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Investigating the Interaction of AURKA and UBE2C in Colorectal Cancer Cells

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INVESTIGATING THE INTERACTION OF AURKA AND UBE2C IN COLORECTAL CANCER CELLS

A

THESIS

Presented to the faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

M. D. Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

by

Apurva M. Hegde, B.E.

Houston, Texas

August 2015

Acknowledgements

Firstly, I would like to thank my mentor and advisor Dr. Subrata Sen for his guidance and support throughout my time as a graduate student in his lab. I would also like to extend my heartfelt thanks to my committee members Dr. Stanley Hamilton, Dr. Scott Kopetz, Dr. Lalitha Nagarajan and Dr. Paul Scheet for providing their time, valuable insight and perspectives, and encouraging me at every step of my project.

A big thank you to all the members of Sen Lab without whose help, support and guidance this project would not have been successful. In particular, I'd like to extend my gratitude to Yvette Gonzales for helping me with experiments and troubleshooting in spite of her busy schedule and making herself available whenever I needed her, and to Dr. Vibhuti Srivastava who is not only a very capable and accomplished scientist but also an exceptional mentor.

A special thanks to my family, I'm indebted to them for their constant encouragement and advice whenever I was feeling low and their unconditional love and support. Lastly, thank you to all my friends for extending a helping hand whenever I needed it and always being my pillars of support.

Abstract

Apurva Manohar Hegde, B.E.

Advisor: Subrata Sen, Ph.D.

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths in the US. Among the many genomic aberrations previously implicated in colorectal cancer, recurrent amplification of chromosome 20q is frequently associated with liver metastasis. Previous research in our lab identified a gene signature on chromosome 20q associated with colorectal cancer progression. In this study, one of the genes in the signature, the ubiquitin conjugating enzyme UBE2C, was identified through preliminary bioinformatics analysis as a candidate for further examination of its role in CRC progression. Co-expression analysis of UBE2C in tumor-normal datasets from the public database Oncomine revealed all the datasets showing its highest co-expression with Aurora kinase A (AURKA) pathway members, i.e. with AURKA and its binding co-factor and activator TPX2, all being localized on chromosome 20q. In addition, preliminary immunoblotting experiments in a panel of ten CRC cell lines showed high positive correlation between endogenous protein expression of UBE2C and AURKA. Overexpression of AURKA led to an increase in UBE2C protein as well as transcript levels, although UBE2C protein levels remained stable when cells were treated with Aurora kinase A inhibitor, suggesting that the mechanism of regulation is independent of the kinase activity. Furthermore, we found evidence of a novel interaction between AURKA and UBE2C protein by co-immunoprecipitation experiments. Finally, we assessed the impact of AURKA expression on the half-life of UBE2C protein using cycloheximide pule-chase assay. In summary, we report a novel relationship between two cell cycle proteins AURKA and UBE2C with potential implications for a new combination therapy in colon cancer.

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Background and Introduction

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells, with disruption of the genetic architecture of cells being one of the hallmarks of the disease. It has emerged as one of the most dreaded diseases known to mankind and poses a formidable challenge in the field of medicine. Cancer of the colon and rectum in particular, is estimated to cause 49,700 deaths in the United States in 2015, the third leading cause of cancer-related deaths for the two sexes separately, and the second leading cause when men and women are combined [Cancer Facts & Figures (2015), Link 1]. Despite increased screening for premalignant lesions contributing to decreased incidence of this disease over the past 20 years, the rate per 100,000 still remains high in the US, with survival rate dropping from 90% in patients with localized disease to only 13% in patients with distant metastatic spread [Global Cancer Facts & Figures (2014), Link 2; Cancer Facts & Figures (2015)]. Therefore, elucidating the underlying mechanisms driving progression of this cancer is essential for developing effective biomarkers and therapeutic interventions.

Colorectal Cancer pathogenesis

Figure 1 shows different pathways known to cause Colorectal Cancers (CRCs) [1].

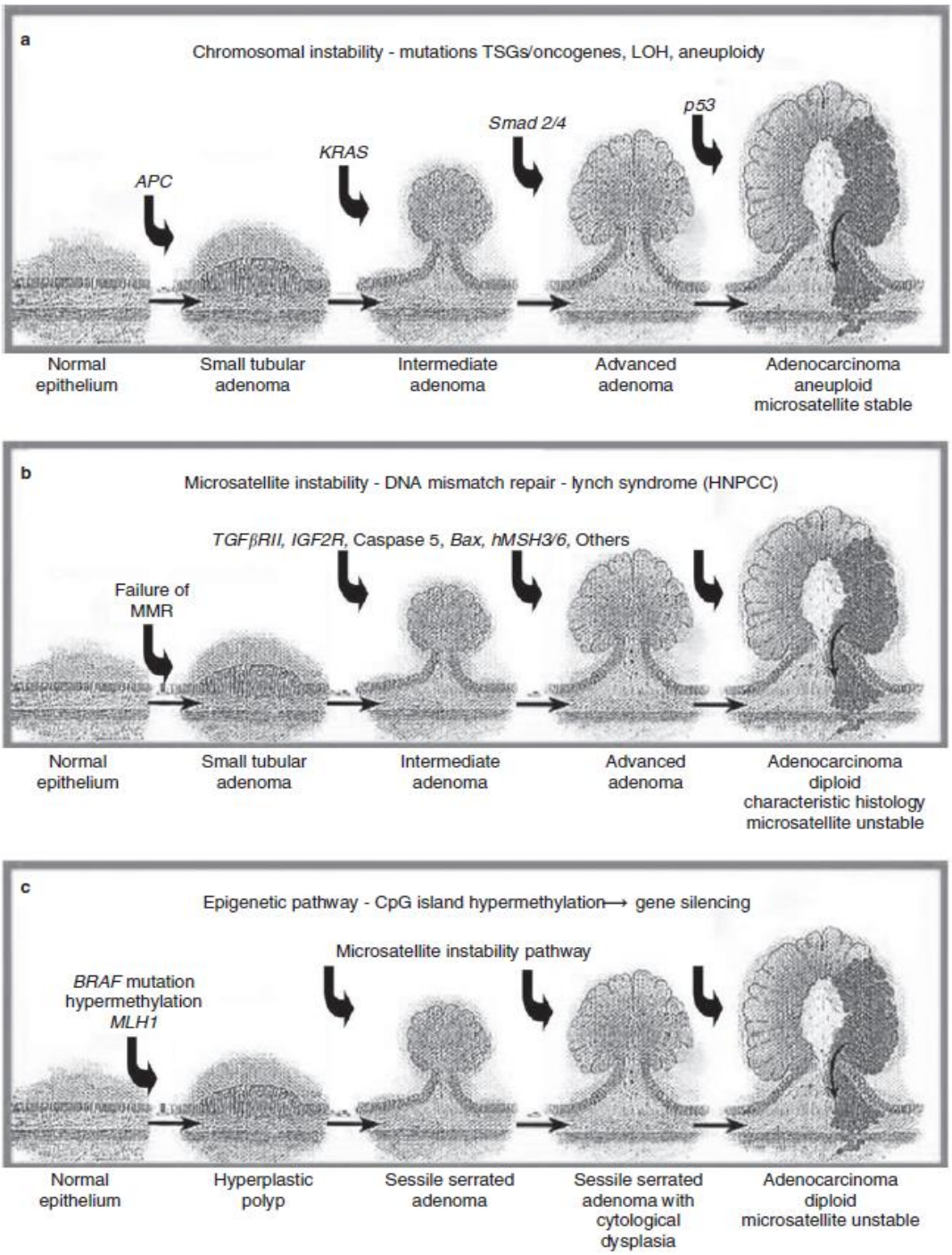

Figure 1. Molecular pathways implicated in colorectal tumorigenesis. The chromosomal instability pathway characterized by sequential loss-of-function and gain-of-function aberrations in tumor suppressors and oncogenes respectively, was first discovered, followed by microsatellite instability pathway that affects DNA mismatch-repair genes. Recently, another pathway that is characterized by epigenetic changes to key mismatch repair genes has been discovered in a smaller set of patients.

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CRCs are diverse in their underlying genetic makeup and are frequently characterized by genetic instability [2], arising due to microsatellite instability (MIN) and chromosomal instability (CIN). These two pathways of CRC pathogenesis are thought to evolve independently. MSI tumors make up about 15% of all CRCs, harboring mutations, mismatched basepairs and/or indels in the repetitive microsatellite sequences present throughout the human genome [3]. These characteristic alterations in the microsatellite sequences have been shown to arise due to defects in DNA mismatch repair genes [4]. The rest of the ~85% of CRCs are characterized by CIN, which is reflected in persistent gain or loss of whole chromosomes or large portions of chromosomes [5].

Although whether CIN is the cause or consequence of tumorigenesis is still debated [6, 7], it is beyond doubt that CIN increases the likelihood of tumorigenesis and drives the progression of the disease. Multiple studies, including a meta-analysis of ~10,000 patients by Walther et al in 2008, have shown CIN to be significantly associated with a worse prognosis and survival in CRC patients [8-10]. Another study found CIN to confer intrinsic multidrug resistance to colorectal tumors [9]. Though the importance of CIN has been well studied in the context of various cancer types, the molecular mechanisms driving the genomic changes that are characteristic of CIN are not yet fully understood.

