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The Role of Chd2 in DNA Damage Signaling

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Abstract

Cancer is a disease of uncontrolled cell growth and abnormal cell proliferation. There are a myriad of genetic modifications that disrupt proper cell functioning and can lead to cancer, and recent studies have suggested that improper chromatin remodeling may be yet another mechanism that leads to uncontrolled cell proliferation. Chromodomain helicase DNA binding proteins (CHD) are a family of proteins that have been associated with chromatin regulation and as a result, genome transcription regulation. Studies on the CHD family could have implications in cancer research because of the proteins' role in chromatin remodeling, gene expression and possibly chromosomal stability.

Research in our laboratory focuses on the Chromodomain helicase binding 2 protein (CHD2). Previously, the lab developed a CHD2 deficient mouse model which is characterized by a high susceptibility for lymphomas. The goal of this research project was to analyze the molecular mechanisms of Chd2 deficiency induced lymphomas and determine if the Chd2 protein is a substrate for ATM kinase, a well known DNA damage signaling kinase. To this end, an HA-tagged version of Chd2 was transfected into human cancer cell lines and exposed to DNA-damaging stimulus. Analysis of the Chd2 peptides for ATM specific phosphorylation indicated that the results so far were inconclusive. Additional experiments to unequivocally determine the phosphorylation of Chd2 by ATM are currently being planned and discussed.

Introduction

Cancer is a disease of uninhibited cell growth and atypical cell proliferation. The stability of the genome, the fidelity of its replication, and the efficiency of DNA replication are crucial to normal cell growth and development. The genetic material is manipulated several times throughout the cell's lifecycle, greatly increasing the chances of incorporating mistakes and developing DNA damage. The enormously lengthy genome must be packaged in order to fit within the nucleus of the cell. In the initial step of the DNA condensing process, the naked double-stranded DNA is wound around an octamer of basic histone proteins, producing a nucleosome. The nucleosome consists of a core of five types of histones, H1, H2A, H2B, H3, and H4, and DNA wrapped about 1.8 times around the core [1]. Each nucleosome occurs at regular intervals along a DNA strand, resulting in a "beads on a string" formation. Further winding and looping results in a chromatin fiber and higher level structures, ultimately forming the characteristically compact chromosome form which can easily fit within the confines of the cell's nucleus. The later steps are dependent on a correctly executed nucleosome configuration; however, by intentionally changing the charge on histones, the cell can render genes active, inactive, or responsive to developmental and external signals [2].

The reverse of this compaction process is equally important. Transcription repression is caused by packaging promoters within nucleosomes, which prevents initiation. It is therefore, very important to unpack DNA to give RNA polymerases and other related proteins access to the genetic material. This allows for genomic transcription and elongation, and further downstream, protein formation. In order to counteract the condensing activity of nucleosomes and free the promoter regions, cells

have chromatin-remodeling complexes which can disrupt DNA-histone interactions by clearing a portion of nucleosome-free DNA in an ATP-dependent manner. Initiation of transcription by chromatin remodeling can occur through a variety of mechanisms including destabilizing the nucleosome itself (SWI/SNF-related complexes), transferring histones octamers to a separate DNA molecule (RSC, a member of the SWI/SNF family), and "sliding" histones to different areas along the same strand of DNA (ISWI complexes) [3]. Elongation, in contrast, can occur regardless of nucleosomes. RNA polymerase (pol) II can continue to transcribe while the cell maintains the nucleosome structure by recruiting a number of elongation, chromatin-related, and chromatin-remodeling factors to the site of elongation [3, 4].

In addition to its role in transcription and elongation, chromatin-remodeling plays a role in other chromatin-based processes including, recombination, regulation of gene expression, and double-strand DNA (dsDNA) repair response. Chromatin remodeling complexes such as the well-studied SWI-SNF family assist in dsDNA break repair more specifically through the homologous recombination pathway. The ATP-dependent complexes, SWI-SNF and RSC, hydrolyze histones or rearrange nucleosomes, allowing greater accessibility of regulatory proteins to the undamaged sister chromosome's DNA [5]. By exposing the homologous donor sequence, the chromatin-remodeling complexes allow homology-searching complexes to work. Defects in chromatin-remodeling complexes show poor cellular response to DNA double strand breaks, resulting in an accumulation of genomic alterations and the potential for cancer development. The well-characterized SWI/SNF family has been long defined as a chromatin remodeler. The lesser known, poorly-studied chromodomain helicase DNA-binding (Chd) family has just

recently demonstrated its importance in transcription regulation and developmental processes [11].

