing cells. Heatmap generated by clustergrammer. (E-F) STRING analysis of proteins that displayed at least a 0.5 fold (E) increase or (F) decrease (Log_2H/L ratio) in ubiquitination in the matched (BCBL1 and 293T) dataset.

Supplemental Figure 1. Time-course of lytic induction. (A-B) Doxycycline induced lytic reactivation of TREx BCBL1 RTA cells resulted in an increase in peptides derived from lytic proteins (viral peptides noted in green in B) and a decrease in peptides of cellular protein origin (cellular proteins of interest noted in red and HLA indicated in blue). (C) RTA transfected 293T cells expressed RTA. (D) Peptides of cellular origin previously reported to be downregulated following lytic induction. (E) Integrin alpha 6 (ITGA6) displayed a modest increase. TREx BCBL1 RTA cells were treated with doxycycline (1ug/ml) for 0, 4, 8, 16, and 24h. 293T cells were transfected with RTA using PEI. $1.8x10^7$ cells were harvested in an 8M Urea buffer. 6ug of protein was reduced, alkylated, trypsin digested, cleaned on C18 stage tips and processed for MS. Data was analyzed using Maxquant, Perseus, and Graphpad Prism.

Supplemental Figure 2. Analysis of the ubiquitin-modified proteome in RTA transfected and KSHV infected cells, related to Figure 1. Scatterplots of (A) 293T RTA and (B) TREx BCBL-1 RTA demonstrating reproducibility between experiments with Pearson correlation. (C) Percent coefficient of variation within each dataset. (D) Shared ubiquitination site scatterplot rank vs log_2 H/L ratio demonstrating behavior of each modification in each dataset. Log_2 H/L ratio >0.5, <-0.5 were considered significant increase or decrease respectively. Blue=TREx BCBL-1 RTA, Orange=293T RTA. (E) DAVID analysis of matched dataset. (F) K63 and K48 linked polyubiquitin chains were increased in RTA expressing 293T cells ((Log_2H/L ratio = 0.57 and 1.49, respectively).

Figure 2. RTA expression reduces the abundance of selected ubiquitinated proteins. (A) 293T cells were transfected with the indicated expression vectors in the absence or presence of RTA. Lysates were analyzed by immunoblot against the indicated epitope tags, RTA, or β-actin as a loading control. (B) TREx BCBL-1 cells were treated with 1ug/mL doxycycline and collected at the indicated time points. Lysates were analyzed by immunoblot for endogenous protein levels, RTA, and β-actin as a loading control. * indicates non-specific bands. Transcript levels of target genes were examined by qPCR in (C) 293T cells transfected with or without RTA and (D) TREx BCBL-1 cells treated with 1ug/mL doxycycline for 24h.

Supplemental Figure 3. RTA expression reduces the abundance of selected ubiquitinated proteins, related to Figure 2. (A) RTA induced degradation of selected targets is dose dependent. Increasing amounts of RTA were co-transfected with 1ug of indicated tagged-protein in 293T cells. Lysates were analyzed by SDS-PAGE followed by immunoblot against FLAG or V5, RTA, or β-actin as a loading control. (B) Cycloheximide chase illustrating RTA dependent reduction of target protein half-life. 293T cells were transfected with 1ug indicated tagged-protein and either empty vector or RTA. 24hr post-transfection, cycloheximide was added and cells were harvested at the indicated timepoints. Lysates were analyzed as described above.

Figure 3. RTA targets SUMO 2/3 for degradation. (A) SUMO2/3 displayed increased ubiquitination in both doxycycline induced BCBL-1 cells and RTA transfected 293T cells (Log_2 H/L ratio). (B) The KSHV latent/lytic ubiquitin-modified proteome is enriched for proteins that are also SUMOylated. Proteins were evaluated for evidence of SUMOylation via PhosphoSitePlus. (C) Inhibition of SUMOylation results in lytic reactivation. Vero rKSHV.294 cells that contain a SeAP reporter gene were treated with increasing concentrations of SUMOylation inhibitor 2-D08. Following 48h incubation, virus containing media was collected and added to 293 MSR Tet-OFF cells. Media was assayed for SeAP activity following 72h incubation. (D) Protein half-life was graphed using a representative western blot (see supplemental figure 3B) and image J. SUMO2/3 monomer bands were normalized to β-actin bands and graphed relative to time 0, empty vector control using Graphpad Prism.