Chromosome 20q in CRC pathogenesis

One genomic aberration that is frequently seen in CIN tumors is gain of chromosome arm 20q. Chromosome 20q is one of the most frequently amplified genomic regions in colorectal tumors [11, 12] and is reported to occur early during tumorigenesis [13, 14]. In 2011, Tabach et al proposed a model of cancer initiation driven by spontaneous 20q amplification, describing the upregulation of expression of genes localized on 20q directly and indirectly altering the role of transcription factors as well as oncogenic signaling pathways, thus causing a change in various cellular functions including cell cycle activity, metabolic pathways and cell-adhesion. This finding was confirmed by the large scale study undertaken by The Cancer Genome Atlas (TCGA) consortium, which identified chromosome 20q13.12 as one of the genomic hotspots associated with tumor aggressiveness [15].

Specifically, several studies have found amplifications on chromosome 20q to be associated with liver metastasis [16-22], one of the predominant sites of metastasis in colorectal cancer [23] . In 2000, Hidaka et al showed that primary CRC tumors with liver metastasis are enriched for amplification in regions of chromosome 20q, with at least 89% of primary tumors with liver metastases and 94% of metastatic lesions showing gains on chromosome 20q and the mean level of relative copy number correlating with tumor progression [19]. More recently, the application of newer technology to study chromosome 20q amplifications have confirmed these findings. In 2010, Sayague´s, J.M. et al utilized data from 500K SNP arrays to confirm that the genetic profile of metastatic CRC was defined by imbalanced gains of chromosomal regions that frequently included chromosome 20q [11]. In the

same year, Bruin S.C. et al described a novel classifier system named LM-PAM predominantly based on chromosome 20q aberrations, to predict patients who were likely to develop liver metastasis with an accuracy rate of 80% [17]. Evidence from such studies suggests that genes on chromosome 20q likely drive the selection for the amplification of this region of the genome in more aggressive tumors.

Previous work in our lab identified a chromosome 20q gene signature associated with CRC progression [Carter, J. et al, unpublished data], by utilizing two *in vitro* colorectal cancer model systems – the SW (SW480 and SW620) and KM12 (KM12C, KM12SM and KM12L4C) series cell lines. The cell line SW480 was derived from the primary colon tumor of a Duke's Stage B CRC patient whereas SW680 is the lymph-node derivative of the same patient with recurrent metastatic disease [24]. In contrast, the highly metastatic KM12SM and KM12L4C cell lines were established from tumors growing in nude mice that had been implanted with KM12C cells, the poorly metastatic parental line derived from a Duke's Stage B colorectal tumor, into the spleen of nude mice or injecting subcutaneously into their cecum [25]. Integrated copy number and mRNA expression analysis of the five cell lines led to the identification of a 4-gene signature localized on the three Minimal Common Regions (MCRs) of amplification on chromosome 20q, suggesting that these amplified genomic regions containing the gene signature are selected for during the metastatic process.

The gene signature consists of 4 genes, namely BMP7, DNMT3B, UBE2C and YWHAB, all of which have been previously implicated in cancer [26-34]. Moreover, when the gene signature was analyzed in a large cohort of patients

from The Cancer Genome Atlas Colorectal Cancer study, the expression profile of the gene signature was found to show significant association with negative prognostic characteristics like lymph node spread and/or distant metastasis, as well as CIN expression subtype, suggesting that gain of function alterations in the gene signature may contribute to disease progression.

Investigation of candidate gene for further examination

The rationale behind this study was to narrow down on one candidate gene from the signature whose functional impact on the progression of CRC could be studied at a molecular level. We used two filtering criteria to prioritize the genes of the 20q signature for further examination. The first criteria included the results of a recent study by Zhang et al in 2014, characterizing the proteogenomic profile of the colorectal sample cohort from the TCGA dataset. This was the first large scale study to integrate the genotype and phenotype information based on proteomic and genomic profiles in CRCs [35]. In this study, the proteomic analysis of 95 CRC samples, performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and covering a total of 7,211 genes, was compared to the corresponding genomic aberrations as well as RNA-seq information reported in the original CRC samples analyzed by TCGA. The findings show that chromosome 20q gain is associated with the largest global mRNA and protein level changes in CRC. 89% of the 79 genes that had quantifiable protein measurements showed significant Copy Number Alteration (CNA)-mRNA correlation, but only 51%

showed significant CNA-protein correlation ($P<0.01$), suggesting that these genes may be preferentially selected for during the tumorigenic process. In particular, of the four genes in the gene signature, it was interesting to note that UBE2C and YWHAB showed significant CNA-mRNA as well as CNA-protein correlation, indicating that these genes may have a direct functional impact on tumorigenesis. It must be noted that the other two genes in the signature – BMP7 and DNMT3B, did not have quantifiable protein measurements that could be utilized for this analysis. Nevertheless, this finding helped narrow down from four to two candidate genes for further examination in our study.

Secondly, we wanted to examine if the candidate genes revealed a pattern of co-expression with previously well-characterized oncogenes. We performed co-expression analysis *in silico* using the public database Oncomine on ten colorectal tumor datasets where we examined genes that co-expressed with UBE2C and YWHAB. While YWHAB did not show a discernible pattern of significant co-expression with any particular cancer-related signaling pathway across the datasets, UBE2C was highly co-expressed with the Aurora A Kinase (AURKA) pathway members, i.e. AURKA and its binding and activating co-factor TPX2, in ten out of ten datasets, suggesting a pattern of co-expression of UBE2C with known cancer driver genes. A representative result is in Figure 2, data used from Tsukamoto et al, 2011[36].

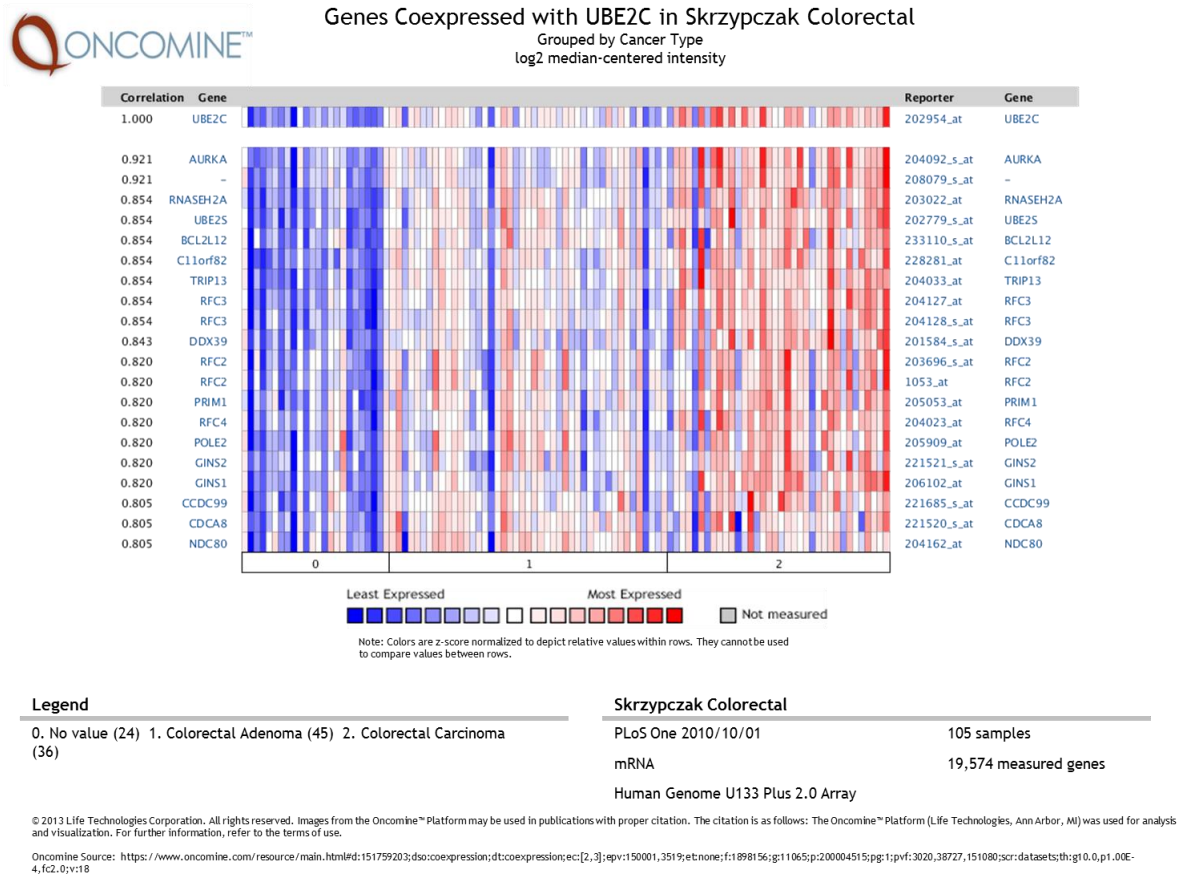
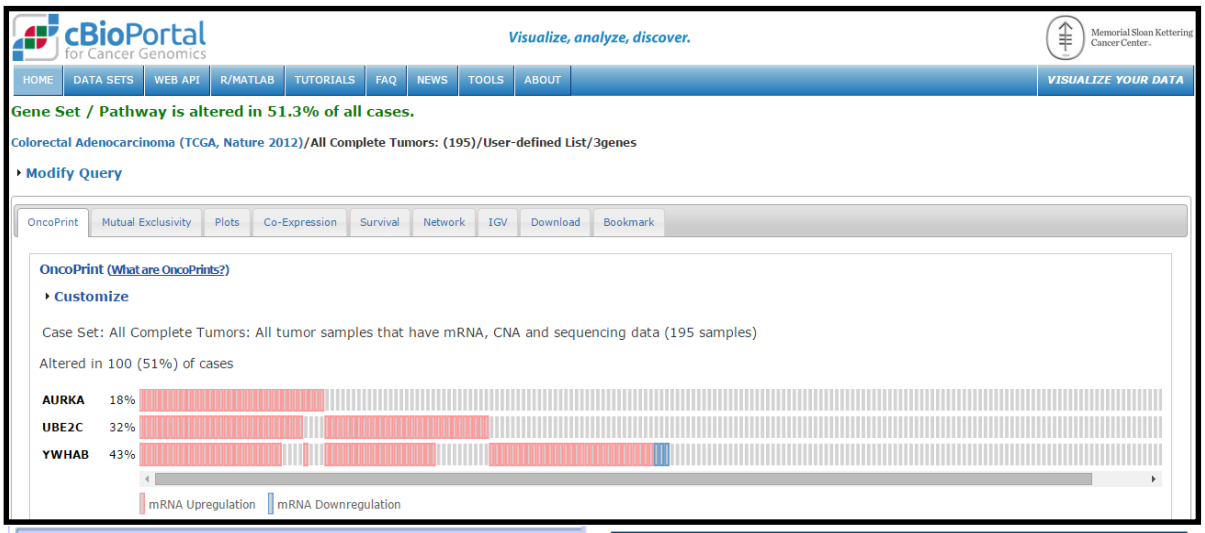
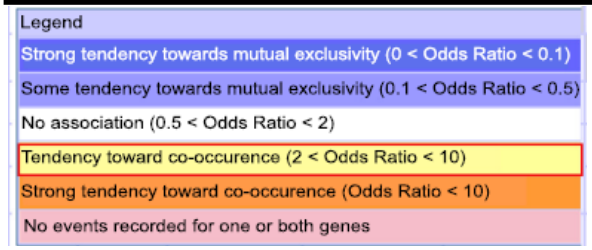