The Chd family

The Chromodomain helicase DNA binding (Chd) family of genes encode a group of ATP-dependent chromatin remodeling proteins. They are part of the SNF2 superfamily of proteins. All members of the Chd family are distinguished by two sequence motifs: tandem chromodomains (Chromatin organization modifier) and SNF2-like ATPase domains. Chd proteins are activated by single-stranded, double-stranded, or nucleosomal DNA and exert their effects on transcriptional activation by aiding in chromatin remodeling. Because the Chd proteins render chromatin into transcriptionally inactive heterochromatic form, it is suggested that Chd could play a role in gene expression as a negative regulator [7].

The chromodomain is a 50 amino acid region. The evolutionarily conserved sequence is found to have a role in chromatin-structure remodeling in an ATP-dependent manner and transcriptional regulation of genes. Chromodomains are characteristically found in proteins that can interact with heterochromatin such as heterochromatin protein 1 (HP1) and polycomb (Pc) proteins [8]. Importantly, the chromodomain and helicase domains have been found to be necessary for appropriate interaction with chromatin. This association is mediated by the chromodomain, which binds the protein directly to RNA, DNA, and methylated H3 histone [6]. Other functions of Chd family members include association with transcriptional repression. In general, Chd family proteins play a crucial role in chromatin-related processes, particularly gene expression.

The Chd family is further broken down into three subfamilies: The Chd1-Chd2 family, the Chd3-Chd4 family, and the Chd5-Chd9 family. Each subfamily has its own identifying structures, domains, and functions. Briefly, the Chd1-Chd2 proteins contain a 229 amino acid region near the C-terminus used as a DNA-binding domain which preferentially binds to AT-rich DNA sequences. The Chd3-Chd4 family most notably lacks the DNA-binding domain. Instead, the proteins have two N-terminal plan homeo domains (PHD) which resembles Zn-finger domains. The subfamily consisting of Chd5-Chd9 has extra domains, including paired Brahma and Kismet (BRK) domains, switch-defective protein 3, adaptor 2, nuclear receptor co-repressor transcription factor IIIB (SANT-like) domain, CR domains, and a DNA-binding domain. As with the Chd1-Chd2 family, Chd5-Chd9's DNA-binding domain shows a preference for AT-rich DNA portions [6].

Of the Chd family of proteins, Chd1 is one of the better characterized. Both mouse Chd1 (MmCHD1) and human Chd1 (HsCHD1) have a pair of chromodomains, a single helicase/ATPase domain, and a DNA-binding domain. Chd1's biggest role seems to be in interacting between transcribed genes and transcription elongation factors, as evidenced by its physical interactions with RNA elongation complexes PAF, FACT, and Spt4/5 [9]. In humans, it has been shown that PAF, FACT, and associated ubiquitylation factors move onto the transcribed regions. Subsequent monouibiquitylation of H2B leads to trimethylation of H3K4 which is theorized to recruit Chd1 [9]. Recent data has shown that Chd1 may play a crucial role in transcription termination with RNA pol II because of its localization to 3'-untranslated regions of genes [10]. Finally, Chd1 is speculated to

have a potential role in replication because of its association with Spt16 and Pob3, which may be involved in replication [10].

The other member of the first subfamily, Chd2, is closely related to Chd1. Chd1 and Chd2 share a high homology between chromodomain motifs (77.9% similarity) and helicase/ATPase domains (92.3% similarity); however, their greatest differences are in the DNA-binding domain (75.1% similarity) [10]. Chd2 has several distinctive structural motifs: several bipartite nuclear localization signals (NLS-BP), a Myb-DNA binding domain, a centrally located helicase/ATPase domain, paired chromodomains near the N terminus, and an A+T hook. The Myb-DNA binding domain is a DNA binding domain (DBD) unique for its consensus sequence and the two-fold manner in which it binds protein and DNA, through contact with either the sugar phosphate backbone or the base itself [11]. This Myb-related DBD has been suggested to play a role in cell differentiation and proliferation [12]. The helicase/ATPase domain putatively allows efficient Chd functioning by altering DNA-protein interactions and unwinding DNA [7]. The A+T hook allows for preferential Chd binding to A-T rich sequences of DNA [7]. The chromodomain, chromatin organization modifier, is a 50 amino acid motif with roles in chromatin remodeling and transcriptional gene regulation [6].

