

# **Genomic Tagging of the Anaphase-Promoting Complex Activator Protein Cdc20 in *S. cerevisiae***

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**Katarzyna A. Koscielska**

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Approved:

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**Professor Destin Heilman, Project Advisor**

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**Professor Kristin K. Wobbe, Co-Advisor**

1. APC/C
2. Cdc20
3. *S. cerevisiae*

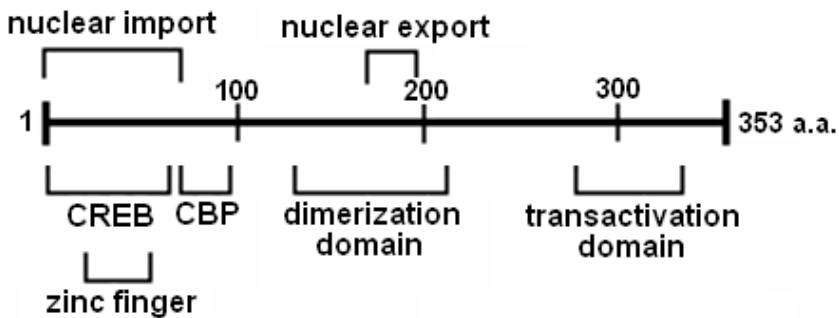
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## Introduction

### *HTLV-1 Tax*

Human T-lymphotropic Virus Type 1 (HTLV-1) is a human oncogenic retrovirus that is the disease agent of adult T-cell leukemia/lymphoma. It was the first human retrovirus discovered (15), and remains of significant scientific interest. The mechanism of HTLV-1-induced cellular transformation is not yet completely understood, but it has been confirmed that of the 14 proteins that are encoded in the genome, Tax is the one that seems to be responsible for the pleiotropic effects that an HTLV-1 infection can have on susceptible cells. Tax is a 351-amino acid, 40-kDa phosphoprotein (3; 18) that functions as a transcriptional activator of the viral genes by recruiting transcriptional coactivators to the long terminal repeats flanking the HTLV-1 genome (8). Structurally, Tax contains a nuclear import and nuclear export signal, numerous leucine zipper-like sequences and other DNA-binding sites (3), along with a suspected dimerization domain and a domain responsible for gene transactivation.



(adapted from Aefantis et al., 2007)

In the cell, Tax is mainly localized in nuclear and perinuclear speckles, and it also colocalizes with the centrosomes (2). Expression of Tax alone is sufficient to induce detrimental

changes in normal T-cells, especially centrosome overamplification (2), aneuploidy, chromosomal instability and micronucleation of cells (11). It is possible that these changes may lead to cellular transformation; however, the exact mechanism of development of the adult T-cell leukemia remains to be elucidated.

