Comparing the Malignant Properties of Parental and a knock-in version of HCT116 cell line expressing the CDK2-mutant of eukaryotic Elongation Factor 2 (eEF2)

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Abstract

Modulation of protein synthesis according to the physiological cues is maintained through tight control of Eukaryotic Elongation Factor 2 (eEF2), whose unique translocase activity is essential for cell viability. Phosphorylation of eEF2 at its Thr56 residue inactivates this function in translation. In our previous study we reported a novel mode of post-translational modification that promotes higher efficiency in T56 phosphorylation. Cyclin A/CDK2-mediated phosphorylation of eEF2 at the S595 residue is required for more potent phosphorylation at the Thr56, suggesting CDK2 takes a role in robust suppression of protein synthesis. In the current study, we analyzed the cell cycle, proliferation, cell death, migration, colony formation, autophagy, and response to Cisplatin properties of the point-mutant variant of HCT116 cells that express the CDK2 mutant (S595A-eEF2) of eEF2. The knocked in S595A mutation resulted in decreased levels of T56 phosphorylation of eEF2, which appears to have similar biological consequences to other experimental manipulations such as silencing the activity of the kinase for the Thr56 residue, eEF2 Kinase (eEF2K). Our findings indicate that interfering with the inhibition of eEF2 results in elevated protein synthesis in HCT116 cells and is associated with the progression of malignancy in the colorectal cancer cell line, where eEF2K activity could provide a tumor suppressive role.

Introduction

De novo protein synthesis is a demanding process in terms of both energy and biomaterial consumption in eukaryotic cells, therefore, its performance must be calibrated based on availability of both nutrient and metabolic energy as well as external physiological cues (Neelagandan et al. 2020). For example, upon acute deprivation in energy or amino acid pools regulatory checkpoints operating at the initiation and elongation stages of translation pause the on-going protein synthesis allowing cells to re-direct energy and biomaterials for stress response processes (Kaul, Pattan, and Rafeequi 2011). Thereby, tight regulation of protein synthesis machinery confers agileness to the cell in re-arrangement of gene expression to changing physiological conditions without requiring de novo transcription (Browne and Proud 2002). Therefore, translational control constitutes an important component of cell viability and proper progression of all cellular processes, including cell division cycle (Kronja and Orr-Weaver 2011).

Somatic cells are known to depend typically on transcriptional regulation to achieve desired changes in gene expression required for the progression throughout the cell cycle (Cho et al. 2001) (Tanenbaum et al. 2015). However, a few studies that emerged in the last two decades have modified this view by highlighting the role of regulation at the translation level in shaping gene expression during cell division (Polymenis and Aramayo 2015) (Aviner, Geiger, and Elroy-Stein 2013). For example, repression of translation reported at the G2/M transition from several studies is the most salient example of translational regulation in the somatic cell cycle (Sivan, Kedersha, and Elroy-Stein 2007) (Sivan and Elroy-Stein 2008) (Tanenbaum et al. 2015). First reports on the existence of a reversible global repression of protein translation prevailing during mitosis assumed that this repression impacted all mRNAs (Fan and Penman 1970) (Bonneau and Sonenberg 1987) (Celis, Madsen, and Ryazanov 1990). Although, functional significance of this dampening in the translation rates for the proper progression of the cell

cycle was not fully understood at the time, a recent study by Tanenbaum and colleagues provides insightful clues for the basis of a translational suppression during mitosis (Tanenbaum et al. 2015). Strikingly, the study points out that a large fraction of a 200-mRNA-pool that undergoes changes in their translational efficiency become suppressed in their translation at the mitotic entry, which reverts at the mitotic exit.

Earlier studies support the notion that mitotic translational repression is achieved via the inhibition of the initiation stage through downregulation of cap-dependent translation (Cornelis et al. 2000) (Pyronnet, Dostie, and Sonenberg 2001) (Qin and Sarnow 2004) (Wilker et al. 2007). On the other hand, more recent work provides evidence that blockade of translational elongation could also be contributing to mitotic translational repression (Sivan, Kedersha, and Elroy-Stein 2007)(Sivan and Elroy-Stein 2008).

Elongation stage of protein translation is controlled via inhibition of the translocase activity of eukaryotic elongation factor 2 (eEF2) (Jørgensen, Merrill, and Andersen 2006). Elongating polypeptide chains depend on the translocase activity of eEF2 in order to move from the A site of the ribosome to the P site to allow the loading of t-RNA's charged with the incoming amino acids in the vacated A site (Browne and Proud 2002). Hydrolysis of GTP is indispensable for eEF2 to execute its translocase activity (Hershey 1991). Since addition of each amino acid to the growing polypeptide chain is one of the most energy consuming processes, reversible inhibition of translation elongation enables cells to switch to an energy-saving mode in response to cellular stress (Hershey 1991). Phosphorylation of eEF2 at the Thr56 residue in its GTP-binding pocket ablates translocase activity through preventing binding of GTP and lowering the affinity of the enzyme for the ribosomes (Ryazanov 1987) (Ryazanov and Davydova 1989). The only known kinase for this inhibitory Thr56 phosphorylation is eukaryotic elongation factor 2 kinase (eEF2K) which is an atypical Ca2+/Calmodulin dependent kinase from the alpha kinase family (Nairn and Palfrey 1987) (Ryazanov et al. 1997).

Activity of eEF2K is fine-tuned as an output from a complex array of multiple phosphorylation events the net effect of which can either augment or attenuate its catalytic activity towards eEF2 (Proud 2015). For example, in response to mitogenic signaling, hormones and amino acids, the mitogen-activated protein kinase (MAPK) and mTOR pathways suppress eEF2K to promote protein synthesis (Knebel, Morrice, and Cohen 2001) (Redpath, Foulstone, and Proud 1996) (X Wang et al. 2001). On the other hand, challenges such as nutrient deprivation, hypoxia, and oxidative stress activate eEF2K to pause protein synthesis via direct phosphorylations by AMP kinase- (AMPK) and protein kinase A/Ca2-dependent signaling (Browne, Finn, and Proud 2004) (Horman et al. 2002) (Redpath and Proud 1993). Strikingly, a wide spectrum of stimuli exert an activating or inhibitory effect on overall translation elongation through relaying the signal to eEF2K rather than eEF2 itself. Therefore, T56 phosphorylation of eEF2 operates as a reversible switch to turn on/off translational elongation.

Coupling of sub-cellular processes to the progression of cell division cycle is achieved through cyclin-dependent kinase-mediated phosphorylation of hundreds of their substrates. By taking advantage of kinase-engineering and mass spectrometric approach we identified over 180 putative targets of Cyclin

A/CDK2, 9 of which curiously executed a key function in protein translation machinery. Given its key regulatory role overseeing the global translation we first validated that eEF2 as a Cyclin A/CDK2 target in vitro (Chi et al. 2008).