Figure 2. Co-expression analysis of mRNA expression data from Oncomine. Example dataset shown is from Tsukamoto et al, Clin Cancer Research (2011) [36]. Correlation of expression of UBE2C and AURKA at the mRNA level is evident across the normal samples and different tumor types, with the expression increasing in carcinomas, compared to adenomas and normal samples.

Similarly, when we analyzed the mutual co-occurrence of overexpression of UBE2C and YWHAB with AURKA in the TCGA CRC cohort, UBE2C and AURKA revealed a higher mutual co-occurrence rate as compared to YWHAB and AURKA (Figure 3). This further confirmed the co-expression analysis results from Oncomine.





Gene	YWHAB	UBE2C	AURKA
YWHAB	---	<0.000001	<0.000001
UBE2C		---	<0.000001
AURKA			---

Figure 3. Mutual exclusivity analysis from cBioPortal website. Overexpression of UBE2C and AURKA shows a stronger tendency of co-occurrence at the mRNA level as compared to that between AURKA and YWHAB. Analysis was performed using TCGA Colorectal Cancer study samples (as of August 2014). Legend is provided for the color scheme represented.

Since the Oncomine co-expression analysis was performed using mRNA expression data, we wanted to validate these findings at the protein level. A preliminary examination of endogenous protein levels of AURKA and UBE2C by western blotting in ten exponentially growing colorectal cancer cell lines revealed that six of the ten cell lines showed a positive correlation between AURKA and UBE2C protein expression, whereas we did not see any correlation between AURKA and YWHAB, as the expression of YWHAB was unchanged in all cell lines.

Given that AURKA and TPX2 have been previously reported to promote 20q-amplicon-driven colorectal adenoma to carcinoma progression [37], it was interesting to note that UBE2C expression was positively correlated with AURKA pathway members at the mRNA and protein levels, suggesting a possible co-operation between the signaling axes in driving tumorigenesis, thus prompting us to further focus on the possible interaction between the two, to investigate their potential impact on CRC pathogenesis.

Hypothesis and aims of the project

Based on the aforementioned observations, the central hypothesis of this project is that functional interaction between AURKA and UBE2C contributes to colorectal carcinogenesis.

To test this hypothesis, I propose the following specific aims

1) AIM 1: To determine the effect of change in AURKA protein expression and activity on UBE2C protein levels
2) AIM 2: To examine the effect of change in AURKA expression on the transcriptional regulation of UBE2C expression.

Methods

Cell lines and tissue culture

A total of ten cell lines were used in this study. Caco-2, SW480, SW620, HT-29, HCT116, RKO and DLD-1 were procured from American Type Culture Collection (ATCC). The KM12 series of cell lines were a gift from Dr Isaiah Fidler's laboratory at UT MD Anderson Cancer Center. Caco-2, HCT116 and RKO cell lines were cultured in MEM media, HT-29 in McCoy's media, DLD-1 in RPMI-1640 and SW series of cell lines in DMEM/high glucose media, all with 10% FBS (15% FBS for Caco-2), 1% L-glutamine and 1% Penicillin/Streptomycin (Corning, 25-005-Cl and 30-002-Cl respectively); KM12 series of cell lines were cultured in MEM media with 1mM sodium pyruvate (Sigma-Aldrich, S8636), 1ml of NEAA per 100ml of media (Lonza, 13-114E), 1% L-glutamine and 1% Penicillin/Streptomycin. All cells were cultured in a CO2 incubator with 5% CO2 at 37^{o}C.

Transfection of DNA, siRNA and Protein isolation

5ug plasmid DNA and 80nm SiRNA were transfected into cells in serum free media at 70-80% confluence using OPTIMEM reduced serum reagent (Life technologies Inc) and Lipofectamine 2000 (Life Technologies Inc.) transfection reagent, according to manufacturer's recommendation, for 24 to 72 hours. Media was changed to normal growth media 6 hours after transfection. SiRNA targeting AURKA (custom SiRNA sequence AUGCCCUGUCUUACUGUCA) and UBE2C (SMARTpool ON-TARGET plus, catalog no. LU-004693-00-0005) were procured from GE Dharmacon. To

isolate protein, cells were washed twice with 100% PBS and lysed with 0.1L buffer (for 50ml buffer: 0.02M Tris HCl (pH 8.0), 0.1M NaCl, 500uM EDTA, 0.05M NaF, 1% NP40, Protease inhibitor (Roche) – 1 tablet, make up the volume to 50ml with ddH20) for co-IP and RIPA buffer (for 50ml buffer: 0.02M Tris-HCl, 0.15M NaCl, 1uM EDTA, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, protease inhibitor – 1 tablet, make up the volume to 50ml with ddH20). Protein concentration was measured using Bradford reagent (Bio-Rad) according to manufacturer's protocol.

Drug treatments

Alisertib (MLN8237) was dissolved in 100% DMSO and was used at 100nM and 500nM concentration for 1 hour and 24 hours. Corresponding volumes of 0.05% DMSO treatment was used as negative control. Total protein was isolated from lysed cells as described above. For co-IP experiments with cells arrested in mitosis, nocodazole treatment was performed at 2.5ug/ml concentration for 16 hours.

Quantitative Real Time PCR

Total RNA was isolated from transfected cells using TRIzol reagent (Invitrogen) and phenol-chloroform precipitation according to manufacturer's recommendation. Extracted RNA was treated with DNase I (Life technologies) to remove DNA contamination. 2-3 ug DNase-treated RNA was converted to cDNA using Superscript II RT kit (Life Technologies) according to manufacturer's recommended protocol. Primer sequences are: AURKA Fwd -

5’-GCCCTGTCTTACTGTCATTCG-3”, Rev – 5’-ACAGAGAGTGGTCCTCCTGG-3’, UBE2C Fwd – 5’-GGACCATTCTGCTCTCCATCC-3’, Rev – 5’- AGCTGTGGGGTTTTTCCAG -3’. mRNA levels were quantified using SYBR green qPCR master mix (Life technologies, catalog no. 4309155) and fluorescent signal was measured by a ViiA 7 Real-Time PCR System (Life technologies). Target specificity was confirmed by melting curve analysis. Relative gene expression fold change values were calculated using the $2^{-\Delta\Delta CT}$method as outlined in Link 3. Variance in the normalized RT-PCR values was calculated using the F-test and statistical significance was calculated using the Student’s t-test. Graphs were created using Graphpad prism.

Co-immunoprecipitation

1mg of protein was pre-cleared by incubation with protein-A agarose beads while rotating for 1 hour at 4^{o}C. The supernatant was incubated with primary antibody overnight at 4^{o}C. The antibody-protein lysate mixture was then incubated with fresh protein-A agarose beads and the unbound supernatant was discarded. The beads were denatured using SDS and heat denaturation by boiling for 5 minutes. These samples were then used to perform SDS PAGE followed by western blotting as described below.