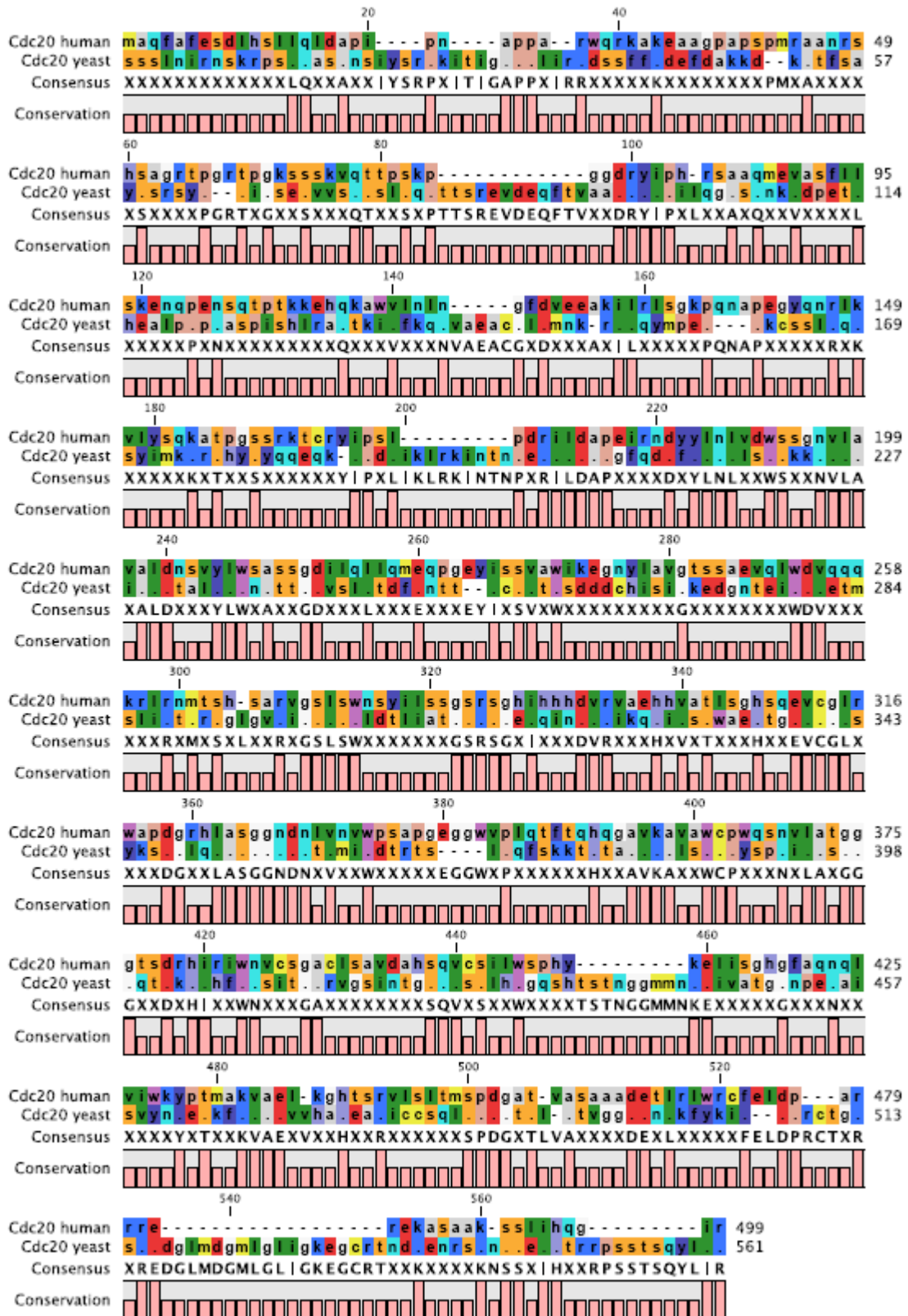
Interestingly, when Tax is introduced into human transformed cells (e.g. HeLa), it shows propensity for drastically deregulating the cell cycle. Findings of Liu et al. (10) indicate that Tax directly interacts with the APC/C<sup>Cdc20</sup>, causing a number of cellular events that in the long run commit the cells to senescence and cause growth arrest. Tax binds and activates APC/C ahead of its regular schedule in the cell cycle, causing premature degradation of cyclin A, cyclin B1, securin and Skp2 during S phase (8; 11). Reduction in the levels of these regulators causes the cells to go through an erroneous cell division cycle and become permanently growth-arrested in G<sub>1</sub> phase. Cells in this condition are virtually undistinguishable from cells in senescence (8). The fact that this effect of Tax can only be observed in transformed cells (and, incidentally, in *S. cerevisiae* cells) makes it an important topic in cancer research.

Although Tax-induced senescence is phenotypically identical to natural cellular senescence, it operates via a different mechanism. Tumor suppressors pRb and p53 are known to be involved in natural cellular senescence (6). However, Tax-induced senescence can occur in HeLa cells, which lack these functioning tumor suppressors (8). It is likely that some other proteins need to be mutated in order to allow Tax infection without inducing senescence.

Cells lacking functioning p27<sup>Kip1</sup> protein can maintain Tax without experiencing senescence (8). A proposed mechanism for Tax-induced senescence is as follows: after initiating expression Tax prematurely activates the APC/C (possibly by binding the Cdc20 subunit); in consequence, Skp2 becomes polyubiquitylated and is degraded from S phase onwards, and