Figure 4. RTA targets cellular proteins for degradation through diverse mechanisms. (A) The RTA ubiquitin ligase domain is partially required for the degradation of some substrates of RTA. 293T cells were transfected with the indicated substrate plus either wild-type or mutant (H145L) RTA or empty vector pcDNA control. Lysates were analyzed for substrate abundance via western blot. Results displayed are from a representative of at least three independent experiments. (B) The RAUL ubiquitin ligase domain is partially required for the RTA induced degradation of MCM7. 293T cells were transfected with the indicated substrate plus either wild-type or mutant (C1051A) RAUL or empty vector pcDNA control. Lysates were analyzed for

substrate abundance via western blot. Results displayed are from a representative of at least three independent experiments.

Figure 5. RTA targets antigen presentation by reducing peptide transport into the ER. (A) Proposed model in which RTA targets ER associated HLA by inhibiting TAP2. Created with Biorender.com. (B) Protein half-life was graphed using a representative western blot (see supplemental figure 3B) and image J. HLA monomer bands were normalized to β-actin bands and graphed relative to time 0, empty vector control using Graphpad Prism. (C) Peptide transport assay. iSLK cells were treated +/- doxycycline (1ug/mL) to induce RTA expression. 48hrs after treatment, cells were harvested, semi-permeabilized and incubated with fluorescent peptides under the indicated conditions. Cells were analyzed using a Guava EasyCyte flow cytometer. (D) RTA expression results in decreased intracellular HLA staining. iSLK cells were treated +/- doxycycline (1ug/mL) to induce RTA expression. Cells were fixed, permeabilized and stained for HLA or CXCR5 as a control. Left histograms = unstained cells, right = stained cells. Cells were analyzed as stated above. (E) Mean fluorescence intensity was analyzed for 3 replicate experiments using a ratio paired t test. *$p<0.05$

Supplemental Figure 4. RTA expression results in loss of TAP2, related to Figure 5. (A) Induced RTA expression in iSLK cells. iSLK cells were treated +/- 1ug/mL doxycycline for 24hr and harvested. Lysates were analyzed by SDS-PAGE followed by immunoblot for RTA and β-actin as a loading control. RTA expression reduces the abundance of TAP2. 293T cells transduced with RTA (B) and BCBL1 cells treated with doxycycline to induce RTA (C) were harvested and lysates were analyzed by SDS-PAGE followed by immunoblot for TAP2, RTA, and β-actin. * Indicates non-specific bands.