Cycloheximide assay

Cells were cultured in 10 cm dishes and transfected either with Flag-tagged AURKA and empty vectors for a period of 24 hours, or siAURKA and

scrambled siRNA for 48 hours, using lipofectamine2000 transfection reagent, using manufacturer's protocol. Cycloheximide reagent at a concentration of 50ug/ml was added to the cells and cells were harvested with RIPA at various time points. Immunoblotting was carried out after protein isolation and quantification as described above and below.

Immunoblotting

Cell lysates or co-IP samples as stated above, were denatured using 1X SDS and by boiling at 100^{o}C for 5 minutes. Denatured cell lysates were run on either 10% or 12% polyacrylamide gels, transferred to nitrocellulose membrane, and incubated with primary antibody (List of primary and secondary antibodies in Table 1) on a shaker at 4^{o}C, overnight.

Antibody	Company (Catalog no).
IAK	BD (610939)
BTAK/STK15	[38]
UBE2C (full length)	Abcam (ab56861)
UBE2C (N-terminal)	Abgent (AP2119a)
UBE2C (C-terminal)	Abgent (AP2119b)
Phospho-AURK A/B/C	CST (29146)
TPX2	Bethyl laboratories (A310-101A)
YWHAB	Santa Cruz (SC-629)
BMP-7	Sant Cruz (SC-9305)
ID1	Santa Cruz (SC-488)
Flag	Sigma (F1804)
Cleaved PARP	CST (9541S)
Cleaved Caspase	CST (9661)
HSP90	Santa Cruz (SC-13119)
ACTB	Santa Cruz (47778)
Anti-HRP (Rabbit IgG)	Genedepot (W3902-500)
Anti-HRP (Mouse IgG)	GE Healthcare (NXA931)

Table 1. List of antibodies used in western blotting.

Results

AURKA and UBE2C protein expression are positively correlated in colon cancer cells

In order to validate the results of the Oncomine co-expression analysis as well as our findings from the TCGA mutual-co-occurrence analysis in colorectal cancer, we examined the endogenous protein levels of AURKA and UBE2C in ten cell lines by western blotting. These were seven MSS cell lines including Caco2, SW480, SW620, KM12C, KM12SM, KM12L4A, HT-29 and three MSI cell lines including HCT116, RKO and DLD-1. We observed a positive correlation between AURKA and UBE2C in six of the ten cell lines (Figure 4). Those cell lines that showed higher endogenous AURKA protein levels also showed high UBE2C expression, cell lines with moderate AURKA expression revealed relatively lower UBE2C levels whereas the cell lines that expressed very little AURKA protein showed the lowest UBE2C levels. In addition, we observed a similar phenomenon with the expression of TPX2, the binding and activating partner of AURKA, showing positive correlation with AURKA and UBE2C. We also examined the expression levels of ID1 and BMP-7 which are localized on chromosome 20q, to confirm that the correlation did not stem due to potential amplification of chromosome 20q, and did not see a correlation of their expression with AURKA.

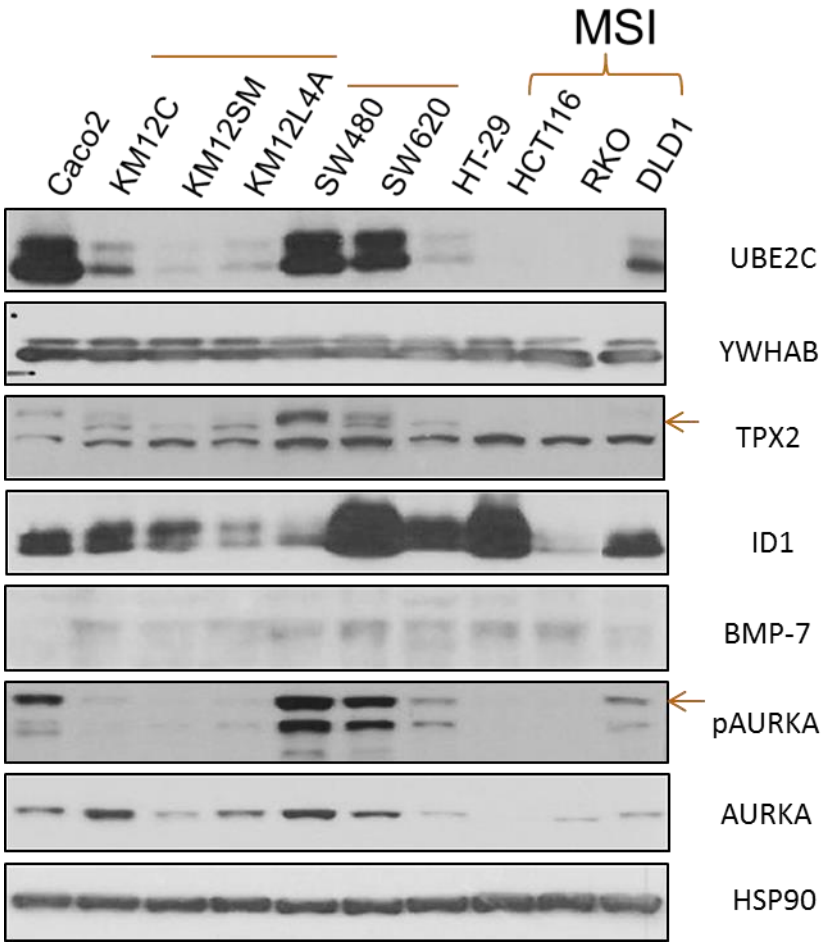


Figure 4. Endogenous expression of UBE2C, AURKA, phospho-AURKA and additional chromosome 20q encoded proteins (YWHAB, TPX2, ID1, BMP-7). Cell lines that show highest AURKA expression also show high expression of UBE2C whereas cell lines with low AURKA expression also express UBE2C at very low levels. Loading control used is HSP90.

AURKA overexpression leads to increase in UBE2C protein levels

We were interested in examining whether change in AURKA expression would effect a change in UBE2C levels too. Three cell lines were chosen for further examination based on their endogenous expression levels of AURKA and UBE2C, their comparable doubling time as well as transfection efficiency, with one cell line, SW480, being consistently used in all experiments. Overexpression of Flag-tagged AURKA by transient transfection led to an increase of UBE2C protein levels as compared to empty vector controls in all three cell lines, indicating a positive correlation between the expression levels of the two proteins (Figure 5). As can be seen from the figure, we confirmed transfection efficiency by checking the intensity of the Flag epitope in the expressed Flag-tagged AURKA protein.

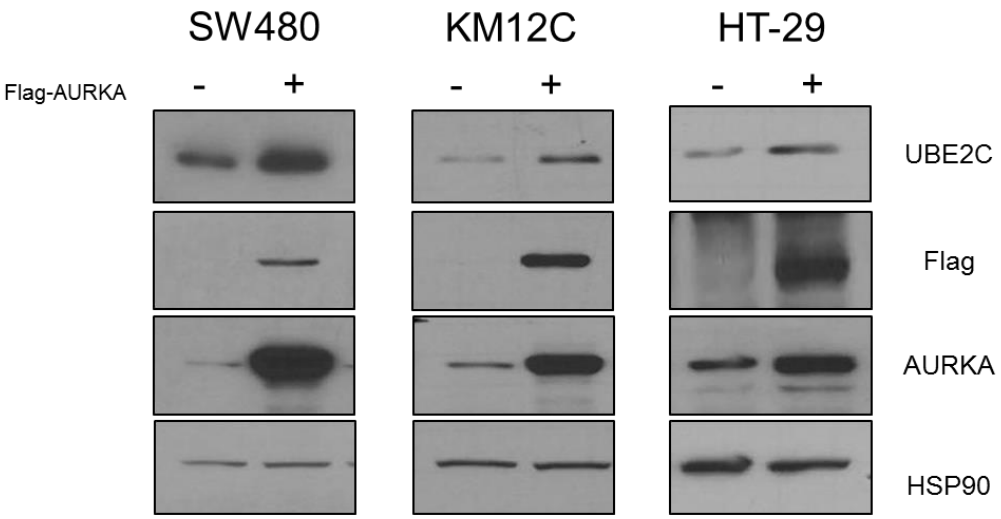


Figure 5. Protein expression of UBE2C when Flag-tagged *AURKA* plasmids are transiently transfected into SW480, KM12C and HT-29 cells, as determined by immunoblotting. Expression of UBE2C increases when AURKA is overexpressed.

AURKA knockdown down-regulates UBE2C expression at the protein level

We wanted to confirm the above findings to see whether UBE2C levels decrease upon knockdown of AURKA expression. We treated three cell lines, SW480, KM12C and HT-29, with siRNA targeting *AURKA* and probed for UBE2C protein using western blotting. siRNA sequences for *AURKA* are provided in the Methods section. We observed a corresponding decrease in UBE2C expression when AURKA expression was down-regulated, confirming the above observation that a positive correlation exists between AURKA and UBE2C in colon cancer cells (Figure 6) and suggesting that AURKA regulates UBE2C expression either transcriptionally or post-translationally.