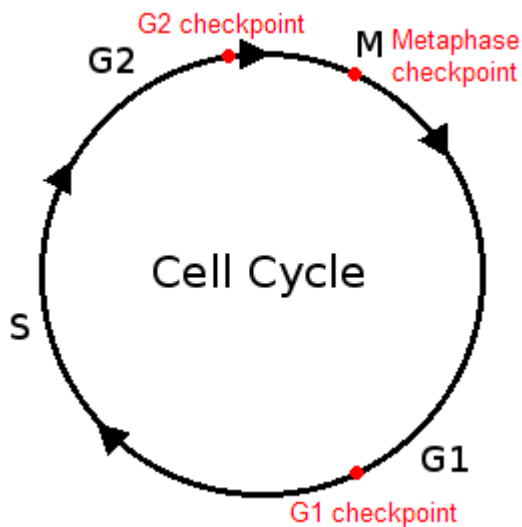
SCF<sup>Skp2</sup> becomes inactivated due to lack of available Skp2. In the end, p21C1p1/WAF1 and p27<sup>Kip1</sup> are stabilized, and this process commits a cell to senescence (11). Finding out whether Tax and Cdc20 physically interact could help verify this theory.

Point mutations of Tax have been assayed for comparison against wildtype activity (11). The phenotypes suggested that the growth arrest binding domain of Tax is different and separate from the transactivation and cell-signaling domains. The location of the APC/C binding domain of Tax is yet to be determined. Tax is thought to bind the APC/C<sup>Cdc20</sup> directly, because these two proteins were shown to co-immunoprecipitate (10). Considering this and the surprising information that Tax induces senescence in both yeast and human cells, it would be logical for the Cdc20 subunit to be a highly conserved protein. In support of this theory, a BLASTP v.2.2.18 sequence alignment of *Saccharomyces cerevisiae* (accession no. BAA03957.1) and *Homo sapiens* Cdc20 protein (accession no. AAH00624.1) revealed 35% identity and 54% similarity (see figure below, generated using CLC Free Workbench). *S. cerevisiae*, as the simplest eukaryotes, are a good model for studying the cell cycle, and with the addition of the fact that Tax induces growth arrest phenotype in yeast cells, they are an important subject of Cdc20 studies.



### ***Anaphase-Promoting Complex/Cyclosome (APC/C)***

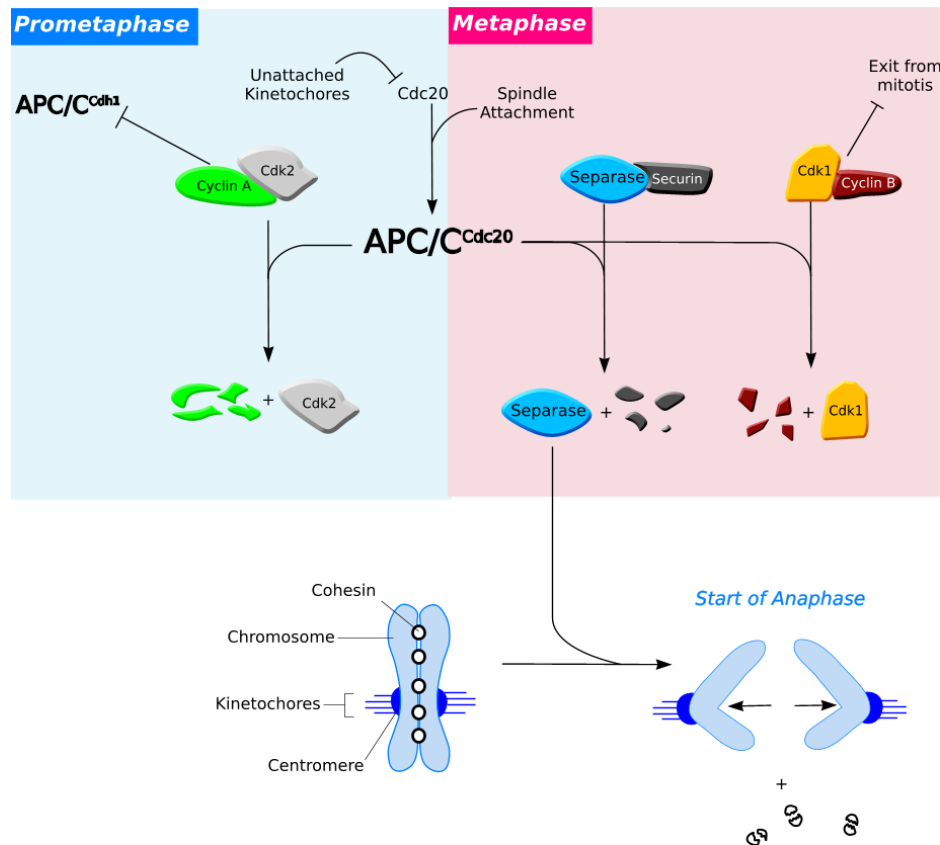
The anaphase-promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase that serves the function of the master regulator of the metazoan cell cycle. It is a multi-subunit protein whose main enzymatic activity is ubiquitylation of other proteins. Ubiquitylation is a process of covalent attachment of small, 76-amino acid, 8.5 kDa protein moieties called ubiquitin onto specific lysine residues of the target protein (17). Addition of only a couple of ubiquitin molecules can have an important role in signaling and post-translational modifications of some proteins; however, addition of four or more units is termed polyubiquitylation and targets the protein for degradation by the 26S proteasome (16). This is exactly the pathway via which APC/C fulfills its most prominent role in the cell: regulation of progression through the cell cycle.