In our follow up study we reported a novel mode of regulation for eEF2 whereby a distinct residue Ser595 in the C-terminal domain revealed as a target for Cyclin A/CDK2 and is required for augmentation of the phosphorylation at T56, suggesting a role for Cyclin A/CDK2 in relaying a more potent inhibitory signal to pause protein synthesis (Hizli et al. 2013). S595 phosphorylation of eEF2, the maximal levels of which is observed in mitosis, also displays sensitivity to CDK2 inhibitors both in in vitro kinase assays and in cells (Hizli et al. 2013). While mutation of S595 to an Alanine residue lowers phosphorylation of eEF2 at T56, mutation of H599 to a Proline completely abolishes the T56 signal (Hizli et al. 2013). In addition, a phospho-peptide spanning the eEF2 S595 region competes better than the unphosphorylated version of the same peptide with eEF2K in phosphorylation of T56 (Hizli et al. 2013). Finally, using purified components we were able to demonstrate that pre-phosphorylation of eEF2 by cyclin A/CDK2 at S595 elevates the efficiency of T56 phosphorylation in the subsequent kinase reaction by eEF2K (Hizli et al. 2013).

All these observations allowed us to speculate whether the positive regulation of the inhibitory T56 phosphorylation by the novel CDK2-targeted site fortifies the inactivation of eEF2 by eEF2K through enhancing the recruitment of eEF2K to its only known substrate. Considering that phosphorylation at Ser595 reaches maximal levels parallel to a similar increase in T56 phosphorylation at the entry to mitosis, this potent break imposed on translation elongation could be contributing to the recently demonstrated repression of translation at mitotic entry.

In order to discern further details of physiology underlying Ser595 phosphorylation of eEF2 we engineered HCT116 cells that encode a Ser595Ala mutant eEF2 using CRISPR technology and compared both cell lines in terms of their cell cycle progression, proliferation, colony formation, migratory properties, and response to Cis-platin. One advantage of producing the phosphorylation mutation in the eEF2 gene in a colon carcinoma cell line is to address the potential contributions of altered translational elongation to tumorigenic processes in addition to studying the differences two cell lines may display in their cell cycle properties. Increasing number of reports have recently pointed to the cytoprotective effects of increased activity of eEF2K in diverse types of cancer, including breast cancer, ovarian cancer, colon cancer, glioma, medulloblastoma, hepatocellular carcinoma, and prostate cancer (Xuemin Wang, Xie, and Proud 2017). Moreover, emergence eEF2K as a pleiotropic regulator of an array of tumorigenic processes such as cell cycle, proliferation, autophagy, apoptosis, angiogenesis, invasion, and metastasis formed a basis to evaluate eEF2K as a potential drug target (Zhang et al. 2021) (Karakas and Ozpolat 2020). Unlike the case seen in the above-mentioned cancer types where decrease in eEF2K activity exerts an anti-tumorigenic efect, our findings suggest that decrease in T56 phosphorylation of eEF2 in the S595A knock in cells is associated with increased proliferation, altered cell cycle properties, and moderately increased migratory and colony formation capacity.

Materials and Methods

Cell Lines and Cell Culture Conditions

Parental HCT-116 (human colorectal adenocarcinoma cell lines) was a kind gift of Dr. Bruce Clurman (Fred Hutchinson Cancer Center). Ser595-to-Alanine mutant version of the HCT116 cells were obtained through incurring the point mutation in the eEF2 gene using CRISPR technology. Both parental and the point mutant versions of HCT-116 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, #41966-029, Invitrogen, Gibco, UK). Each medium was supplemented with %1 Penicillin/Streptomycin/Amphotericin (PSA, Invitrogen, Gibco, UK) and %10 fetal bovine serum (FBS, #10500-064, Invitrogen, Gibco, UK). Cells were maintained at 37°C and 5% CO2 in a humidified incubator.

Cytotoxicity Assay

Effects of Cisplatin (Koçak Farma, Turkey) on cell viability of HCT-116 cells were tested. HCT-116 WT and KI cells were cultured in 96-well plates at a density of 5000 cells/well. The next day, cells were treated with Cisplatin (doses ranging from 80μM to 0.5μM). After culturing for 72 hours at different concentrations of the compound, cell viability was assessed via MTS assay (3-(4,5-dimethyl-thiazol-2)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolim salt (MTS) (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK) according to manufacturer's instructions. Treatment containing medium was removed and an MTS solution (PBS solution included %10 MTS and 4.5g/L D-glucose solution) was added followed by 90 minutes of incubation at 37°C. Then, their absorbance was measured at 490 nm by using an ELISA plate reader (Biotek, Winooski, VT). IC50 values were calculated by the GraphPad prism software.

Cell Cycle Analysis

Cells were seeded into T25 flasks at a density of 50x103 and were further cultured for 72h at 37°C. Then, they were harvested by trypsinization, washed with PBS and fixed with 70% ice-cold ethanol to be kept at -20°C for least two hours. Fort he flow cytometric analysis cell pellets were permeabilized with 0.1% triton-X-100 and incubated with 20 μg/ml RNase at room temperature for 30 minutes. Finally, cells were stained with PI and then immediately analyzed by a 488 nm single laser emitting device within 15 minutes.

Real-Time PCR

Total RNA was isolated by using RNA isolation kit (#740955.250, Macherey-NAGEL, Düren, Germany) according to the user's manual. After that, isolated total mRNAs were converted in cDNAs with QuantiTect Reverse Transcription Kit (#205313, QIAGEN, Hilden, Germany). RT-PCR was performed using SYBR Green (#4309155, Thermo Fisher, Waltham, ABD) and assayed in triplicate using iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA). The expression levels were normalized with respect to RPL30 (Ribosomal Protein L30) gene (F: 5'-ACAGCATGCGGAAAATACTAC-3' R: 5'-AAAGGAAAATTTTGCAGGTTT-3') levels. Genes and their corresponding primer sequences used in this study as follows; Tumor protein 53 (TP53) (F: 5'-GCCCAACAACACCAGCTCCT-3' R: 5'-CCTGGGCATCCTTGAGTTCC-3') baculoviral inhibitor