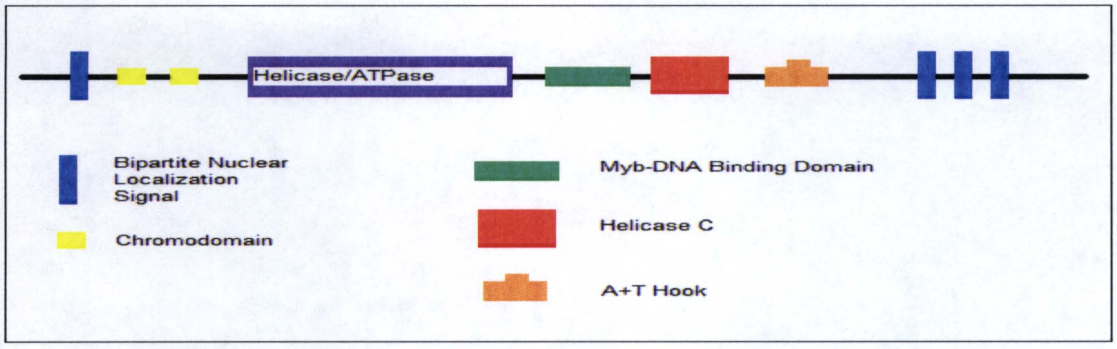

Figure 1. Schematic diagram of the Chd2 protein structure representing various domains.

Putative functions of Chd2 include a role in development and differentiation through cell-cycle progression [13]. Data shows that Chd2 deficient heterozygous mouse models survived past perinatal stages, but sustained multiple organ anomalies [13]. Chd2 deficient homozygous mouse models did not survive either because of embryonic lethality or post-birth infanticide [13]. Research into Chd2 and its functions is still in its infancy. Not much is known about the protein, but due to the Chd family's role in chromatin regulation, it is suggested that Chd2 may have a role in cancer prevention because of its role in chromosomal stability.

Ataxia telangiectasia mutated protein (ATM)

Ataxia telangiectasia mutated (ATM) is a protein kinase that is integral in several double strand DNA (dsDNA) breaks. ATM coordinates a series of complicated, multi-passage system of pathways that react to dsDNA breaks. These pathways then momentarily halt the cell cycle and organize to repair the DNA damage. Named for the mutated gene in the human disorder ataxia telangiectasia (AT), ataxia telangiectasia mutated (ATM) is part of a protein family that has kinase activity at serine/threonine motifs [14]. ATM is a 3056 amino acid protein with sequential FAT domain, PI(3)K domain, and FATC domain at the carboxy-terminus [15]. The FAT and FATC domain functions have not yet been clearly elucidated. The PI(3)K domain is named for its homology with phosphotidylinositol-3-OH-kinases' catalytic sequences and contains the motifs for the catalytic site of the protein [16].

ATM and related protein ATR, when activated, have the potential to bring about G1 arrest, G2 arrest, cell repair, and/or activate stress response genes. Through

phosphorylation of substrates such as Brca1, NBS1, p53, Chk1/2, and Mdm2, ATM can help promote cell genome stability [16, 17].

ATM is primarily activated by double strand DNA breaks caused by IR. Because of ATM's impressively quick response to DNA breaks, the mechanism for ATM activation has been highly contended. It has yet to be determined whether ATM is directly activated by DNA damage and physical contact with the break or indirectly activated through chromatin alteration. Research has shown that ATM may autophosphorylate a dimeric inactive form of itself. Constitutively, ATM is proposed to exist in some form of multimer, held in check by the FAT domain. Post DNA damage, ATM responds by phosphorylating the other units in the FAT domain, freeing all units involved. The ATM protein can then become active as a monomer [16].