The cell cycle phases are shown on the diagram above. The most heavily regulated points in the cycle are termed the checkpoints, and their function is to halt the cell cycle before progression into the next phase until all the processes occurring in the previous phase have been completed. In case of the APC/C, the checkpoint of most interest is the one that regulates

progression from prometaphase into anaphase (see diagram below). During mitosis, the APC/C exists as two different complexes: APC/C<sup>Cdc20</sup> (when bound to the activator cell division cycle 20 homolog, or Cdc20), and APC/C<sup>Cdh1</sup> (when associated with the activator Cdh1). The APC/C<sup>Cdh1</sup> is responsible for regulating exit from mitosis, and is also involved in G<sub>1</sub> and possibly G<sub>2</sub> phase. Cdc20 is a 55 kDa protein that plays a major role in the regulation of the cell cycle through its association with and activation of APC/C during prometaphase and metaphase. Upon attachment of the kinetochores to their respective spindle poles, Cdc20 binds the APC/C in order to initiate anaphase (12). Cdc20 is usually kept away during prophase and metaphase by the mitotic checkpoint complex (MCC) in order to prevent its premature release. In prometaphase, cyclin-dependent kinase 1 (Cdk1) is activated by cyclin B and phosphorylates proteins needed for mitosis (13). Afterwards, APC/C<sup>Cdc20</sup> starts targeting cyclin B for degradation throughout anaphase and telophase. After the degradation is complete, Cdk1 becomes deactivated (5; 7; 14). This process is necessary to exit mitosis (13).





(adapted from Heilman, 2006 by Oliver J. Salmon)

The APC/C<sup>Cdc20</sup> also initiates the main event of anaphase: the rapid, total and irreversible separation of sister chromatids. It polyubiquitylates securin, a protein inhibitor of the cysteine protease separase (14). Upon securin degradation, separase is activated and degrades a protein complex called cohesin. The breakdown of cohesin initiates the separation of sister chromatids. However, it has been determined that the degradation of cohesin alone is not sufficient to ensure proper chromosome separation.

### ***Current Study***

The suspected interaction of APC/C activator Cdc20 with the HTLV-1 protein Tax make it an important target of scientific investigation. The goal of this study is to create a genomically tagged version of the *cdc20* gene, so that the protein is expressed with an easily detectable

epitope tag covalently attached to it. Tagged Cdc20 will then be used in protein-protein interaction studies, like immunoprecipitation, to determine whether it indeed directly interacts with Tax.

## Materials and Methods

### PCR

Genomic recombination constructs were generated via PCR using Phusion High-Fidelity DNA Polymerase (Cat. No. F-530, 20U at 2U/ $\mu$ l) and Phusion HF Buffer at 98°C for 4 min, then 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1.5 min. Plasmid pFA6a-GFP(S65T)-TRP1 (9) was used as a template. The forward primer (see sequences below) was designed to anneal to the beginning of the T<sub>ADHI</sub> region (regular font) and contained the last 40 nt. from *cdc20* 3' end excluding the stop codon (italics), and 27-nucleotide HA epitope sequence (bold font) ending with a TGA stop codon (underlined). The reverse primer was designed to anneal to the 3' end of the TRP1 gene on the plasmid template and contained 40 nt. of reverse complementary sequence to the beginning of the 5' UTR of the *cdc20* gene (italics). The PCR products were resolved on a 0.9% agarose gel, bands were excised and purified with a gel purification kit (Promega Wizard Cat No. A7170) according to the manufacturer's protocol.