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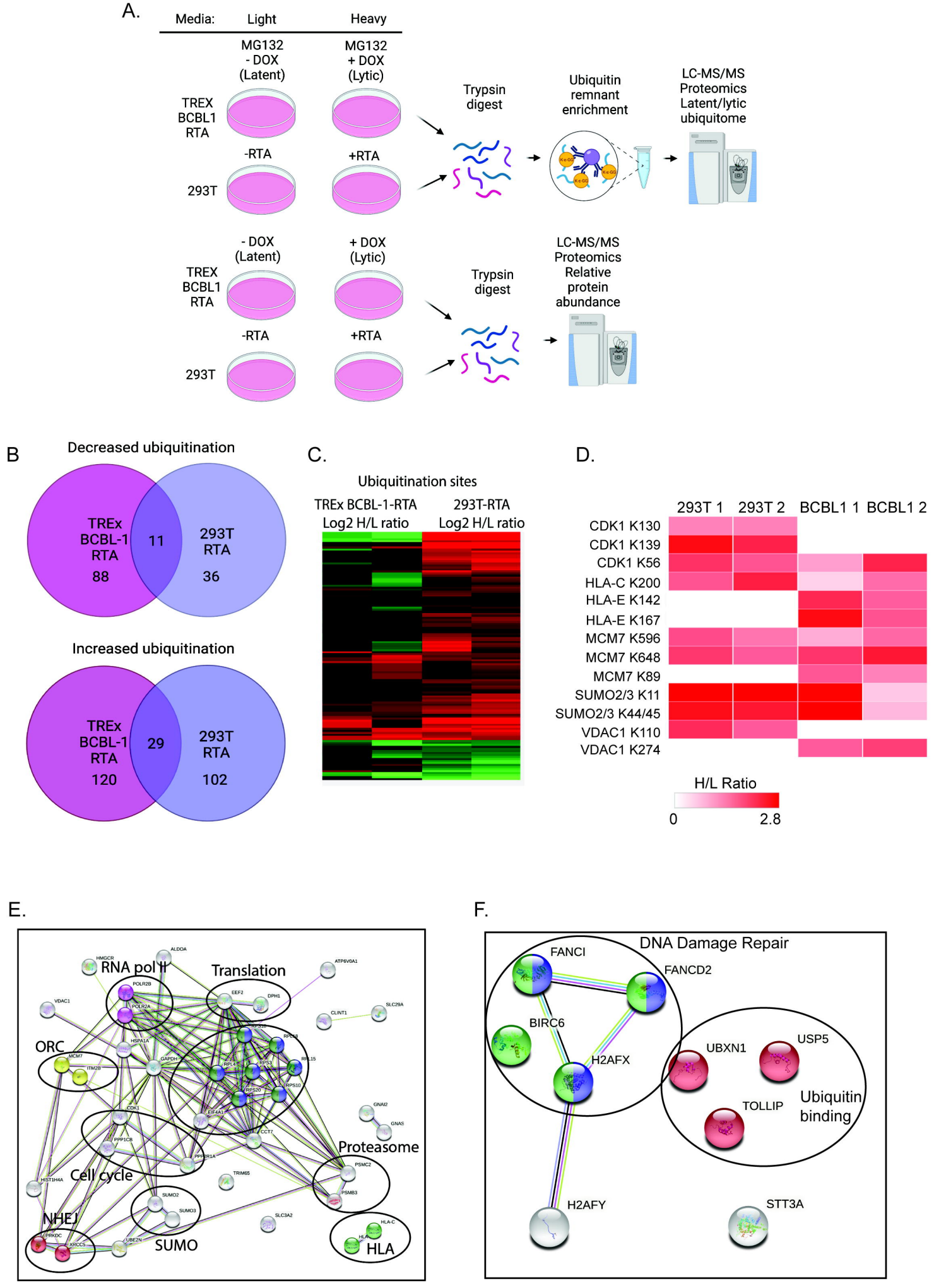
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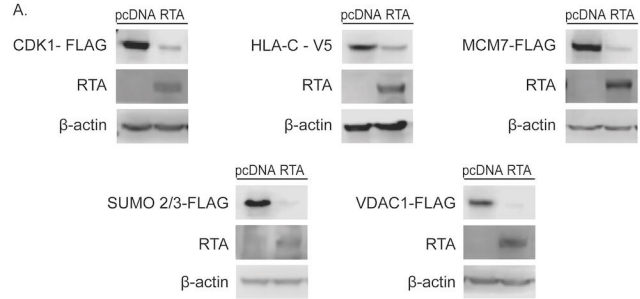
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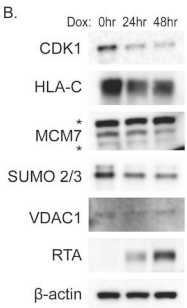
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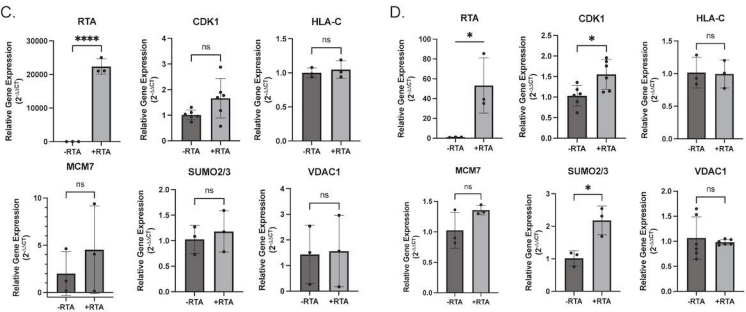
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A.