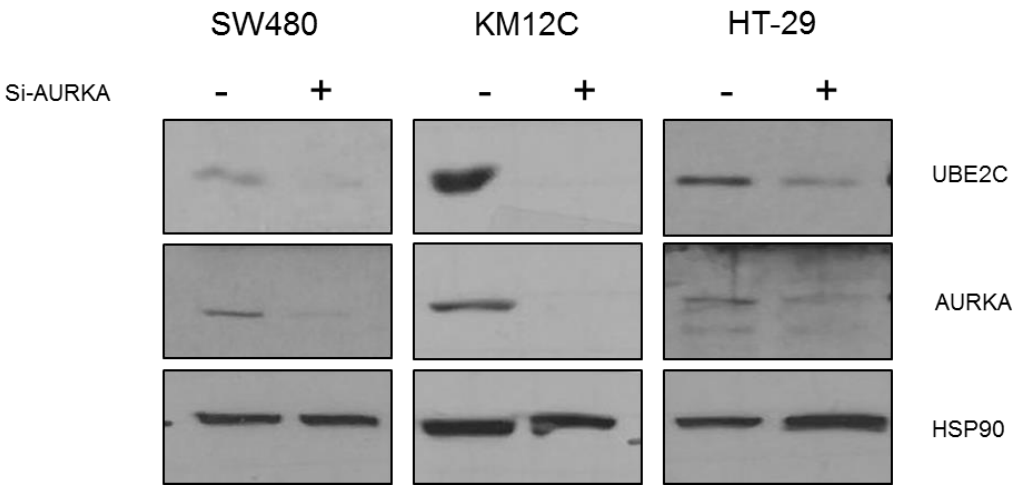


Figure 6. Protein expression of UBE2C when AURKA is knocked down by siRNA treatment in SW480, KM12C and HT-29 cells, as determined by immunoblotting. Expression of UBE2C also decreases when AURKA levels are diminished.

Positive correlation between AURKA and UBE2C expression is reflected at the mRNA level

We were curious to see whether the above phenomenon was limited to the protein level, thus indicating a solely post-translational mechanism of regulation of UBE2C expression, or whether it was also reflected at the transcript levels. We overexpressed Flag-tagged *AURKA* in SW480 and KM12C cell lines and checked for the expression of *AURKA* and *UBE2C* mRNAs using quantitative RT-PCR. Primer sequences for the PCR assay can be found in the Methods section. We observed a statistically significant increase in the expression of *UBE2C* mRNA in the presence of higher expression of *AURKA* (Figure 7).

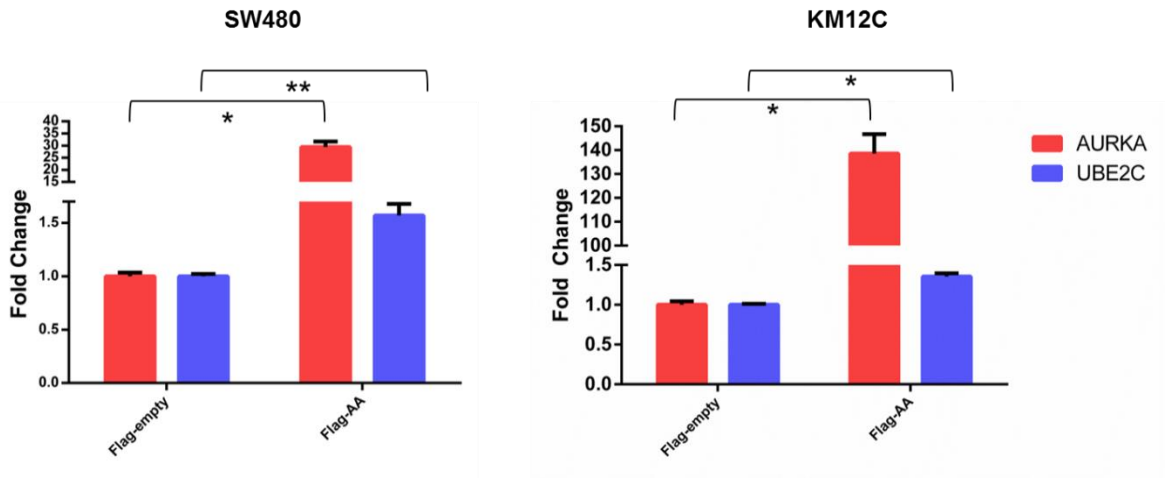


Figure 7. mRNA expression of *UBE2C* when Flag-tagged *AURKA* is overexpressed, as determined by quantitative RT-PCR (* p<0.01, ** p=0.02). Overexpression of *AURKA* leads to a moderate increase in *UBE2C* levels.

We further wanted to confirm these results by investigating the effect of knockdown of *AURKA* on *UBE2C* mRNA levels. RNAi-mediated down-regulation of *AURKA* led to a statistically significant decrease in *UBE2C* transcript levels, as seen in Figure 8.

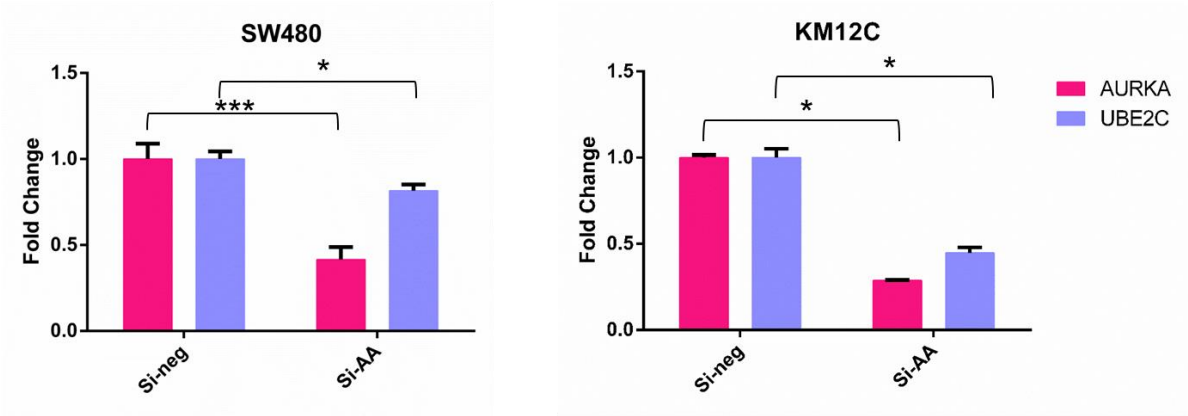


Figure 8. mRNA expression of *UBE2C* when *AURKA* is knocked down by siRNA treatment, as determined by quantitative RT-PCR (* $p<0.01$, *** $p<0.05$). Knock-down of *AURKA* leads to a moderate decrease of *UBE2C*.

The above results suggested the possibility that AURKA may be involved in transcriptional regulation of *UBE2C* expression. Searching for potential DNA-binding or transactivation sites in the AURKA protein sequence using multiple prediction softwares did not yield any predicted sites. We were also curious to see whether known targets of AURKA may be acting as potential transcription factors and helping regulate UBE2C levels. However, potential transcription factor binding sites in the UBE2C promoter region from ENCODE did not reveal any known AURKA downstream targets in the list of most relevant predicted transcription factor binding sites (Link 4). The findings, nonetheless, raise the interesting possibility that transcription factor(s) regulating *UBE2C* expression may be substrate(s) of AURKA, which remain to be identified. Besides, AURKA appears to be regulating UBE2C protein expression at the translational level.

Regulation of UBE2C expression is independent of AURKA kinase activity

Since Aurora A functions primarily as a serine/threonine kinase, we wanted to examine the change in UBE2C expression when kinase activity of AURKA was inhibited using the drug Alisertib (MLN8237). We treated the cell lines SW480 and KM12C with increasing doses of the drug, each for 1 hour and 24 hour durations, inducing the repression of phospho-AURKA expression. As seen in Figure 9, we did not see a change in UBE2C levels between treated and untreated controls at 100nM concentration of the drug, though at the 500nM concentration of Alisertib treatment, we did see a decrease in the protein levels of UBE2C. However, at this concentration we also saw decrease in activated AURKB and AURKC. Since it is known that Alisertib shows >200 fold higher selectivity for AURKA over the other Aurora kinases [39], it leads us to believe that the change in UBE2C may be due to off-target effects of the drug and may not truly reflect the change caused by inhibition of AURKA alone.