5' - TACAAGGAGGCCCTCTAGTACCAGCCAATATTTGATCAGG**TATCCATATGATGTCCCAGATTATGCT**  
TGAGGCGCGCCACTTCTAAA-3' (forward)

5' - ATTATATGCCTTGACATGAACTTTTATTTTTTTTATTTTAGAATTTCGAGCTCGTTTAAAC-3' (reverse)

### *S. cerevisiae* Transformation and Growth

12  $\mu$ l of the PCR product was transformed into Y187 strain of *S. cerevisiae* using a lithium acetate protocol (resuspension in 100mM LiOAc; transformation in 34% w/v PEG, 1M LiOAc, 0.14mg/ml single-stranded carrier DNA). The starter culture (50ml) was incubated with shaking at 30°C overnight and the large-scale culture (300ml) was incubated with shaking at 30°C until the absorbance at 600nm reached 0.6. 150 $\mu$ l of the transformation product (pellet resuspended in 300 $\mu$ l of sterile H<sub>2</sub>O) was plated and selected on tryptophan deficient (Trp<sup>-</sup>) YPD

plates (1% w/v yeast extract, 2% peptone, 2% glucose/dextrose, 2% agar). Several transformants were selected and replated to re-confirm selection.

### ***Yeast Colony PCR***

To confirm the correct recombination locus, PCR was performed using a forward primer (see sequences below) that annealed approximately 500 nt from 3' end of *cdc20* gene, and reverse primers that annealed either within the T<sub>ADH</sub> region, or within the *trp1* gene, using Taq polymerase with Novagen 10X NovaTaq Buffer with MgCl<sub>2</sub> (Cat. No. 71037) at 95°C for 2 min, then 40 cycles of 95°C for 30 sec, 50°C for 30 sec, and 60°C for 4 min. The PCR products were resolved on a 0.9% agarose gel.

5' -ACAGGTGCACGAGTTGGCTC-3' (forward)

5' -TTTAGAAGTGGCGCGCC-3' (T<sub>ADH</sub> reverse)

5' -TACATCAACACCAATAACGCC-3' (*trp1* reverse)

### ***Western Blot***

Cellular extracts were prepared by cracking the yeast cells by vortexing at top speed for 10 minutes with glass beads, and resuspending the cells in SDS-containing 6X protein loading buffer (300mM Tris-HCl, pH 6.8; 0.01% w/v bromophenol blue; 15% glycerol; 6% w/v SDS; 0.01% β-mercaptoethanol). Protein samples were resolved on a 12% polyacrylamide gel, and transferred via a wet transfer for 1 hour at 200mA onto a nitrocellulose membrane using a Bio-Rad apparatus. TBS buffer composition: 25mM Tris-HCl; 137mM NaCl; 2.7μM KCl; pH adjusted to 7.2 with 6M HCl. The membrane was blocked using 5% milk solution in TBS-T (TBS, 0.5% Tween-20). First blot was performed with mouse anti-HA primary antibody diluted in TBS-T (for one hour on an agitator) and then washed 5 times for at least 5 minutes in TBS-T. Afterwards a second blot was performed using an α-mouse secondary antibody diluted in TBS-T