ATM plays an important role in genome protection and genomic stability. The best-studied ATM activity is the response to double strand DNA breaks. The almost immediate response is effective because of ATM's activation strategies. ATM stimulates its many substrates through several different mechanisms, ensuring activation of the necessary downstream targets. This is demonstrated by p53, a defined tumor repressor and ATM substrate. ATM can directly phosphorylate p53, phosphorylate CHK2, which in turn phosphorylates p53, or phosphorylate MDM2, which leads to the degradation of p53. These changes in p53 arbitrate the G1-S stage of the cell cycle. ATM also adopts an "all roads lead to Rome" strategy in which it works from different pathways to a common end point. The S-G2 cell cycle checkpoint is controlled by ATM through several different pathways; the BRCA1, CHK2, and SMC1 substrates all activate separate paths that have the common end of halting the cell cycle at the S phase. Finally,

ATM utilizes a "domino effect" which can activate substrates into kinases themselves. The resulting kinases can phosphorylate downstream targets, increasing the efficiency with which ATM responds to DNA damage [16].

Some notable ATM substrates and pathways include p53, CHK1/2, SMC1, BRCA1, NBS1, and MDM2. All pathways result in some form of cell cycle control at various checkpoints. Cell cycle checkpoints are important in normal replication and cell growth. In response to abnormal signals, checkpoints can temporarily halt the cell cycle or signal cell death. There is redundancy within the pathway intermediates and endpoints, demonstrating ATM's efficiency at responding to DNA strand breaks [17]. ATM also has other potential mechanisms by which it protects a cell. ATM has been suggested to participate in cell cycle checkpoint activation and may play a role in preserving telomeres, the protective genome sequence at chromosome ends [16]. Because of ATM's integral role in genomic stability, there is strong link between ATM mutations and cancer.

Rationale

ATM is known to phosphorylate serine/threonine (S/Q) motifs. Research has shown that in order for phosphorylation to occur, hydrophobic amino acids must be present at N-1 and N-3 positions [11]. Analysis of the mouse Chd2 amino acid sequence revealed ten S/Q motifs as potential ATM phosphorylation sites. Further analysis of the S/Q motif sequences showed five out of the ten possible phosphorylation sites had a hydrophobic amino acid at either the N-1 or N-3 positions, and only one out of ten had both. The central hypothesis of this project is that Chd2 is an ATM substrate and functions at the chromatin level to affect DNA damage responses including DNA damage

specific chromatin remodeling and repair. This is based on our previous work with the Chd2 mouse model that are susceptible to lymphomas and the Chd2 mutant cells that accumulate spontaneous DNA strand breaks.

1 mmrnkdk**sq**e edsslhsnas srsaseevsg sdsg**sq**sese qgsepgsghg sesnsssess
61 e**sq**seseses agsk**sq**pvlp eakekpaskk eriadvkkmw eeypdvygvr rsnrsrqeps
121 rfnvkgeass gsesgspkrr gqrqlkkqek wkqdpsedeq eqgtsaesea eqkkgkarrp
181 vprrtvpkpq vkkqpkiqrg krkkqessdd dddddeapkr qtrrraaknv sykedddfet
241 dsddliemtg eggdeqqdns etiekvldsr lgkkgatgas ttvyaveang dpsddfdter
301 eegevqylik wkgwsyihst wesedslqqq kvkglkklen fkkkedevkq wlgkvspedv
361 eyfscqqela selnkqyqiv erviavktsk stlgqtdfpa hsrkpapsne peylckwmgl
421 pysecswede aligkkfqnc idsfhsrnns ktiptrecka lkqrprfval kkqpaylgge
481 slelrdyqle glnwlahswc ks(nsvilade mglgktiqti sflsylfhqh qlygpflivv
541 plstltswqr efeiwapein vvvyigdlms rntireyewi h**sq**tkrlkfn alittyeill
601 kdktvlgsin waflgvdeah rlknddslly ktlidfksnh rllitgtp)lq nslkelwsll
661 hfimpekfef wedfeedhgk grengyqslh kvlepfllrr vkkdvekslp akveqilrve
721 msalqkqyyk wiltrnykal akgtrgstsg flnivmelkk ccnhcylika pedseresgq
781 evlqslirss gklilldkll trlrergnrv lif**sq**mvrml dilaeyltik hypfqrldgs
841 ikgeirkqal dhfnadgsed fcfllstrag glginlasad tvvifdsdwn pqndlqaqar
901 ahrigqkkqv niyrlvtkgt veeeiierak kkmvldhlvi qrmdttgrtv lennsgrsns
961 npfnkeelta ilkfgaedlf keiegeesep qemdideilr laetrenevs tsatdell**sq**
1021 fkvanfatme deeeleerph kdwdeiipee qrkkveeeer qkeleeiyml prirsstkka
1081 qtndsdsdte skrqaqrssa sesetddsdd dkkpkrrgrp rsvrkdlveg ftdaeirrfi
1141 kaykkfglpl erleciarda elvdksvadl krlgelihns cvsamqeyee qlkestsegk
1201 gpgkrrgpti kisgvqvnvk siiqheeefe mlhksipvdp eekkkycltc rvkaahfdve
1261 wgveddsrll lgiyehgygn weliktdpel kltdkilpve tdkkpqgkql qtrvdyllkl
1321 lrkglekkgt vasgeeaklk krkprvkken kaprlkdehg lepasprhsd npseegevkd
1381 dgleksptkk kqkkkenken kekpvssrkd regdkerkks kdkkekvkgg dgkssskskr
1441 **sq**gpvhitag sepvpigede dddldqetfs ickermrpvk kalkqldkpd kglsvqeqle
1501 htrncllkig driaeclkay sdqehiklwr rnlwifvskf tefdarklhk lykmahkkrs
1561 qeeeeqkkkd dslggkkpfr peasgssrds li**sq**shtshn lhpqkphlpa shgpqmhghp
1621 rdnyshpnkr hfsnadrgdw qrerkfnygg gnsapwggdr hhqyeqhwyk dhhygdrrhm
1681 dahrsgsyrp nnmsrkrpye qynsdrdhrg hrdyydrhhh dskrrrsddf rpqnyhqqdf
1741 rrmsdhrptm gyhgqgpsdh yrsfhtdklg eykqpmpslh talsdprspp **sq**ksphdsks
1801 pldhrspler sleqknnpdy nwnvrkt