of apoptosis repeat-containing 5 (BIRC5 or Survivin) (F: 5'-TCTTCACCGCTTTGCTTTC-3' R: 5'- CGCACT TTCTCCGCAGTTTC-3'), Bcl-2-associated X protein (BAX) (F: 5'- TGCAGAGGATGATTGCCGCCG-3' R: 5'-ACCCAACCACCCTGGTGTTGG-3'), Tyrosine-protein kinase (ABL-1) (F: 5'-TACCCGATTGACCTGTC-3' R: 5'-CGATTTCAGCAAACGACCCC-3'), proliferating cell nuclear antigen (PCNA) (F: 5'-CAAGTAATGTCGATAAAG AGGAGG-3' R: 5'-GTGTCACCGTTGAAGAGAGTGG-3') Yes1 associated transcriptional regulator (YAP) (F:5'-CACAGCATGTTCGAGCTCAT-3' R:5'-GATGCTGAGCTGTGGGTGTA-3') Salvador Family WW Domain Containing Protein 1 (SAV1) (F:5'-CCTGTGCTCCTAGTGTACCTC-3' R:5'-GCGTAAACCTGAAGCCAGTC-3') Neurofibromin 2 (Merlin) (F:5'-GACAGCTCTGGATATTCTGCAC-3' R:5'-CTGCAAGGTGAGTTTGAGGG-3' (LATS2) (F:5'-ACAAGATGGGCTTCATCCAC-3' R: 5'-CTGACATGGCTCCCTTTCTG-3') (Ki-67) (F: 5’ GAAAGAGTGGCAACCTGCCTTC 3’ R: 5’ GCACCAAGTTTTACTACATCTGCC 3’) ATG5 (F: 5’AAAGATGTGCTTCGAGATGTGT3’ R: 5’CACTTTGTCAGTTACCAACGTC3’) LC3B (F: 5’AAGGCGCTTACAGCTCAATG3’ R: 5’CTGGGAGGCATAGACCATGT3’) BRCA1 (F: 5’GAACCAGGAGTGGAAAGGTCA3’ R: 5’GCTGTTGCTCCTCCACATCA3’) BR CA2 (F: 5’AGACTGTACTTCAGGGCCGTACA3’ R: 5’GGCTGAGACAGGTGTGGAAACA3’) CDK1 (F: 5’ CACTTGGCTTCAAAGCTGGCTC 3’ R: 5’ ATGGGTATGGTAGATCCCGGC 3’) CDK2: (F: 5’ CTGGACACGCTGCTGGATG 3’ R: 5’ ATGCCAGTGAGAGCAGAGGC 3’) CDK4: (F: 5’ GTCTATGGTCGGGCCCTCTG 3’ R: 5’ CAGATCAAGGGAGACCCTCACG 3’) CDK6 (F: 5’ GTCTGATTACCTGCTCCGCGA 3’ R: 5’ TCCAGAATCATTGCACCTGAGGG 3’) E2F4 (F:5’GCATCCAGTGGAAGGGTGTG3’ R: 5’ACGTTCCGGATGCTCTGCT3’ GADD45A (F: 5’GATGCCCTGGAGGAAGTGCT3’ R: 5’GAGCCACATCTCTGTCGTCGT3’) MST1 (F:5’CCTCCCACATTCCGAAAACCA3’ R:5’ GCACTCCTGACAAATGGGTG3’) KIBRA (F:5’GCATTAAAGGTGGACAAAGAGAC3’ R: 5’ TCTTAGAGCGGATGATGGTG 3’). The fold changes for each sample were determined using the 2(-Delta Delta C(T)) method.

Cell Migration (Scratch) Assay

HCT116 WT, and HCT116 KI cell lines were seeded in the six-well plate (#CLS3512, Corning Plasticware, Corning, NY) and their densities are $2x10^5$ cells/well. They were incubated for 24 hours in the incubator. After 24 hours, the cells were scratched. Then, the media was aspirated and the cell was washed with 1 ml PBS. fresh media was added and each good photograph was taken every 24 hours for 3 days.

Colony Formation Assay

To reveal the long-term effects of HCT116 WT and HCT116 KI cell lines, a Colony formation assay (CFA) was performed. Briefly, HCT116 WT and HCT116 KI cells were plated into six-well plates (#CLS3506, Corning Plasticware, Corning, NY) as 1000 cells/well was seeded and media was changed with fresh media every 48 hours. After 8–10 days, wells were washed with PBS and fixed by using 4 percent Paraformaldehyde (PFA). After fixation, crystal violet treatment was applied. Then, the dye was cleaned from the wells, and photos were taken with a luminometer device (Biorad, USA) and colonies were counted.

5-Ethynyl-2'-deoxyuridine (EdU) Assay

EdU is a 5-ethynyl-2'-deoxyuridine analog that is absorbed into dividing cells during DNA synthesis. As a result, EdU inclusion is a marker for cell proliferation. As suggested by the manufacturer EdU Staining Proliferation Kit (iFluor 647) (Abcam, Cambridge, MA, UK; ab222421). HCT116-WT and -KI cells were seeded in 4 wells (Millicell® EZ Slide, 4-well), and after 72h, cells were treated with a culture medium containing 20 μM EdU reagent. Next, cells were incubated for 2 h and were fixed with paraformaldehyde. Nuclei were stained with DAPI. The percentage of EdU-positive cells was quantified and analyzed using time-lapse fluorescence microscopy.

Immunocytochemistry (ICC) Assay

HCT116-WT and -KI cells were seeded in 4 wells (Millicell® EZ Slide, 4-well), and after 72h, they were fixed with 2% parafolmaldehyde for 30 minutes at 4°C. After, 0.1% Triton-X100 was added for each well for 10 minutes to permealize cells at room temperature. 10% FBS added to each well to block cells. After that, Cell were incubated with primary antibody 1:1000 SAV-1 (ab105105), MST-1 (ab51134) LC3B (ab192890) and ATG-5 (ab108327) overnight. Then, cells were incubated with secondary antibody (1:200) for 2 hours. DAPI (1:1000) were added to each well for 20 minutes at 4°C. The percentage of stained cells were quantified and analyzed using fluorescence microscopy.

Caspase 3/7 Activity Assay

Caspase activity in HCT116 cells was measured for 72h by using Caspase-Glo® 3/7, assay systems (Promega, Madison, WI) according to manufacturer's instructions. Shortly, cells were cultured in white 96-well plates and caspase 3/7 activity was measured for 72 h. Caspase levels were measured at different time points (30-60-90-120-150 minutes) by a luminometer (Varioscan Thermo Fisher).

Protein Isolation and Immunoblotting

Isolation of protein samples from HCT116-WT and HCT116-KI cells grown under normal conditions was performed as descrided previously (Chi et al. 2008) (Hizli et al. 2013). Immunoblotting was performed as described in the standard immunoblotting protocol applied in our earlier study using phospho-T56-eEF2 (Cell Signalling Cat no 2331) and total eEF2 (Cell Signalling Cat No 2332).

Statistical Analysis

All data are shown as the means ± standard errors. The statistical analysis of the results was performed with unpaired t test, and graphs were drawn using GraphPad Prism 5 software. Statistical significance was determined at $p < 0.05$.