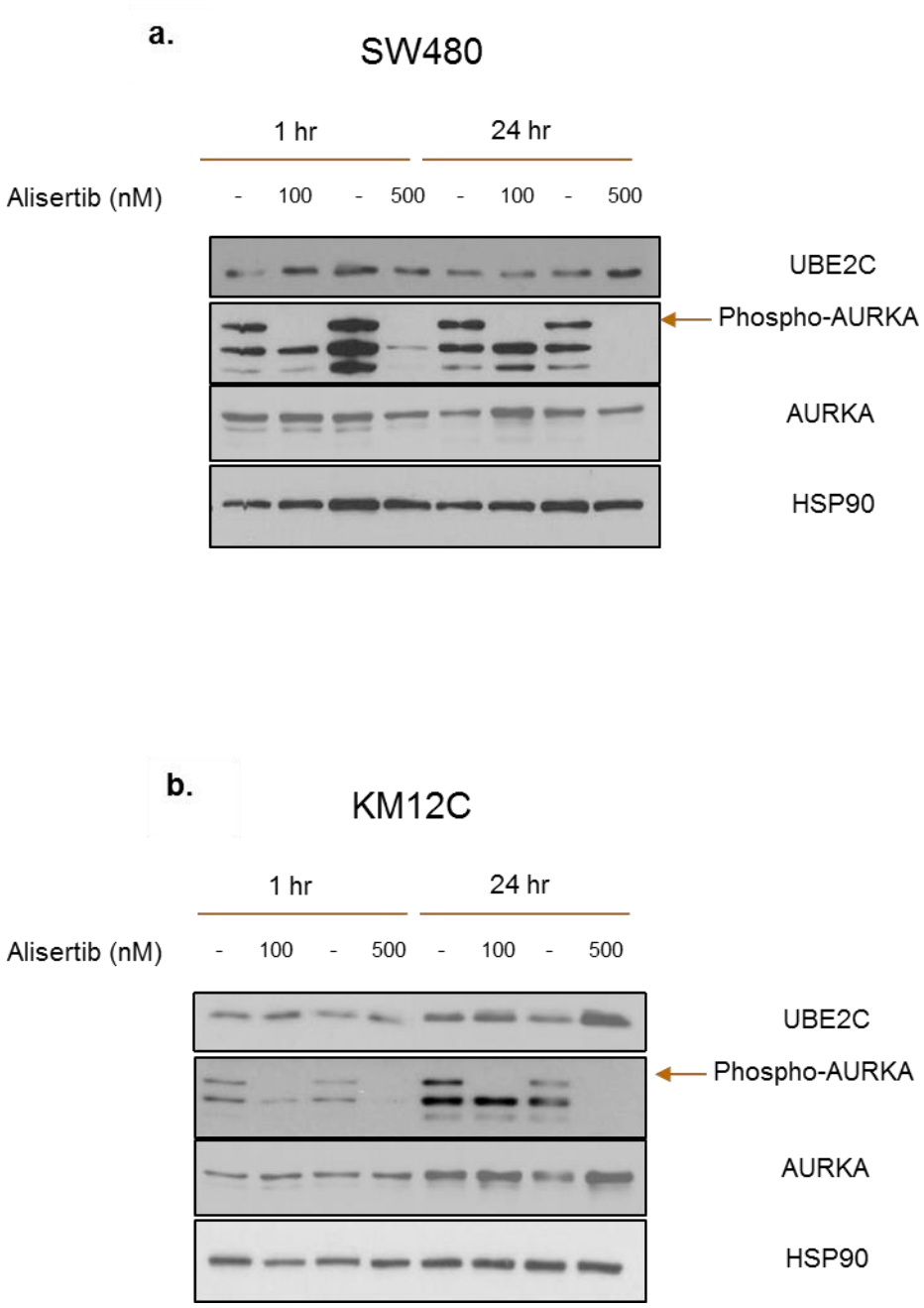


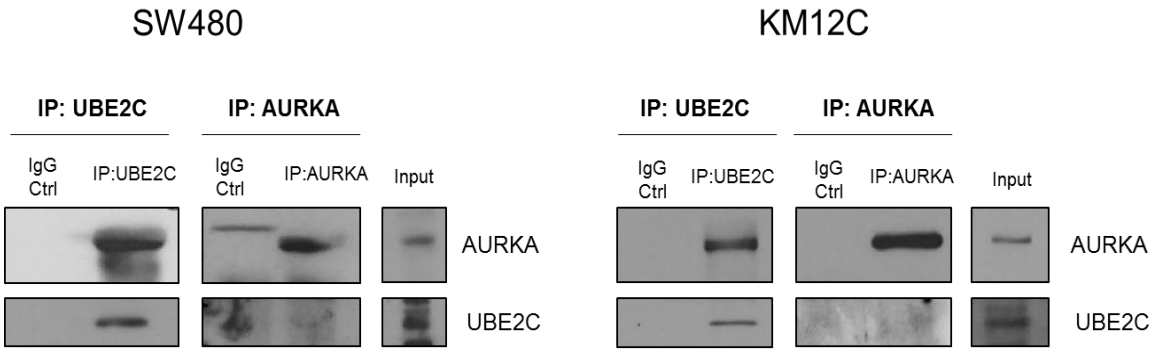
Figure 9. Protein expression of UBE2C after treatment of SW480 (a) and KM12C (b) cell lines with AURKA inhibitor Alisertib, as determined by immunoblotting. Decrease of activated AURKA does not show significant change in UBE2C protein expression.

Co-immunoprecipitation shows evidence of binding between UBE2C and AURKA

Keeping in view the recent study showing kinase-independent regulatory function of AURKA in neuroblastoma cells [40], we sought to examine whether AURKA may be binding to UBE2C in the cell and thus playing a role in increasing its expression. On performing co-immunoprecipitation of AURKA and UBE2C in exponentially growing SW480 and KM12C cells, we observed UBE2C binding to AURKA (Figure 10 a). Interestingly, in both cell lines we were able to visualize the binding only when UBE2C antibody was used for immunoprecipitation and AURKA was probed in the immunecomplex by immunoblotting. However, we failed to detect UBE2C protein in immune-complex precipitated with AURKA antibody. .

AURKA is a mitosis regulatory enzyme that peaks expression during G2-M phase of mitosis. In order to rule out the possibility that low expression of AURKA in exponentially growing cells limits its availability for binding with UBE2C, we treated SW480 cells with nocodazole, which is an anti-neoplastic agent that arrests cells in mitosis by inhibiting microtubule polymerization. This time, we did detect UBE2C binding to immunoprecipitated AURKA, although the interaction was weak (Figure 10 b).

a.



b.

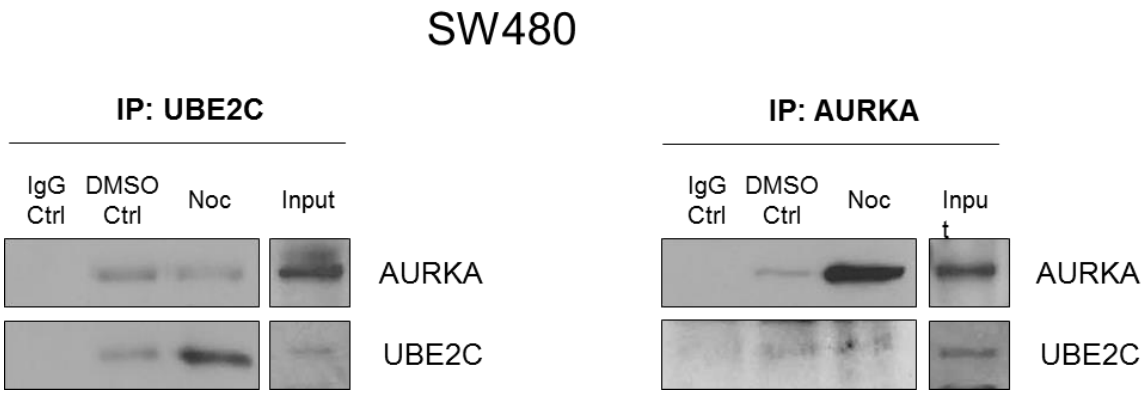


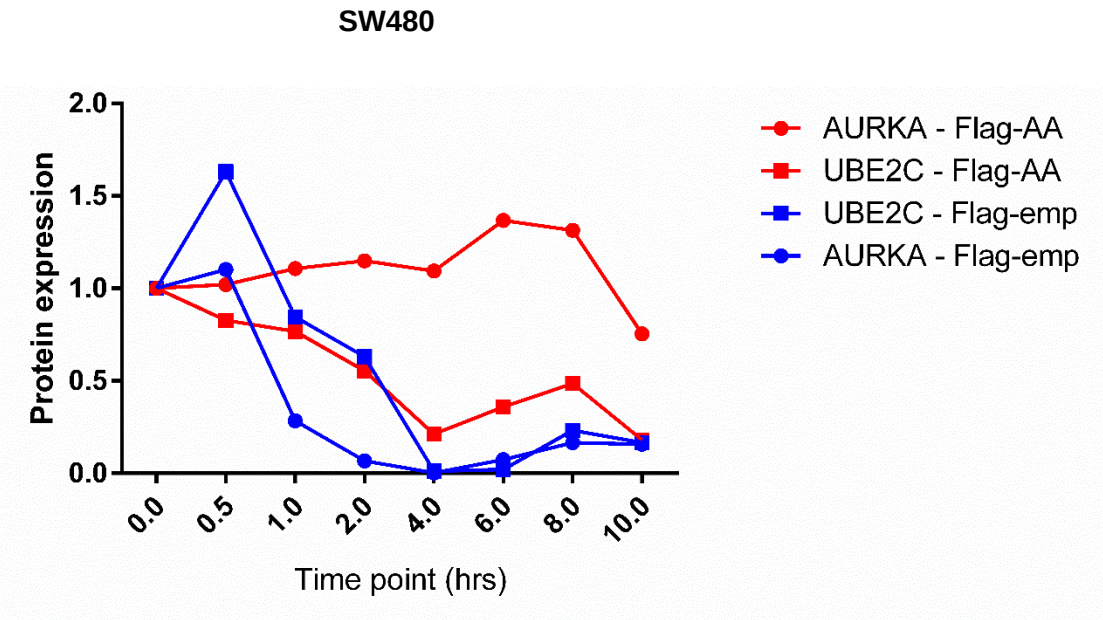
Figure 10. Co-immunoprecipitation of AURKA and UBE2C in a) exponentially growing SW480 and KM12C cells and b) Nocodazole treated SW480 cells. AURKA binding is visible in samples where UBE2C has been immunoprecipitated and probed for the presence of AURKA.