Uniprot ID	GENE NAME	BCBL-1 H/L A	BCBL1 H/L B	293T RTA H/L 1	293T RTA H/L 2
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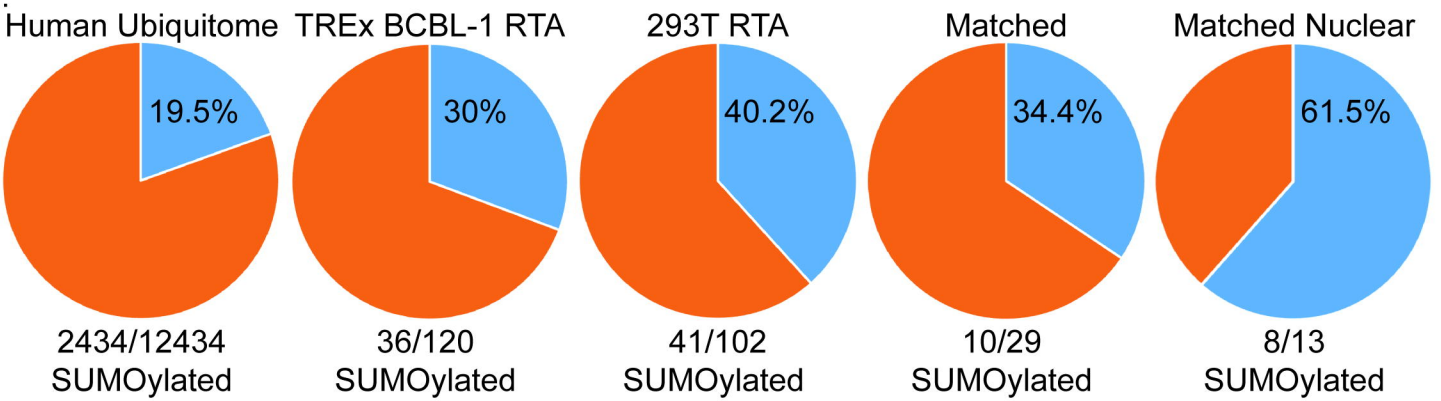

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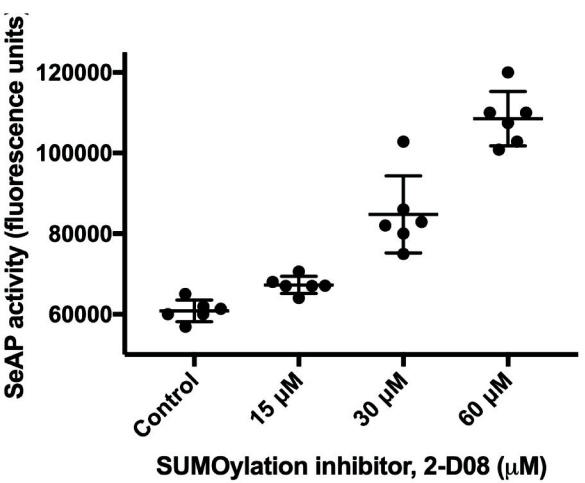