Results

3.1. Comparison of HCT116-WT and HCT116-KI Cell Cycle Profiles and Expression of Cell Cycle Regulators

First we confirmed reduced levels of endogenous phospho-T56-eEF2 levels on protein samples isolated from parental and point-mutant HCT116 cells. As expected, knock in cell line that express a S595A-eEF2 point-mutant displayed decreased T56 phosphorylation (Fig. 1a). To understand whether there are any differences in cell cycle distribution of HCT116-WT and HCT116-KI cells in various phases of the cell cycle, we performed a flow cytometric application measuring the DNA content of fixed cell populations that had been cultured under normal growth conditions. There were no significant differences between the G0/G1 subpopulation of HCT116-WT and HCT116-KI cells. On the other hand, a significant accumulation of HCT116-KI cells in the S phase (41.64%) was noteworthy compared to that of HCT116-WT cells (15.77%). In terms of G2/M population, parental cells had a significantly higher number of cells in this phase (24.43%) compared to HCT116-KI cells (7.28%) (Fig. 1b and Fig. 1c).

To explore the molecular basis underlying the differential cell cycle distribution between the WT and KI cells, we compared the mRNA levels of key cell cycle regulators using real time PCR. Gene expression levels of each regulator was normalized to the abundance of RPL30 transcript. There was no significant difference in the levels of G1 cyclin-dependent kinases (CDK) CDK4 and CDK6 between the HCT116-WT and HCT116-KI cells. Likewise, levels of CDK2 and PCNA appeared to be essentially the same between the WT and knock in cells. Although, lack of difference in the CDK2 expression between the two cell lines did not explain the reason why HCT116-KI cells present with a higher cell number in the S phase, the increase in their levels of Ki-67 was in support of increased S phase population. Finally, the lower expression of the mitotic cdk, CDK1, in the knock in cells compared to the parental cell line was consistent with the reduced G2/M population seen in the mutant cell line (Fig. 1d).

3.2. Comparison of HCT116-WT and HCT116-KI Proliferation Property.

In order to acquire further information on how the proliferation of eEF2 mutant cells compares to that of wt cells, both cell lines were analyzed in a 5-EdU-incorporation assay. Both HCT116-WT and HCT116-KI cells were cultured in presence of 5-EdU to label DNA, allowing incorporation of the tag at Thymidine bases. As shown in Fig. 2, the HCT-116-KI cells displayed a higher proliferative ability by 3 fold compared to that of parental HCT116 cells confirming flow cytometric analysis of the increased S phase population for the HCT116-KI cells.

3.3. Comparison of Baseline Cell Death between HCT116-WT and HCT116-KI cells

To evaluate the baseline cell death in HCT116-WT and HCT116-KI cell lines, caspase activity was used as a read-out where activities of Caspase 3/7 were measured using Caspase-Glo 3/7 Assay System (Promega). Caspase 3/7 activity of HCT116-KI cells that were cultured for 72 hours is represented relative to that in HCT116-WT cells in 30 minute time intervals (Fig. 3). At all timepoints, Caspase 3/7 activity levels were higher in HCT116-WT cells compared to those HCT116-KI cells. Also, Caspase 3/7 activity remained the same throughout the time course of the measurement in HCT116-WT cells, while a gradual increase in the HCT116-KI cells was observed reaching a maximum in Caspase 3/7 activity at 150 minutes (Fig. 3b).

Next, levels of apoptotic facilitators and regulators such as BRCA1, BRCA2, Bax, BIRC5, and p53 were measured using real time PCR. In terms of amounts of transcript present in the untreated asynchronously growing HCT116-WT and HCT116-KI cell populations, there were no major differences in BRCA1, BRCA2, Bax, and BIRC5 expression. However, in the HCT116-KI cells there was a significant induction of the nuclear transcription factor, p53, that has a key pro-apoptotic role (Fig. 3a).

3.4. Comparison of Migratory Properties between the WT and HCT116-KI cells

To understand the effects of S595A point mutation in eEF2 on the migration ability of cells, a scratch assay was performed. Results from this in vitro cell migration assay revealed an increased migration ability of HCT116-KI cells. In the HCT116-WT cell line, 48.32 percent closure was detected in the gap in 72 hours whereas the gap was completely closed in the HCT116-KI cells in Fig. 4a and Fig. 4b.

3.5. Comparison of Anchorage Independent Growth Properties between WT and HCT116-KI cells

To measure differences in anchorage-independent growth properties between HCT116-WT and -KI cells, colony formation assay (CFA) was performed to examine how the mutation in eEF2 affects their colony formation capacity. CFA results have revealed that HCT116-KI cells have acquired an increased colony formation ability. Furthermore, while an average 99 colonies were counted in the HCT116-WT cell line, 150 colonies were counted in HCT116-KI cell line. When these results are taken together, S595A-eEF2 mutation in the HCT116-KI cells has contributed to the enhancement in the malignant properties of the parental line (Fig. 5a and Fig. 5b).

3.6. Changes in Autophagy between the parental and HCT116-KI cells

Since the mutation at the S595 to an Alanine mitigates the extent at which eEF2 translocase activity can be ceased, we hypothesized these cells could have an increased need for amino acids to keep up with a heightened protein synthesis process. To investigate whether HCT116-KI cells could up-regulate autophagy to meet an elevated need for biomaterial, gene expression levels of the two key factors in autophagy (ATG-5 and Beclin-1) were analyzed by real-time PCR. As seen in Fig. 6, there was an increase in the Atg5 and LC3B positive cells in the HCT116-KI variant according to the ICC analysis (Fig. 6a and Fig. 6c). Meanwhile, immunofluorescence signal for LC3B revealed no difference in the protein levels between the parental and HCT116-KI cells. Although there was no change in the level of ATG-5 expression at the mRNA level in the HCT116-KI cells, a striking increase in another important regulator of autophagy, Beclin-1 was evident (Fig. 6b).

3.7. Potential Contribution of Hippo Pathway to the Increased Malignancy of HCT116-KI cells

A complex crostalk between the Hippo pathway and autophagy have recently been reported (D. Wang et al. 2020). It turns out that the Hippo-mediated autophagy can promote two potential outcomes that involve contributing either to survival or cell death-related processes depending on the context of the

signaling cues (Tang and Christofori 2020). Due to our observation that autophagy is elevated in HCT116-KI cells, the potential contribution of the Hippo pathway was investigated by monitoring expression levels of various upstream members of the pathway and targets of downstream transcriptional coregulator-Yes associated protein (YAP). In order to understand the impact of the S595A eEF2 mutant on the activation status of the Hippo pathway, HCT116-WT and HCT116-KI cells were analyzed for the expression of the various factors in the cascade. The results indicated that expression levels of YAP and YAP target genes (CY61, CTGF and GADD45a) were significantly decreased in HCT116-KI cells suggesting suppression of the YAP pathway upon reduction in T56 phosphorylation of eEF2 (Fig. 7a). Similarly, significant reductions in the mRNA levels of MST-1 an KIBRA were recorded in the point mutant cells compared the levels of these HIPPO cascade factors in the parental cells. Furthermore, significantly increased protein levels of MST1 and SAV1 (upstream members of Hippo pathway,) were detected in HCT116-KI cells compared to HCT116-WT cells supporting a contribution by the Hippo pathway including upstream members (Fig. 7b).