UBE2C protein levels are stabilized by AURKA expression

As shown above, the results of the AURKA-overexpression and knockdown studies indicated a correlation in the expression of AURKA and UBE2C. In addition, we also found AURKA binding to UBE2C, suggesting the possibility that AURKA may be involved in stabilization of UBE2C levels in the cell. We performed cycloheximide pulse-chase assay to determine the effect of change in AURKA expression on half-life of UBE2C. Cycloheximide treatment was performed on SW480 cells that had undergone AURKA overexpression for 24 hours as compared to empty vector controls, following which cells were harvested at various time points to examine the levels of protein expression. Conversely, we also studied cells in which AURKA had been knocked down for 48 hours using RNAi and compared them to cells expressing scrambled Si controls for protein expression levels in the presence of cycloheximide.

In the first case, we found that UBE2C as well as AURKA expression in empty-vector treated cells was diminished at the 4 hour time point , whereas cells that showed overexpression of AURKA also showed higher expression of UBE2C at this time interval, with expression returning to basal levels after decrease in AURKA expression at 10 hours (Figure 11 a). In contrast, in cells where AURKA had been knocked down using siRNA, UBE2C levels diminished at the 6 hour time point, whereas scrambled Si transfected cells showing higher AURKA levels did not show diminished UBE2C expression, although its expression did change in correlation with that of AURKA (Figure 11 b). These results indicate that AURKA protein level is involved in regulating the protein half-life of UBE2C.

a.



b.

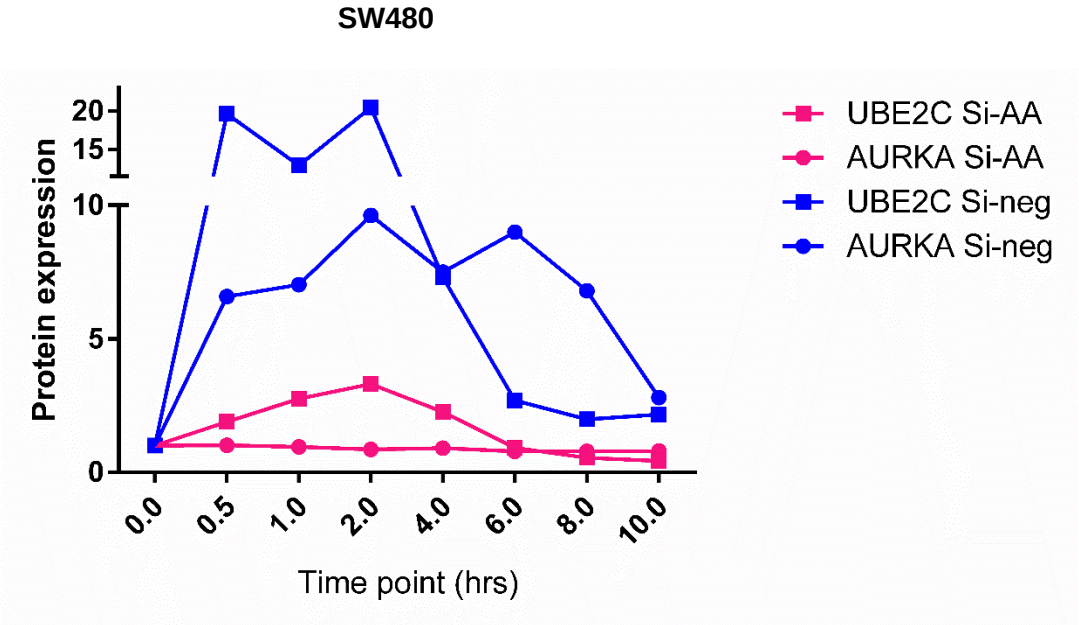


Figure 11. AURKA and UBE2C expression as determined by densitometry-based quantification of immunoblot signals performed after cycloheximide pulse-chase assay in SW480 cells. Higher AURKA expression shows higher levels of UBE2C for longer intervals of time.

Summary and Discussion

The focus of this work was to explore a possible novel interaction between cell cycle enzymes Aurora A Kinase (AURKA) and ubiquitin conjugating enzyme E2C (UBE2C) in colon cancer cells, both encoded on chromosome 20q and displaying similar elevated expression profiles in large colon cancer sample cohorts reported in publicly available datasets, such as, Oncomine and TCGA. . Implication of chromosome 20q amplification in colorectal cancer progression has been well documented, but attempts at narrowing down the genes that drive the selection for this amplification seen in CRCs, especially in tumors that metastasize to the liver have so far been limited. The significance of this study lies in the possibility that disruption of this plausible interaction may help in developing new therapeutic strategies for CRC patients who overexpress these proteins.

The Aurora kinase gene was first discovered in *Drosophila melanogaster* as being responsible for the formation of defective, monopolar spindles when mutated [41]. Since then, three homologous proteins of the kinase family have been discovered in humans – Aurora A, B and C kinases (AURKA, AURKB, AURKC). AURKA has been well studied as a cell cycle enzyme that predominantly localizes at the centrosomes during mitosis and at the spindle poles through to the M phase of the cell cycle, regulating important functions like centrosome maturation, microtubule formation and spindle assembly [42].

The association of *AURKA* with cancer was first reported two years after the gene was discovered. In 1997, Sen et al was the first group to report AURKA (BTAK) as amplified and overexpressed in breast cancer cells [43]. Since then elevated levels of AURKA have been reported in multiple cancers,

including almost 94% of invasive ductal carcinoma of the breast showing enhanced expression of AURKA [43] and it's overexpression was shown to cause tumorigenic transformation both *in vitro* and *in vivo* [38, 44].

Chromosome 20q13.2, the region that AURKA maps to, is a known hotspot for amplification in many human cancers. Owing to its function as a cell cycle regulatory protein, amplification and overexpression of AURKA has important implications for deregulation of normal cellular growth. Elevated levels of AURKA aid in overriding the spindle checkpoint that is activated when cells are treated with chemotherapeutic agents causing defective spindle assembly as well as microtubule destabilization [45, 46]. Moreover, *AURKA* overexpression leads to centrosome amplification resulting in defective chromosome segregation and failure of cytokinesis, thus giving rise to aneuploidy [38] and contributing to carcinogenesis.

Other molecular mechanisms through which AURKA contributes to disease progression have also come to light. In 2004, Katayama et al reported that increased AURKA expression leads to the phosphorylation and subsequent Mdm2-mediated degradation of p53, thus shutting down an important tumor suppressive pathway in cancer cells [47]. In the same year, it was also reported that AURKA-mediated phosphorylation of p53 at Ser215 abrogates p53's DNA-binding and transactivation functions, thus leading to downregulation of the downstream tumor suppressor transcriptional targets of p53 like p21 and *PTEN* [48]. In addition to the degradation of p53, AURKA also circumvents the p53-mediated apoptotic pathway by the suppression of p73 [49, 50].

Given these important findings suggesting involvement of AURKA in carcinogenesis, it is apparent that AURKA is a potential therapeutic target for cancer. Different AURKA inhibitors have been developed, including MLN8054, which was terminated in phase I clinical trials as off target toxicities were reported [51]. More selective second generation drug MLN8237 (Alisertib), has shown improved efficacy in causing tumor regression by induction of senescence on the one hand and activation of apoptotic pathways on the other [52, 53] . It is currently in clinical trials for some solid tumors both as a single agent as well as in combination with other drugs; however in recent clinical trials in gastrointestinal tumors, a small percentage of patients have shown a partial response to the drug [54]. Stratification of patients based on other biomarkers and combination therapy of AURKA inhibitors with other therapies is expected to yield better response among patients.

The ubiquitin conjugating enzyme E2C (*UBE2C*) gene was first cloned by Townsley et al in 1997, as a human homolog of the E2C ubiquitin carrier protein found in yeast [55]. The *UBE2C* gene is located on chromosome 20q13.12 and is reported to have seven transcript variants generated through alternative splicing. UBE2C forms an important component of a large multi-subunit ubiquitin ligase complex called the Anaphase Promoting Complex/Cyclosome (APC/C), which is required to ensure timely degradation of cell cycle molecules like Securin and Cyclin B1 in order for the cell to transition from metaphase to anaphase [56]. Specifically, it is involved in the initiation of mono- and poly-ubiquitination of APC/C targets within the cell [57, 58]. It further promotes the ubiquitination of mitotic checkpoint proteins,

therefore helping in the inactivation of the spindle checkpoint allowing chromosome segregation to occur [59].

Perhaps due to its important functions in cell cycle progression, elevated levels of UBE2C have been observed in various cancers, including colon, liver, bladder, lung, gastric, breast, ovarian, etc. [60-63]. Its high expression has been associated with poor survival in lung cancer, high risk breast cancer patients, ovarian cancer, etc. [64-67]. *In vitro*, *UBE2C* overexpression was shown to impair mitotic arrest in cancer cells in the presence of spindle damaging agents like nocodazole [68], while *in vivo*, overexpression of *UBE2C* led to chromosome missegregation and aneuploidy due to increased APC/C activity [31]. It had also been shown earlier that when primary oral Squamous Cell Carcinomas (SCCs) were immortalized in culture, there was a dramatic increase in UBE2C protein levels [69], suggesting that UBE2C overexpression may play a role in evasion of senescence and increased cell proliferation.