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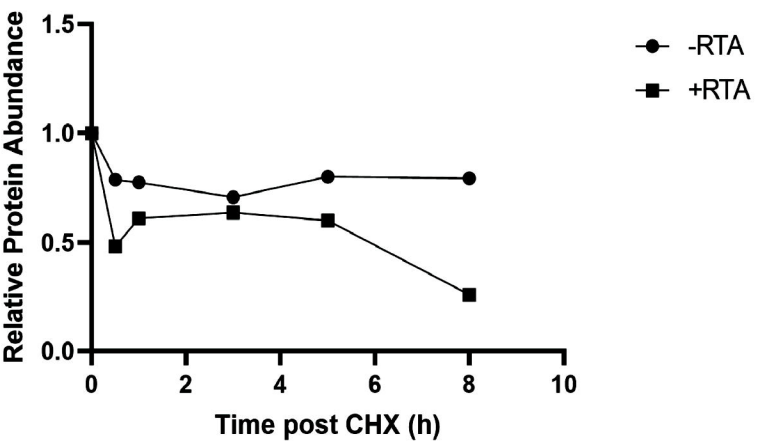

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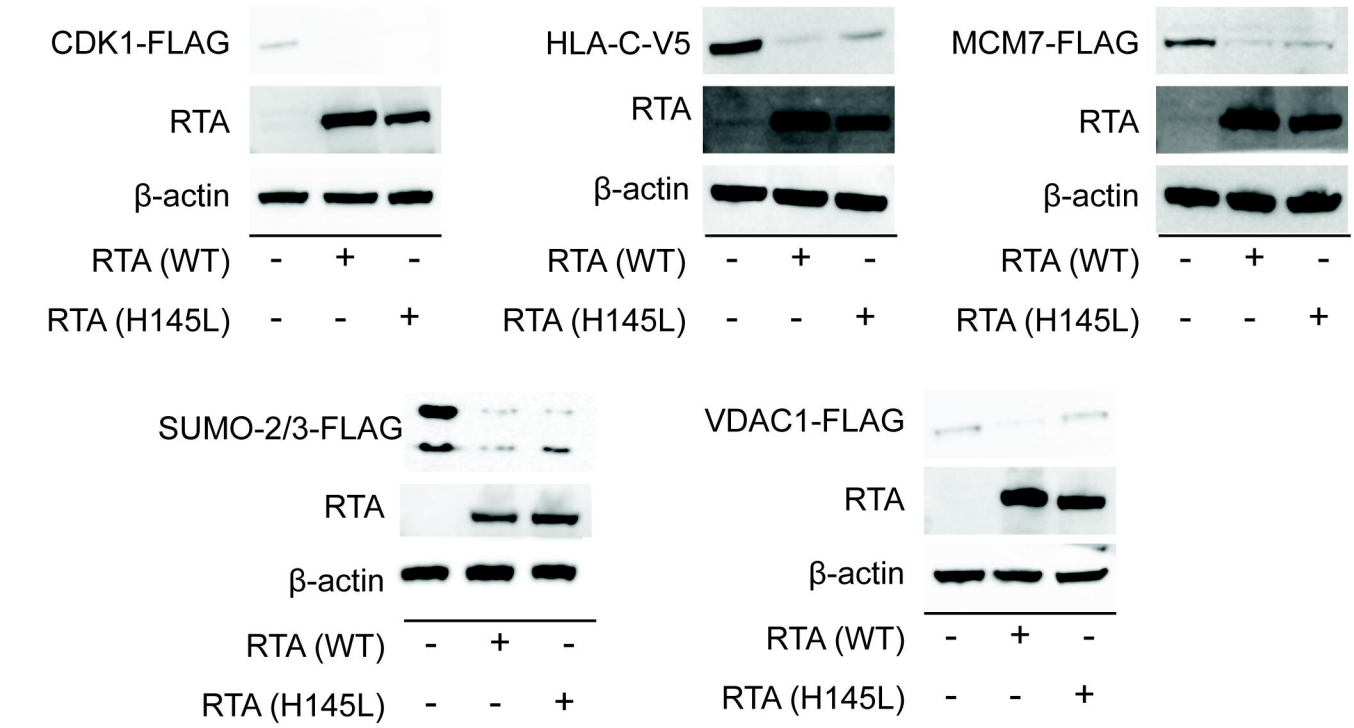
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RTA
β-actin
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RTA (H145L) - - +