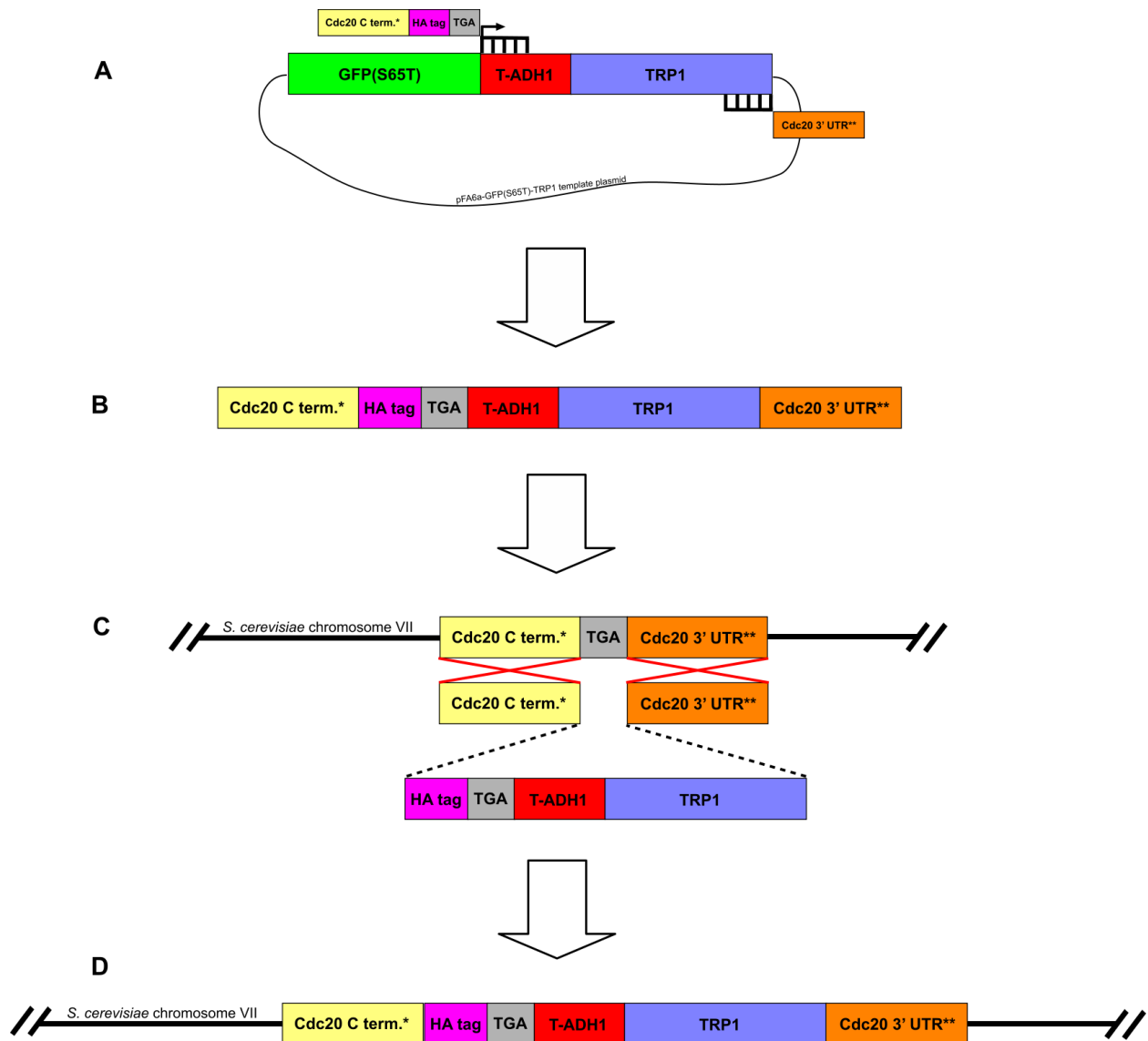
(for one hour on an agitator), and then washing 5 times for at least 5 minutes in TBS-T and 2 times for at least 5 minutes in TBS. The bands were developed for 1 minute in ECL reagent from Pierce (Cat. No. 34077) under a covering and then exposed to film in the dark room.

## Results

In order to establish a yeast strain with genomically tagged Cdc20 protein, genomic recombination constructs were generated using PCR, consisting of the *cdc20* gene, a 27-nucleotide sequence coding for a 9-amino acid hemagglutinin (HA) epitope, and a 40-nucleotide homologous recombination arms at each end. PCR was performed from a pFA6a-GFP(S65T)-TRP1 plasmid template to obtain a linear product engineered to contain both arms of homologous recombination in the *cdc20* locus on *S. cerevisiae* chromosome VII (as shown in Figure 1A). Primers were designed to anneal at the 5' end of the T<sub>ADH1</sub> region (forward) and at the 3' end of the *trp1* gene (reverse).

The finished product consisted of a 40-nucleotide sequence from the 3' end of the *cdc20* gene without the stop codon (the left arm of homologous recombination), a 27-nucleotide HA tag sequence, a stop codon, an ADH1 terminator sequence, *trp1* gene sequence, and first 40 nucleotides of the 3' untranslated region of the *cdc20* gene (the right arm of homologous recombination) (see Figure 1B). T<sub>ADH1</sub> is responsible for correct termination of the *cdc20* construct and for expression of the *trp1* gene, which in turn is necessary for subsequent selection of successful recombinants.