More recently, in 2011, Bavi et al demonstrated that RNAi-mediated knockdown of UBE2C in colon cancer cells led to a decrease in cell growth [70]. They also observed downregulation of UBE2C on treatment with proteasome inhibitor Bortezomib, which further sensitized the cells to Bortezomib- and oxaliplatin-mediated apoptosis, implying that inhibition of UBE2C leads to suppression of cell growth *in vitro*. This was confirmed using xenograft models where Bortezomib-mediated downregulation of UBE2C led to increased shrinkage in tumor volumes in mice treated with Bortezomib and Oxaliplatin combination therapy. These observations suggest UBE2C to be a promising potential therapeutic target in colon cancer.

In view of the above observations, our data point towards an interesting possibility of a yet unknown novel functional interaction between AURKA and UBE2C proteins that are known oncogenes. The mRNA data from TCGA and Oncomine revealing that overexpression of AURKA and UBE2C are closely tied was confirmed by our observation of a similar phenomenon at the protein level in exponentially growing CRC cells. The absence of correlation of expression of other 20q genes indicates that this phenomenon may not solely be due to overall 20q gain but more selective to AURKA and UBE2C. Interestingly, we found that UBE2C expression level is upregulated with increase in AURKA levels and is downregulated when AURKA is knocked down, both at the protein as well as mRNA levels. This reveals the possibility of a novel signaling axis that may be promoting disease progression in a subset of CRC patients who show overexpression of the two genes.

The absence of predicted DNA-binding and transactivation sites on AURKA as well as transcription-factor binding sites on UBE2C, coupled with the limited increase in UBE2C transcript levels even after 25 to 140-fold increase in AURKA levels indicates that perhaps a more potent post-translational mechanism of regulation of UBE2C may be occurring in the cells. In this context, the 2009 study by Otto et al, that found AURKA to stabilize N-MYC in a kinase-independent manner in neuroblastoma cells by binding to it and protecting it from proteasome-mediated degradation becomes relevant [40]. Our observation that UBE2C levels remain constant in spite of AURKA inhibition by the drug Alisertib led us to investigate a potential kinase-independent mechanism of AURKA-mediated UBE2C regulation. Interestingly, in the co-IP studies, we found AURKA binding after

imunoprecipitating UBE2C from the cell extracts, although failed to detect UBE2C in the immune complex when we pulled down with AURKA antibody in both the cell lines. The absence or very low binding of UBE2C detected in the immune complexes pulled down with AURKA antibody even after treatment with a mitotic-arrest agent implies that this phenomenon may not be dependent on the level of expression of AURKA, since AURKA shows highest expression during mitosis. This may indicate the possibility of preferential binding of AURKA with other proteins in the cell or the IP antibody competing with UBE2C to bind to AURKA, which warrants further experiments with antibodies against various different epitopes on AURKA protein.

Further, the evidence of binding between the two proteins prompted us to examine whether AURKA may play a role in stabilizing UBE2C levels in the cell. Cycloheximide assay shows higher levels of UBE2C when AURKA is expressed in high amounts, with both AURKA and UBE2C expression decreasing coordinately after several hours of cycloheximide treatment, indicating protein degradation. In both the samples, protein expression decreased in parallel at the 4 hour time point but rose again, suggesting that the cycloheximide may have been metabolized by the cells by this time point. The overall higher amounts of UBE2C in the scrambled Si-treated cells shows that it would take longer for it to be degraded in the presence of AURKA expression as compared to when AURKA levels are knocked down. Taken together, these studies are suggestive of the positive impact of AURKA signaling on the expression of UBE2C. However, it would have to be further determined whether AURKA regulates UBE2C stabilization directly by binding

to it, by repeating these experiments using deletion and mutation constructs of UBE2C and AURKA.

In addition, the novel relationship between UBE2C and AURKA highlighted in this study has the potential to impact different cellular processes regulated by UBE2C. As mentioned earlier, UBE2C is involved in the initiation of mono- and poly-ubiquitination of APC/Cyclosome targets within the cell. While mono-ubiquitination has been reported to play a role in different processes, including epigenetic regulation of gene expression as well as modification of proteins that regulate DNA repair pathways, poly-ubiquitination is generally employed by the cell to target proteins for proteasomal degradation [71-74]. Overexpression of UBE2C has been reported to cause cancer cells to enter mitosis even in the presence of spindle damaging agents like nocodazole [59]. AURKA-mediated overexpression of UBE2C could potentially have an additive effect on the oncogenic characteristics displayed when AURKA is overexpressed, by facilitating the deregulation of additional cellular processes that may be tumor suppressive in nature. This augmented effect could result in cancer cells exhibiting additional hallmarks of cancer, thus resulting in more aggressive tumors. Further work to delineate the effects of AURKA-mediated UBE2C overexpression on potential change in ubiquitination of its downstream targets will lay the foundation for discerning whether these changes impact the progression of disease.

In summary, we have successfully uncovered a novel interaction occurring between the cell cycle enzymes AURKA and UBE2C in colorectal cancer cells. We have confirmed the correlation of expression of the two proteins as well as potential regulation of UBE2C by AURKA that may have important

implications in promoting the aggressiveness of colorectal cancers, in view the well-characterized oncogenic properties of the proteins. Further investigation on the effects of abrogation of this interaction as well as elucidation of other signaling pathways possibly being affected by this interaction will help determine the importance of therapeutically targeting this novel signaling axis in CRC patients overexpressing the two proteins.

Future directions

The demonstrated interaction between AURKA and UBE2C has not been reported by earlier studies, and therefore has immense potential to be studied in the context of cancer pathogenesis and aggressiveness of the disease..

The 2009 study by Otto et al elucidating the kinase-independent function of AURKA in stabilization of N-Myc by binding to it has expanded the diverse role that AURKA plays in promoting tumorigenesis. Since we have seen the indication of a similar phenomenon in our studies, it would be helpful to determine the binding motifs of the two proteins, using first deletion constructs, then followed by mutation constructs of UBE2C and AURKA to narrow down on the exact residues involved in this interaction. This will further help us determine whether binding of the two proteins is crucial in determining the half-life of UBE2C in the cell or whether increase in UBE2C half-life is merely the result of activation of AURKA downstream signaling pathway.

The functional impact of the interaction of AURKA and UBE2C on CRC cells needs to be elucidated. Important cellular functions deregulated in cancer, like invasive and migratory capacity of cells, could be examined by wound healing assays as well as other migration-invasion assays using stably generated cell lines that overexpress both AURKA as well as UBE2C as compared to cell lines overexpressing UBE2C but low level of AURKA. Determination of the impact of this signaling axis on the ability of anchorage-independent growth and development of additional cancer relevant phenotypes including the role of AURKA in maintaining stemness, as reported by Lee et al in 2012 [75] will be extremely important in designing effective

therapeutic strategies for AURKA and UBE2C over expressing subset of human cancers.

Crosstalk between the AURKA pathway and important oncogenic signaling pathways including Wnt/β-catenin has been reported in multiple myeloma, MYC in neuroblastoma and BMI1 in head and neck cancer [40, 76, 77]. It is likely that interaction between AURKA and UBE2C facilitates and may even prove to be vital for the activation of other such oncogenic pathways. An overview of the changes in expression of well-characterized oncogenes and oncoproteins could be assessed by microarray analysis and Reverse Phase Protein Array analysis of the cells overexpressing AURKA and UBE2C as compared to cells with low AURKA expression. Integrating this data may provide important leads on potential signaling pathways being affected by the interaction between AURKA and UBE2C.

Although we have observed mutual co-occurrence of overexpression of AURKA and UBE2C in publicly available datasets at the mRNA level as well as in cell lines at the protein level, it is important to investigate the phenomenon in tumor tissues as well. IHC staining of the two proteins in CRC tissues would not only help validate our observations in the cell lines but also shed light on the clinical significance of this signaling axis in disease prognosis and therapeutic response of colorectal cancer patients.

Unlike AURKA which has been the focus of many clinical trials in hematologic as well as solid tumors, there is as yet no clinical trial targeting UBE2C, even though its potential as a therapeutic target has been previously discussed by Bavi et al [70]. This can be first attempted in a pre-clinical

setting *in vitro*, by treating cells with a combination of AURKA inhibitor like Alisertib and proteasome inhibitor like Bortezomib, shown to downregulate UBE2C, and observing the effects on cell proliferation, migration-invasion, cell death and chemoresistance, in comparison with these drugs as single agents. If the results are promising, subsequent pre-clinical *in vivo* studies with mouse xenograft models could be undertaken with patient tumors that overexpress both AURKA and UBE2C to investigate the effect of combination therapy on tumor response. Ultimately, stratification of patients based on co-expression of AURKA and UBE2C could be developed as an important biomarker-based selection process for administering this combination therapy to patients in clinical settings. .

Online Links

Link 1:

http://www.cancer.org/acs/groups/content/@editorial/documents/document/acspc-044552.pdf

Link 2:

http://www.cancer.org/acs/groups/content/@research/documents/webcontent/acspc-042151.pdf

Link 3:

http://dharmacon.gelifesciences.com/uploadedfiles/resources/delta-cq-solaris-technote.pdf

Link 4:

http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19&position=chr20%3A44440170-44446682&hgsid=429541681_dnRwkuwLu7Ajo61eCv1V3e2oynre

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