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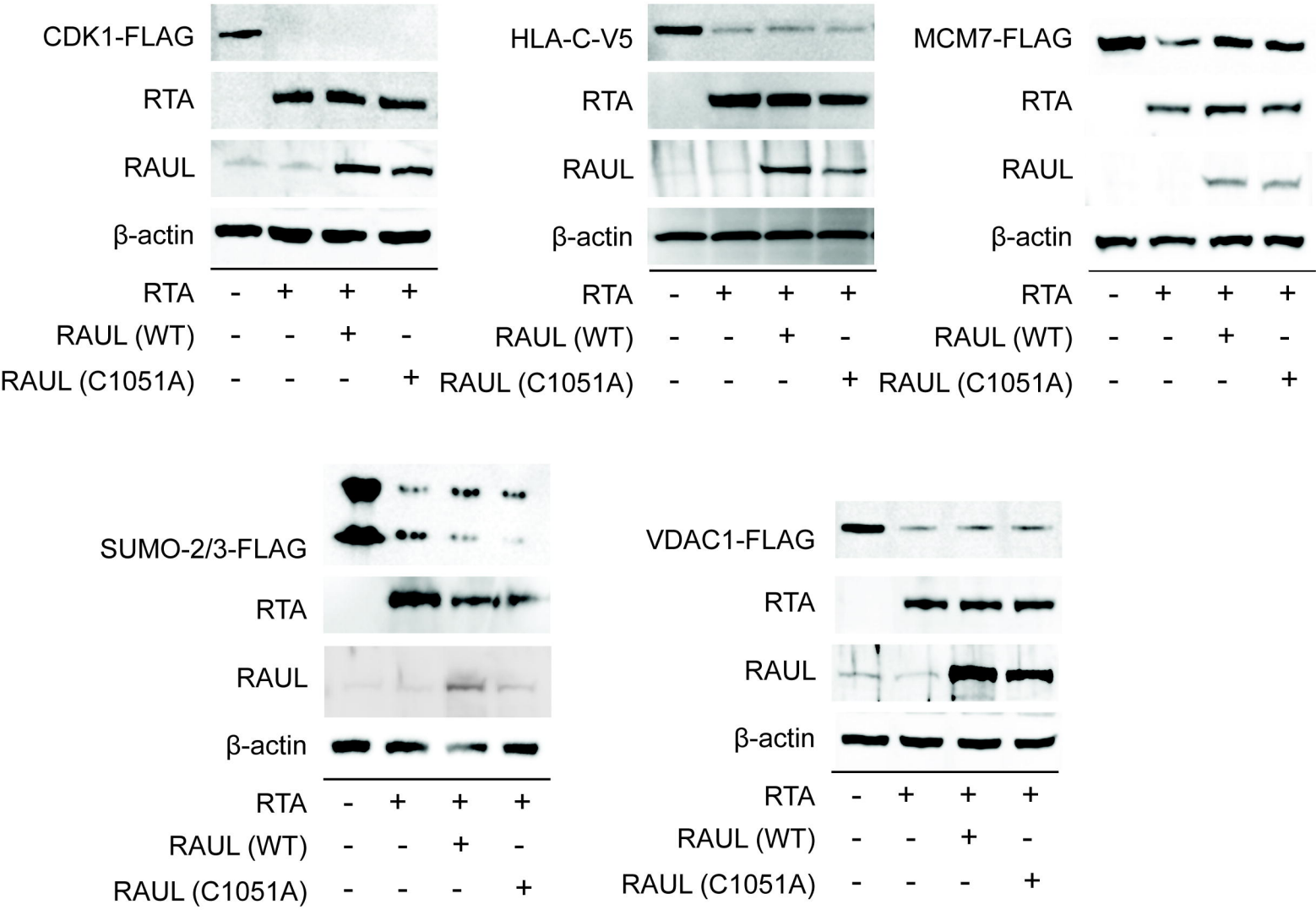
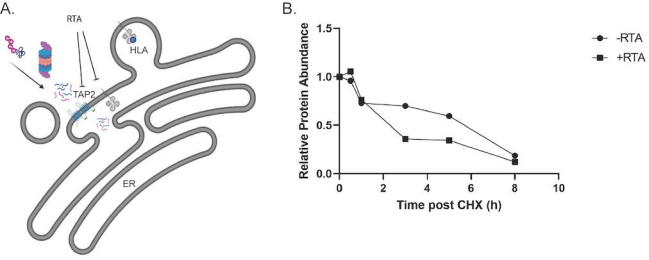
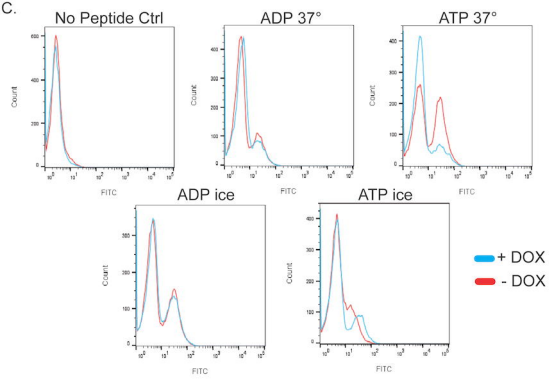
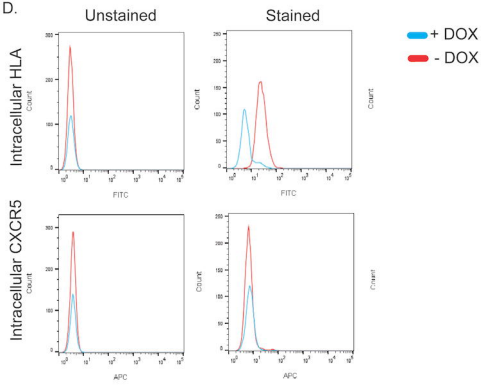
RTA
β-actin
RTA (WT) - + -
RTA (H145L) - - +
SUMO-2/3-FLAG
RTA
β-actin
RTA (WT) - + -
RTA (H145L) - - +
VDAC1-FLAG
RTA
β-actin
RTA (WT) - + -
RTA (H145L) - - +