Figure 2. The mouse Chd2 amino acid sequence. The ten SQ motifs are shown in bold. The mouse Chd2 peptide sequence was obtained from the Ensemble database (version m36).

Materials and Methods

Sequence analysis

Computational analysis of the mouse Chd2 protein sequence was performed to establish the number of SQ-potential phosphorylation sites, locate the SQ sites, and evaluate the amino acids in the N-1 and N-3 positions. Calculations were then made to determine which sites were most viable to ATM phosphorylation.

Cloning of murine Chd2 gene segments

Previous work in the lab created a nearly full length, 1395 amino acid Chd2 clone containing an HA (hemaglutanin) tag. To create this clone, a series three of commercially available (Open Biosystems) cDNA clones that were homologous to the Chd2 protein were used. It was found through a blast comparison that mouse Chd2 and human Chd2 had 94% homology, so mouse Chd2 was used. The first clone, M2021 in a PT7T3D-PacI vector, contained the base pairs for the first 140 amino acids. Clone 2, named M5007, was in a pSport6 mammalian vector and contained 1143.3 amino acids after the M2021 clone. There was an 80 base pair overlap between the end of M2021 and the start of M5007. Restriction sites were added in order to cleave the clones at appropriate sequences. M2021 clone was introduced into the pSport6 vector through cloning and digestion with enzymes XhoI and NotI. The M5007 clone was cut with SalI and NotI and the two cut clones, M2021 and M5007, were ligated together to form temporarily named clone PChd3KJ.

C-terminal HA-tagging of 1395 aa of Chd2

To create the HA-tagged portion of the final clone, the last of the three commercial cDNA clones, M6213, was used. Forward primer 5007EF began by

overlapping part of the M5007 region and the CTHAX reverse primer coded for an HA-tag and Xba1 restriction site. The resulting product contained a portion of Chd2, an HA-tag, and an Xba1 site. This, and the previously created PChd3KJ were cut with Xba1 and ligated together within the pSport6 vector. The final clone PChdKHA(CT) was finished. It consists of part M2021, part M5007, and part M6213, and codes for two chromodomains, the helicase/ATPase domain, and several bipartite nuclear localization signals within the Chd2 protein.