3.7. Effect of Cisplatin on the cell cycle, Cell Survival and death

To determine whether HCT116-KI cells would respond to the Cisplatin treatment differentially compared to HCT116-WT cells, the half maximal inhibitory concentration (IC50) was determined in both cell lines using an MTS-based cell viability assay. Asynchronously growing populations of both parental and mutant cells were treated with increasing concentrations of Cisplatin (in the range of 0.5 μM-80 μM) for 72 h. The IC50 values at 72 h of Cisplatin treatment were determined as 10 μM for both HCT116-WT and HCT116-KI cell lines (Fig. 8a).

Due to the well-known effect of Cisplatin in inducing a strong S phase arrest in the HCT116 cells, we next evaluated the changes in cell cycle regulatory gene expression in response to the Cisplatin treatment at its IC50 concentration of 10μM using the Quantitative RT-PCR method. Gene expression was normalized using RPL30 transcript and shown as the relative expression in both cell lines. Both CDK-1 (~ 9 fold) and CDK-2 (2 fold) expression levels were significantly increased in HCT116-KI cells compared to HCT116-WT, while expression of CDK-4 and CDK-6 were decreased. In the meantime, there was no significant change in the levels of Bax, BIRC5, p53, and ABL1, but there was a 3 fold increase in the levels of Ki67 .

Discussion

Translocation activity of eEF2 is essential for cell viability due to its unique role in enabling polypeptide chain elongation. For example, irreversible inhibition of eEF2 by the Diphtheria Toxin infection, which inactivates eEF2 through permanent ADP-ribosylation of Histidine 715, results in cell death (Drazin, Kandel, and Collier 1971). Therefore, reversible inhibition eEF2 is an important part of the crisis management mechanism of the cell for its adaption to the acute cues of cellular stress.

Curiously, there have been no functionally-activating post-translational modifications described for maximal eEF2 activity. Rather, ever since its discovery in 1988, the phosphorylation of the major Thr56

and two other minor Threonine residues in the GTP-binding domain is known as the switch^{OFF} (stalled protein synthesis) and, conversely, dephosphorylation of the same residue is known as the switch^{ON} (resumed protein synthesis) for the eEF2-dependent translocase activity. In our previous study, we described a novel mechanism for the regulation of eEF2, whereby phosphorylation of a distinct Ser595 residue in the C-terminus domain by Cyclin A/CDK2 is required for the augmentation of Thr56 phosphorylation, promoting a more robust and efficient inhibition of the translocase activity (Hizli et al. 2013).

In order to acquire further insight into the physiological role of Ser595 phosphorylation, we generated a point mutant variant of the HCT116 cells that express a Ser595Ala point mutant of eEF2. Several properties of the parental HCT 116 cells were compared to those of HCT116-KI cells, including proliferation, cell cycle progression, colony formation, migratory properties, and response to Cisplatin treatment. Cell cycle analysis indicated that there is no significant difference in the abundance of G0/G1 population between asynchronously growing HCT116-WT and HCT116-KI cells. However, the mutant cells displayed a significantly higher accumulation of cells in S phase with a concomitant decrease during G2/M population compared to the parental cells. A 3-fold-reduction in the number of HCT116-KI cells at the G2/M border reconciles with both our previous finding that maximal levels of both Ser595 and Thr56 phosphorylation are seen at the entry of mitosis. Moreover, the decrease in the G2/M population in HCT116-KI cells suggests that Cyclin A/CDK2-dependent augmentation of the T56 phosphorylation is required for the proper cell cycle progression and that AK2-dependent accumulation of S595-eEF2 could be underlying the suppression of protein synthesis during mitosis reported by other groups (Fan and Penman 1970) (Celis, Madsen, and Ryazanov 1990) (Sivan and Elroy-Stein 2008) (Tanenbaum et al. 2015). On the other hand, the basis for the accumulation of HCT116-KI cells in S phase is not well understood in the sense that hyperphosphorylation of Ser595 and Thr56 was rather observed in prometaphase cells than S phase cells.

With respect to the expression levels of cell cycle regulators an obvious reduction in CDK1 levels in the HCT116-KI cells supported the decrease in G2/M population. Molecular evidence for the increased S phase population of the knock-in cells clearly did not involve changes in the levels of key regulators of G1/S transition, such as CDK4 and CDK6, nor in E2F4, PCNA and the S phase regulator, CDK2. Nevertheless, a moderate increase in the Ki-67 levels in the knock-in cells was consistent with the higher proliferation propensity of the mutant cells.

In agreement with the finding of an increased S phase population in the HCT116-KI cells, there was a 3-fold-increase in their incorporation of EdU stain, which is indicative of higher levels of DNA synthesis, compared to HCT116-WT cells. The comparison of baseline apoptotic index with respect to their Caspase 3/7 activity between the parental and mutant cell lines revealed that the HCT-116-KI cells have reduced, but progressively increasing levels of cell death, whereas HCT116-WT cells have a higher and constant apoptotic index throughout the measurement. Lack of significant difference in the levels of major apoptosis regulators such as BRCA1, BRCA2, Bax, and BIRC5 did not explain reduced levels of apoptosis

in the HCT116-KI cells. However, a remarkable increase in the expression of p53 by the mutant cells was noteworthy.

To address the potential involvement of the HIPPO Pathway in mediating the increased autophagic flux in the S595A-eEF2-expressing mutant cells, mRNA levels of the several factors of the HIPPO pathway such as TAZ, YAP1, MST-1, Merlin, SAV-1, CY61, CTGF and GADD45a were analyzed. There was a significant decrease in YAP1 mRNA levels and those of its target genes, such as CY61 and CTGF. Increase seen in the autophagy levels could be a direct consequence of heightened protein synthesis in the mutant eEF2-expressing cells. On the other hand, the decrease in YAP expression in the S595A-eEF2 mutant cells, which is concomitant with their improved malignant properties, could be due to the dual role of YAP both as an oncogene and tumor suppressor reported in colorectal cancer (Ou et al. 2017). In other words, YAP could act as a tumor suppressor in the context of the HCT116 cells, where its activity as a transcriptional co-activator is in charge of the upregulation of pro-apoptotic gene expression (Lamar et al. 2012). In this respect, HCT116-KI cells could reflect the pro-apoptotic capacity of YAP, the decrease of which in the knock-in cells could account for the decreased caspase activity measured in these cells. While there was no significant change in the mRNA levels of TAZ and Merlin, the drop in MST-1 transcript levels was at a lower magnitude compared to the drop in the message levels of SAV-1. Intriguingly, these changes in the mRNA levels were co-existent with a striking increase in MST-1-dependent fluorescent signal (modest decrease in transcript level) and a modest increase in SAV1 foci (potent decrease in transcript level) as altered features of the HIPPO pathway in the S595A-eEF2 HCT116 cells compared parental cells.