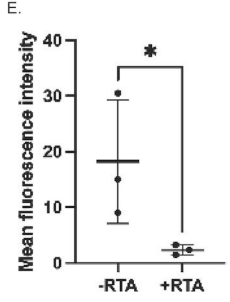
B.


CDK1-FLAG
RTA
RAUL
β-actin
RTA - + + +
RAUL (WT) - - + -
RAUL (C1051A) - - - +
HLA-C-V5
RTA
RAUL
β-actin
RTA - + + +
RAUL (WT) - - + -
RAUL (C1051A) - - - +
MCM7-FLAG
RTA
RAUL
β-actin
RTA - + + +
RAUL (WT) - - + -
RAUL (C1051A) - - - +
SUMO-2/3-FLAG
RTA
RAUL
β-actin
RTA - + + +
RAUL (WT) - - + -
RAUL (C1051A) - - - +
VDAC1-FLAG
RTA
RAUL
β-actin
RTA - + + +
RAUL (WT) - - + -
RAUL (C1051A) - - - +


A.
RTA
HLA
TAP2
ER
B.
Relative Protein Abundance
Time post CHX (h)
-RTA
+RTA


C.
No Peptide Ctrl
ADP 37°
ATP 37°
ADP ice
ATP ice
Count
FITC
+ DOX
- DOX


D.
Unstained
Stained
Intracellular HLA
Intracellular CXCR5
Count
FITC
APC
+ DOX
- DOX


E.
Mean fluorescence intensity
*
-RTA
+RTA