Immunofluorescence:

Two 2-well chamber slides were seeded with 25,000 U2OS cells per chamber. Immunofluorescence was done after transfection with the PChdKHA(CT) clone to confirm localization of the clone to the nucleus. Briefly, the chamber slides were fixed in acetone: methanol, rinsed with Tris buffered saline, then incubated with antibody dilution buffer (3%BSA, 10% goat serum, 0.05% Triton X-100 in PBS pH 7.4) ADB, for an hour. Primary antibody, mouse anti-HA (1:1000 in ADB) was applied for 4 hours in a humidity chamber at room temperature. After 3 x 2ml/10min washes of working ADB, secondary antibody was added. Fluoroisothiocyanate (FITC) conjugated goat α-mouse (1:250 in ADB) was incubated in the humidity chamber for 1 hour at room temperature. The chamber walls were removed and each slide was washed with PBS-Photoflo (0.2%), PBS-Triton (0.05%), and PBS-Photoflo (0.2%) for 10 minutes each. The DNA was stained with DAPI (4',6-diamidino-2-phenylindole), and after completely removing the final stain, each slide was sealed with a cover-slip. Each slide was viewed using immunofluorescence microscopy.

A western blot was also performed to confirm the success of the transfection. Cell lysates were prepared with cold lysis buffer containing protease and phosphotase inhibitors, then sonicated. The lysates were loaded into a 5.5% SDS-PAGE mini-gel. Post-electrophoresis, the gel was transferred onto a 0.45μm PVDF membrane overnight. The membrane was blocked in 5% blotto for several hours, then rinsed with three appropriate volumes of TBS/Tween at 5 minutes each, at room temperature. The primary antibody, mouse α-HA (1:1000 in 2% blotto), was added to the membrane for 2 hours at room temperature. The membrane was then washed with TBS/Tween for 10 minutes, followed by another 10 minutes in 2% blotto, and a final 10 minute wash in TBS/Tween. The secondary antibody, α-mouse HRP (1:2000 in 2% blotto), was applied for 1 hour at room temperature. The membrane was then rinsed three times in TBS/Tween for 20 minutes each. 2 ml of SuperSignal West Pico Chemiluminescent Substrate (Pierce) was added to the membrane for 3 minutes. The film was developed after a 5 minute exposure.

Expression and phosphorylation of Chd2:

293T cells were plated in antibiotic free media and cultured overnight. Transfection was performed using the Lipofectamine kit according to amufacturer's suggestions (Invitrogen). Fresh media was added 4 hours and cells were treated with different DNA damaging agents after 48 hrs of post-transfection and incubated for specific times as shown in table 1.

1	2	3	4	5	6	7
Untransfected and untreated control	X-ray 50j 30 min	X-ray 50j 2 hr	0.1% MMS 30 min	0.1% MMS 2 hr	UV 40gy 30 min	UV 40gy 2 hr

Table 1. DNA damage treatment scheme. Table shows the plate and specific treatment of the cells expressing Ha-tagged Chd2 peptides.

After treatment, cells were collected, washed in media, and resuspended in cold lysis buffer (150mM NaCl + 50mM Tris pH 8) containing protease and phosphotase inhibitors. The lysates were then subjected to sonication to shear the DNA. After the shearing step, 250 µl of cell lysate was incubated with 1 µl DNase and 2.5 µl 1M MgSO4 and rocked at room temperature for 30 minutes. 250 µl NET gel buffer with 2mM EDTA and 20 µl of HA beads was added. The complex was rocked at 4°C overnight. The following day, after a short centrifugation, the bead-protein complex was washed 3 times with 500 µl cold NET gel buffer containing protease inhibitor/phosphotase inhibitor each time. To the pellet, 1X SDS loading buffer was added and samples were boiled at 95°C for 3 minutes, and loaded onto a 5.5% SDS-PAGE mini-gel. After electrophoresis, the gel was transferred onto a 0.45 µm PVDF membrane and blocked for several hours, then rinsed with three 5 minute washes of TBS/Tween at room temperature.

Western blots were performed with antibodies specific for α-ATM/ATR phosphorylated SQ/ST antibody (1:1000 in 2% blotto overnight at 4°C) and secondary antibodies α-rabbit HRP (1:2000 in 2% blotto, 1 hr at room temperature). The membrane was rinsed three times in TBS/Tween washes of 20 minutes each between incubations. SuperSignal West Pico Chemiluminescent Substrate was used to develop the membrane. The film was left for two exposures, at 30 minutes and 1 hour. After stripping the membrane with washing buffer (1XTBS + 0.5M $NaCl_2$ + 0.2M SDS), the membrane was reblocked with 5% blotto. The subsequent western blot was performed using primary antibody α-HA (1:1000 in 2% blotto) incubated for 1.5 hours and secondary antibody α-mouse HRP (1:2000 in 2% blotto) incubated for 1 hour and developed as described above.