In terms of the response of the two cell lines to chemotherapeutics, there is no difference in the IC50 value of Cisplatin between the parental and HCT116-KI cells. However, striking increases in the levels of S (CDK2 2 fold) and G2/M cdk's (CDK1 7 fold) as well as Ki67 (3 fold) accompanied with decreases in G1 cdk's (CDK4 and CDK6) in the HCT-116-KI cells compared to the HCT116-WT cells are noteworthy in the sense that while unchallenged mutant cells appear to be underpopulated in the G2/M phase, they appear to reciprocate expression pattern of the cell cycle regulators in response to genotoxic stress. Nonetheless, further investigation of cell cycle analysis and cell death upon Cisplatin treatment are needed to conclude on the differences of the knock HCT116 cells when they respond to the drug.

When increased proliferation and decreased baseline apoptosis of HCT116-KI cells are put in perspective together with their enhanced migratory and colony formation capacity, expression of the S595A-eEF2 mutant by the knock-in cells could be associated with the progression of malignant properties of HCT116 parental cells. Since S595A-eEF2 mutant expressing knock-in cells have reduced T56 eEF2 phosphorylation and display increased malignancy, these observations are in agreement with the tumorigenic effects seen upon eEF2K knock down by Xie and colleagues in HCT116 cells (Xie et al. 2014). In other words, S595A-eEF2 expressing HCT116 cells phenocopy the mild increase in the malignant properties seen when the endogenous eEF2K levels are knocked down using siRNA-based approach in HCT116 cells as an in vitro model of colorectal cancer in that study.

More interestingly, our observations on the resultant impact of an eEF2-mutant with reduced Thr56 phosphorylation on autophagy also overlap with the elevated levels of autophagy these authors report upon silencing of eEF2K (Xie et al. 2014). Mechanistically, this study reports that the reduced inhibition of protein elongation through eEF2K knock down promotes autophagic response by upregulating both Beclin 1 and Atg7 protein levels independent of changes in mRNA levels of these genes, while there is no change in the levels of Atg5 (Xie et al. 2014). On the other hand, reduced inhibition of protein elongation through expressing an eEF2 mutant with dampened Thr56 phosphorylation levels promoted an induction of Atg5 and no significant change in LC3B foci formation, while significantly increasing Beclin 1 expression both at mRNA (5 fold increase) (Fig. 6b) and protein levels (data not shown). These findings confirm eEF2K as a negative regulator of autophagy in colon carcinoma cells and highlight that inactivation of its function directly liberates eEF2 translocase activity promoting heightened protein synthesis that could trigger activation of autophagy due to elevated demand on amino acids. Finally, increased colony formation upon eEF2K knock down in HCT116 cells reported in this study is also in agreement with our finding from the same assay as confirmed by the increased colony formation ability of the HCT11-KI cells. Clearly, expression of an eEF2 mutant that is incapacitated in its inhibition somehow contributed to the progression of malignant properties of colorectal cancer cells.

Increased eEF2K expression in the context of colon cancer appears to contrast to the cases of breast, ovarian, colon, glioma, medulloblastoma, hepatocellular carcinoma, and prostate cancer where increased eEF2K activity exerts a cytoprotective effect on cancer cells through acquisition of adaptation ability to the hypoxic, nutrient-deprived, and acidic tumor microenvironmet (Leprivier et al. 2013) (Leprivier et al. 2015). In the in vitro and in vivo models of colorectal cancer, manipulations that increase eEF2K activity promotes antitumorigenic effects. For example, Faller and his colleagues demonstarted that anti-tumorigenic activity of rapamycin is exerted through re-activation of eEF2K and decreased protein synthesis in *Apc*^{min} model of colorectal cancer (Faller et al. 2015). Similarly, De Gassart and colleagues provide evidence that anti-tumorigenic activity of a drug used in HIV treatment Nelfinavir (inhibitor of HIV aspartyl protease) is strictly dependent on presence of potent eEF2K activation in the sub-clones of HeLa cells and wt and knock out eEF2K MEFs where the drug becomes ineffective in reducing tumors in the absence of active eEF2K levels (De Gassart et al. 2016). Taken together with the evidence from colorectal cancer tumors from patients, where low expression of eEF2K is associated with poor survival outcome compared to patients whose tumors express higher levels of eEF2K, epxression of this gene elicits differential consequences on malignant properties of the cancers originating from different epithelia (Ng et al. 2019). It will be interesting to fully understand the molecular basis for eEF2K's differential role in terms of contributing to malignancy in different cell systems. A major limitation of this study to demonstrate the role of S595-phosphorylation of eEF2 in normal cell cycle progression. Therefore, the role of S595-phosphorylation of eEF2 in proper G2/M transition as well as in other types of cellular stress such as DNA damage, nutrient deprivaton, and hypoxia should be investigated in a non-transformed cell line model where the S595A mutation in eEF2 gene is knocked-in.

Declarations

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Author Contributions: Conceptualization, F B.Y.,N.T., F.S. and A.A.H.D.; methodology, B.Y.,N.T., F.S. and A.A.H.D.; validation, B.Y., N.T. and A.A.H.D; formal analysis, B.Y., N.T. and A.A.H.D.; investigation, B.Y., N.T. and A.A.H.D; data curation, B.Y., N.T. and A.A.H.D ; writing—original draft preparation, B.Y., N.T. and A.A.H.D.; writing—review and editing, B.Y.,N.T., F.S. and A.A.H.D. All authors have read and agreed to the published version of the manuscript.

B.Y.,N.T., F.S. and A.A.H.D. designed the study. B.Y.,N.T. and A.A.H.D.collected and analyzed the data. B.Y.,N.T., F.S. and A.A.H.D. contributed to results interpretation and the manuscript preparation.

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request.

Ethics Approval: N/A The study does not involve any human or animal subjects.