In order to obtain a genomically recombined yeast strain, the PCR product obtained above was transformed into *S. cerevisiae* strain Y187 using lithium acetate protocol. The yeast genome was expected to homologously recombine with the PCR construct at the sites flanking the original stop codon (TGA) at the *cdc20* C terminus (as shown in Figure 1C). After recombination, the yeast genome was expected to contain the PCR construct incorporated seamlessly into the sequence of chromosome VII at the *cdc20* locus (Figure 1D).



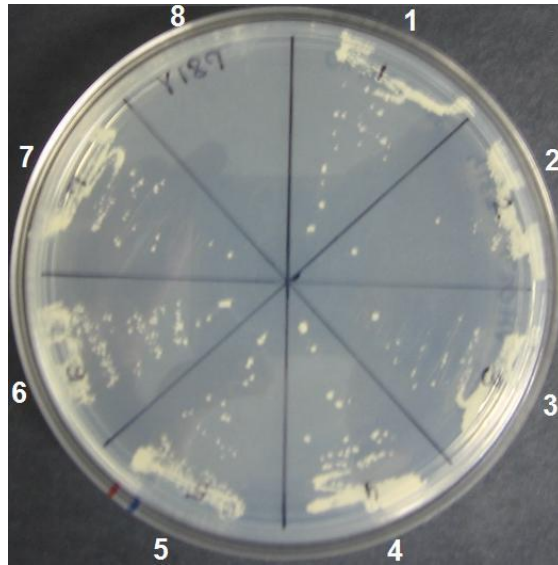
**Figure 1. *S. cerevisiae* genomic recombination.** This figure depicts the steps leading to obtaining genomically tagged *S. cerevisiae*. (A) PCR was performed from a plasmid template to obtain a linear product engineered to contain both arms of homologous recombination in the *cdc20* locus on *S. cerevisiae* chromosome VII. (B) The finished product. (C) The PCR product was transformed into *S. cerevisiae* strain Y187. (D) Yeast genome after recombination. \*40-nt. stretch of homologous sequence. \*\*40-nt. stretch of reverse complement sequence.

In order to verify that genomic recombination took place, yeast colonies were screened for *trp1* gene presence by using Trp<sup>-</sup> selection; transformants were plated on tryptophan-deficient dropout media and incubated for 36 hours. Multiple recombinants were able to grow after plating on Trp<sup>-</sup> media, which suggests that the recombination was successful. Seven of the largest colonies from the screening plates were re-streaked on a common plate and incubated for 36 hours for growth rate comparison and to confirm the selection (see Figure 2, panels 1-7). Panel 8 contains the parental strain Y187, which was not transformed with the PCR product and did not survive the selection due to the lack of *trp1* gene.

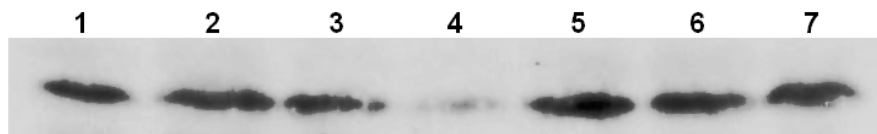
In order to determine presence of HA epitope tag in the positive yeast recombinants, a western blot was performed using a mouse hemagglutinin antibody and an  $\alpha$ -mouse antibody. Strong specific signal was obtained in all lanes, containing recombinants 1-7 (corresponding to panels 1-7 from Figure 2). The probes detected a protein of a size appropriate for Cdc20 bound to the HA tag.

Yeast colony PCR was also performed in order to confirm the correct localization of the tag at the 3' end of the *cdc20* gene, using primers designed to anneal within the *cdc20* locus (forward), and either within T<sub>ADH1</sub> or *trp1* region (reverse) in order to amplify a continuous stretch of sequence incorporating *cdc20* c terminus with the HA tag and T<sub>ADH1</sub> and *trp1* gene (data not shown). The PCR conditions need to be readjusted and the experiment repeated.





**Figure 2. *S. cerevisiae* transformation and TRP1 selection.** Transformed Y187 *S. cerevisiae* were plated on Trp- dropout media to select for stable genomic recombination. Positive transformants were selected (as seen in panels 1-7). Panel 8 contains the untransformed parental strain Y187, which did not survive the selection.