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Figures

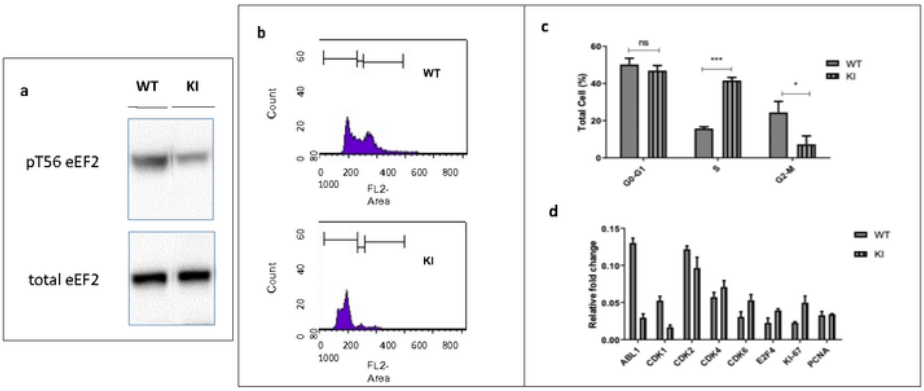

Fig1. ***Comparison of the T56-eEF2 levels, Cell Cycle Profile, and Cell Cycle Regulatory Genes between HCT116-WT and HCT116-KI cells*** (a) Western Blot Aalysis of the Whole Cell Lysates collected from HCT116-WT and HCT116-KI cells using phosho-T56-eEF2 and total eEF2 Antibodies (b) Histograms from the flow cytometric analysis of cell cycle progression in HCT116-WT and HCT116-KI cells (c) Quantitation of the cell cycle data in (b) (d) Expression levels of ABL1, CDK1, CDK2, CDK4, CDK6, E2F4, Ki-67, and PCNA transcripts determined in real time PCR Analysis

Figure 1

See image above for figure legend

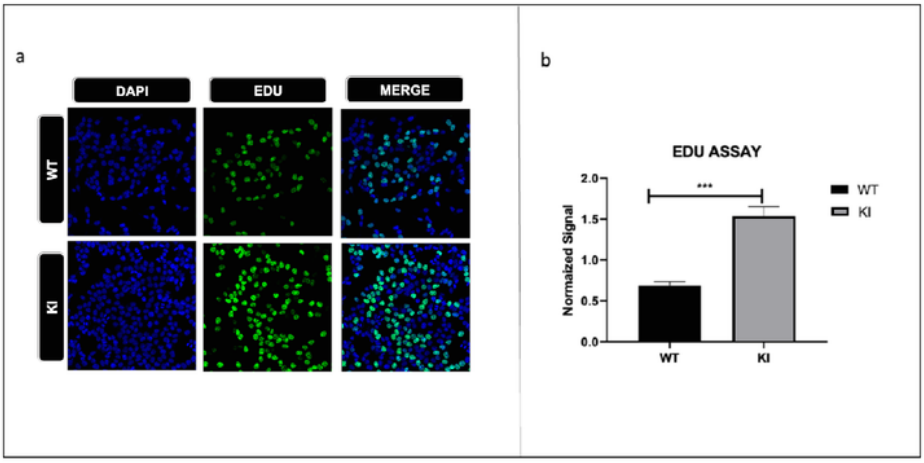

Fig2 ***Changes in Cell Proliferation in HCT116-KI cells*** (a) Images from the EdU-incorporation assay and DAPI staining for HCT116-WT (upper panel) and KI (lower panel) cells. (b) Quantitation of the merge signal from the EdU and DAPI stains.

Figure 2

See image above for figure legend

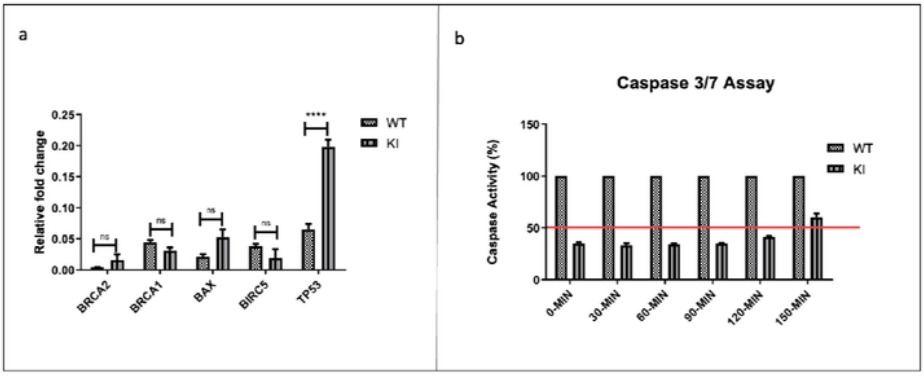

Fig3 *Changes in Cell Death Properties* (a) Expression levels of BRCA2, BRCA1, BAX, BIRC5, and TP53 transcripts determined in real time PCR Analysis (b) Caspase 3/7, activation detected by Caspase-Glo 3/7 luminescence assay

Figure 3

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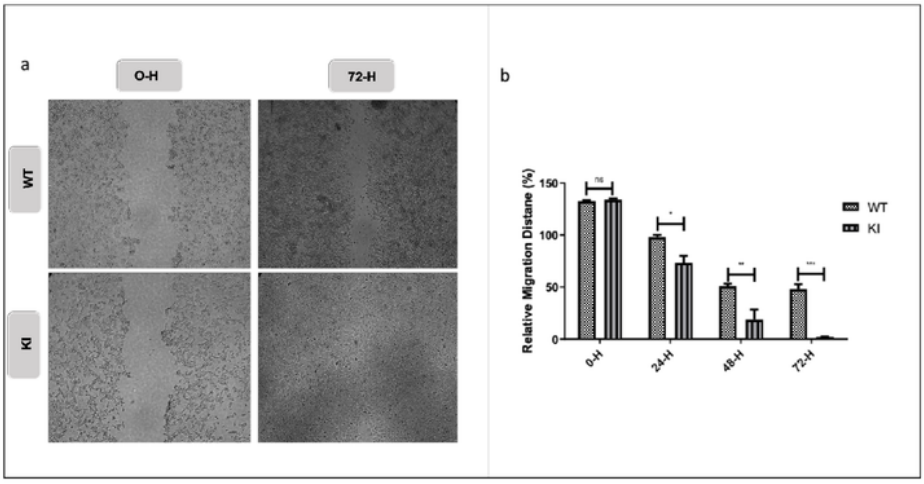

Fig4 ***Changes in Migratory Properties*** (a) Closure of the gap created by scratch in HCT116-WT (upper panel) and -KI cells (lower panel) at 0 and 72 h timepoints. (b) Quantitation of the distance closed by the two cell lines

Figure 4

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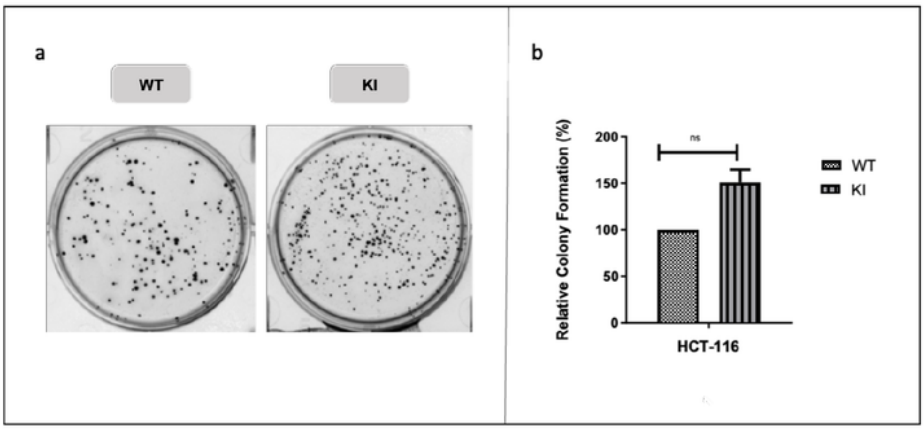

Fig5 Changes in the Anchorage Independent Growth and Colony formation Ability (a) HCT116-WT and -KI cells seeded at low density (500 cells/well) in 6 well tissue culture plates and cultured for 10 days to allow the formation of colonies (b) Quantitative representation for the number of colonies formed in parental and S595A-eEF2-mutant HCT116 cells

Figure 5

See image above for figure legend

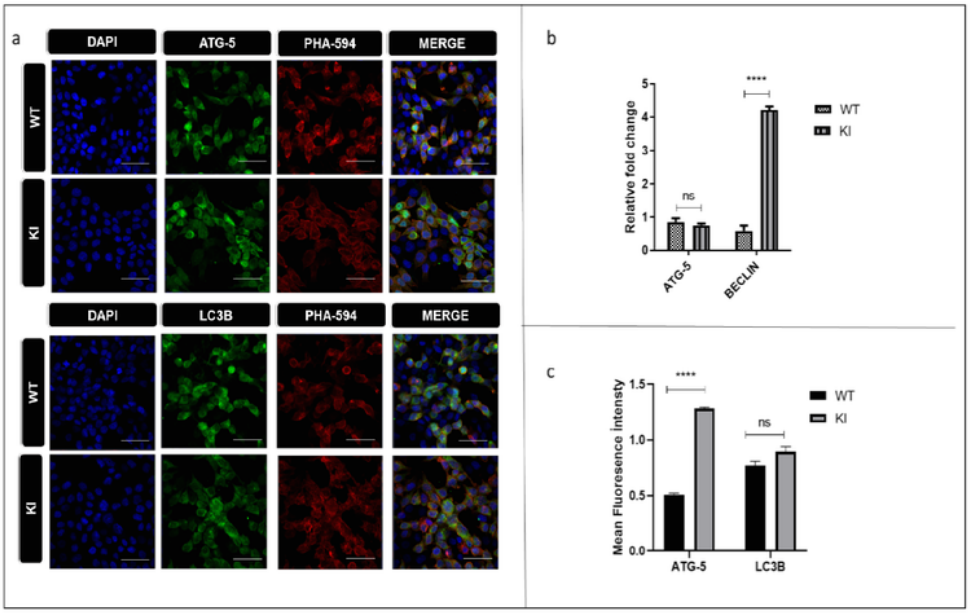

Fig6 *Changes in Autophagy* (a) Representative immunofluorescence images showing ATG-5 and LC3B staining in HCT116-WT and HCT116-KI cells, Magnification:40x and Scale bar: 100 µm (b) Expression levels of ATG-5 and Beclin transcripts determined in real time PCR Analysis (c) Quantitative representation of the ATG-5 and LC3B fluorescence signals

Figure 6

See image above for figure legend

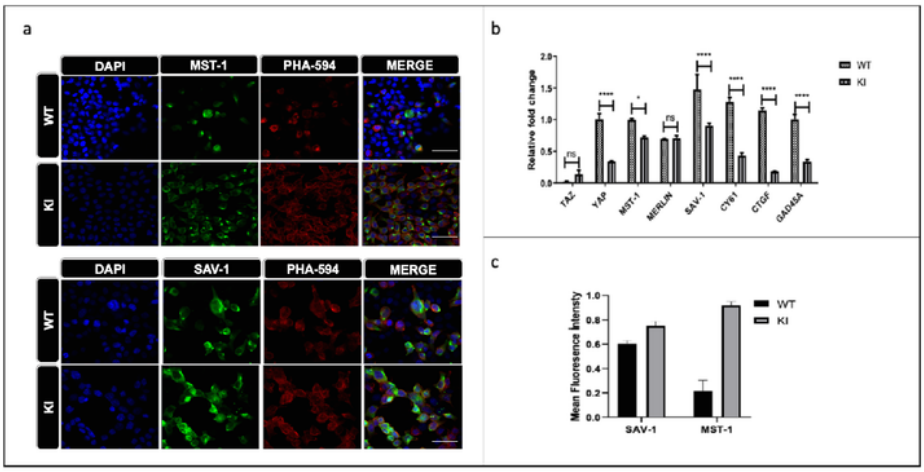

Fig7 ***Changes in Hippo Pathway*** (a) Representative immunofluorescence images showing MST-1 and SAV-1 staining in HCT116-WT and HCT116-KI cells, Magnification:40x and Scale bar: 100 µm (b) Quantitative representation of the SAV-1 and MST-1 fluorescence signals (c) Expression levels of TAZ, YAP, MST-1, KIBRA, MERLIN, SAV-1, LATS-2, CYR61, CTGF, and GAD45A transcripts determined in real time PCR Analysis

Figure 7

See image above for figure legend

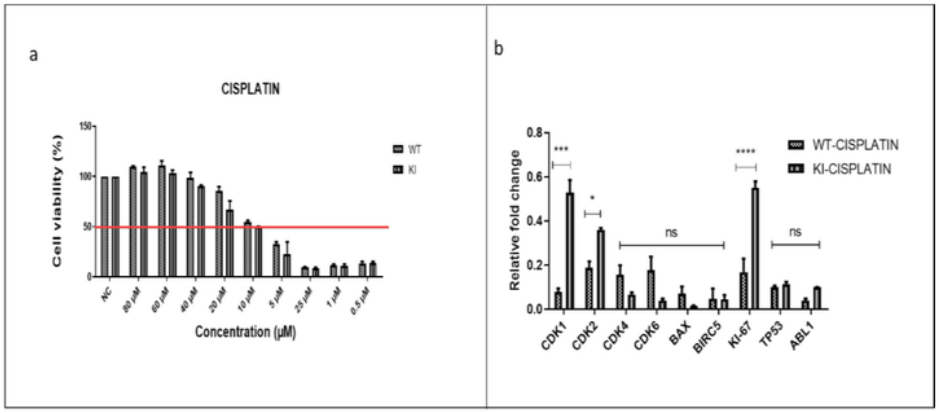

Fig8 Response of HCT116-WT and -KI cells to Cisplatin Treatment (a) The cell survival rates (percent) of HCT116-WT and HCT116-KI cell line at 72nd hours after treatment with Cisplatin (b) Expression levels of CDK1,CDK2, CDK4, CDK6, BAX, BIRC5, KI-67, TP53 and ABL1 transcripts determined in real time PCR Analysis after Cisplatin treatment

Figure 8

See image above for figure legend