**Figure 3. Western blot detecting HA epitope tag.** A western blot was performed to determine the presence of HA epitope tag in the positive yeast recombinants. Strong specific signal was obtained in all lanes, representing recombinants 1-7 (as in Figure 2). The probe detected a protein of size appropriate for Cdc20 bound to the HA tag.

## Discussion

A yeast strain with genomically tagged Cdc20 protein was established in order to facilitate interaction studies between Cdc20 and HTLV-1 protein Tax. It can be inferred that tagging Cdc20 on the C terminus with a small epitope tag does not affect its binding to the APC/C and normal cell cycle regulation is retained, because viable phenotype was obtained in the recombinants, as can be determined from growth visible in Figure 2 panels 1-7. This is an important finding, because some sources (e.g. the *Yeast GFP Fusion Localization Database* maintained by UCSF) have indicated that placing a large tag on the C terminus of Cdc20 does not yield a viable phenotype. This effect is most likely caused by the tag disrupting or masking the region responsible of binding of Cdc20 to the APC/C, which in turn makes cell cycle progression impossible and leads to cell arrest and death. A small tag like hemagglutinin, on the other hand, does not seem to be disrupting normal Cdc20 function.

Trp<sup>-</sup> selection was successfully obtained, as seen in Figure 2; therefore the metabolic marker is present in the cells and integrated into the genome. Stable integration is confirmed by the fact that the resistant phenotype was passed on to the daughter cells and an abundance of colonies was observed. Yeast colony PCR was attempted to confirm the integration at the correct locus, but it will need to be repeated after optimizing the conditions.

Strong specific signal on the western blot in Figure 3, lanes 1-7 confirms that HA epitope tag is present in the cells, and shows that it is covalently bound to a much larger protein, since by itself the tag would be too small to be visible on the gel. This protein is most likely Cdc20, since the genomic tagging construct was specifically engineered to append an HA epitope to the C terminus of Cdc20. The protein runs on a polyacrylamide gel at a size approximately appropriate for Cdc20 combined with the HA tag. Lane 4 shows a weaker signal than all the other lanes,

which could be due to a difference in the quality of yeast cell extracts used for the experiment.

After the correct recombination locus is confirmed using yeast colony PCR, the HA-tagged Cdc20 will be useful for association studies with HTLV-1 protein Tax. Previous genetic experiments in *S. cerevisiae* showed that Tax binds APC/C<sup>Cdc20</sup> and suggested that Cdc20 can be the main association target. Co-immunoprecipitation (co-IP) studies using hemagglutinin and Tax antibodies should be able to determine whether Tax in fact directly binds to Cdc20. In order to perform such studies, the yeast strain with genomically tagged Cdc20 that was created in this study would have to be transformed with a plasmid containing Tax under the control of an inducible promoter. If the suspicion mentioned above is correct, after inducing Tax expression and immunoprecipitating it with a specific antibody a protein gel blotted with HA antibody would show strong specific signal close in size to Tax combined with Cdc20, marking the presence of hemagglutinin-tagged Cdc20. Such results would provide direct evidence that Tax directly interacts with Cdc20 to perform its function and possibly shed more light on the mechanism by which Tax is capable of inducing cell cycle arrest. A study like that would be further facilitated by creating epitope-tagged Tax constructs and using antibodies against the epitope tags, after affirming that tagging Tax does not interfere with its function.

If Tax does not co-precipitate with Cdc20, then it would suggest that one of the other APC/C subunits may be interacting directly with Tax. There are also other possibilities; for instance, it's possible that Tax directly interferes with the correct function of the mitotic checkpoint complex (MCC), which consists of Mad2, BubR1 and Bub3 proteins, and whose function is to sequester Cdc20 away from the APC/C until anaphase is ready to be initiated (12). To confirm this suspicion, co-IP studies of Tax with any of these proteins would need to be performed to provide evidence. Another option would be that Tax actively brings and tethers

Cdc20 to the APC/C ahead of schedule, either directly or indirectly. Further interaction studies would be needed to verify this speculation. Any of these findings will be able to propel forward the investigation of Tax-induced senescence and oncogenesis, and will further the understanding of the mechanisms by which it interferes with cell cycle regulation.

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