Results

Computational analysis of Chd2 sequence for SQ motifs

Computational analysis of the mouse Chd2 amino acid sequence showed a total of ten sq motifs. 5/10 SQ motifs had a hydrophobic amino acid at either the N-1 or N-3 position; 1/10 SQ motifs had a hydrophobic amino acid at the N-3; 3/10 SQ motifs had a hydrophobic amino acid at the N-1 position; finally, only 1/10 SQ motifs had the necessary hydrophobic amino acids at the N-1 and N-3 positions. (See Fig. 3).

SQ Sequence Motifs:

1. nkdk**sq**e
2. sdsg**sq**s
3. esse**sq**s
4. agsk**sq**p
5. ewih**sq**t
6. vlif**sq**m
7. dell**sq**f
8. kskr**sq**g
9. dsli**sq**s
10. rspp**sq**k

Figure 3. Comparison of ten prospective ATM phosphorylation site S/Q motifs. Each sequence includes the SQ motif, four preceding amino acids, and one following amino acid. Green highlights denote the N-1 and N-3 positions; red highlights denote hydrophobic amino acids in the N-1 and/or N-3 position.

Expression and nuclear localization of HA-Chd2 peptide

To determine the expression levels and the sub cellular localization of the HA-tagged Chd2 peptide, we performed western-blot and immuno-fluorescence assays. The western blots confirmed the optimal expression of the HA-tagged 1395 amino acid Chd2 clone as shown in figure 4a. Immunofluorescence analysis demonstrated the localization of the PChdKHA(CT) clone within the nucleus of the transfected U2OS cells. As shown in figure 4b, expression analysis of HA-Chd2 using anti-HA antibodies indicated that the HA-Chd2 peptide was confined only to the nucleus. The overlapping of nuclear staining

with DAPI and HA-Chd2 signal with FITC conjugated secondary amino acids signified the localization of the expressed peptide to the nucleus (Figure 4b). This is consistent with the fact that the HA-tagged Chd2 peptide contains four bipartite nuclear signals within its sequence.

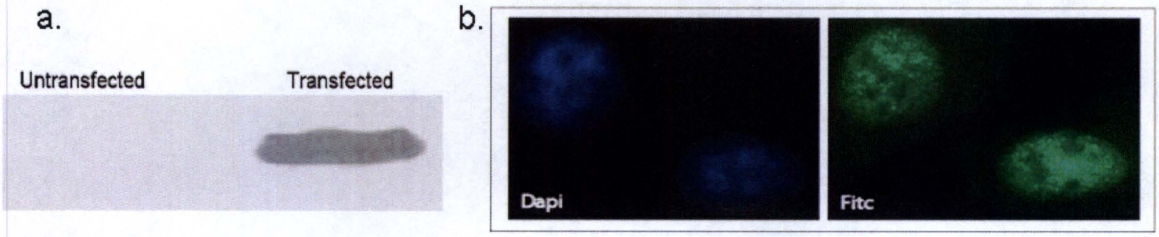

Figure 4. Expression and subcellular localization of HA-tagged Chd2. The representative western blot using α-HA antibody to demonstrate expression of HA-tagged Chd2 into cells is shown in panel a. The fluorescently stained images on the left (panel b) show the nuclear staining by the DNA binding dye, DAPI. The right panel shows expression of HA-tagged Chd2 that is visualized by FITC staining.

ATM phosphorylation of HA-Chd2

To determine, if the exogenously expressed HA-Chd2 peptide was phosphorylated after DNA damage, we performed immunoprecipitation analysis followed by immunoblot analysis with antibodies specific for phosphorylated SQ/ST motifs. The western blots with α-ATM/ATR phosphorylated SQ/ST antibodies were inconclusive. The α-HA film showed expected bands for our HA-tagged 1395 amino acid Chd2 clone at ~160 Kd (figure 5). Importantly we did not detect any phosphorylated bands around 160 Kd indicating that the HA-tagged Chd2 peptide is not phosphorylated under the conditions that were analyzed. The α-ATM/ATR phosphorylated SQ/ST blot, however, produced a non-specific band at a lower molecular weight (~130 kD) than the expected molecular weight of HA-Chd2 peptide in all the

transfected and un-transfected samples. The presence of the novel band in the un-transfected samples also suggested that the novel band may be a non-specific cellular protein bound by the anti-HA sepharose beads.

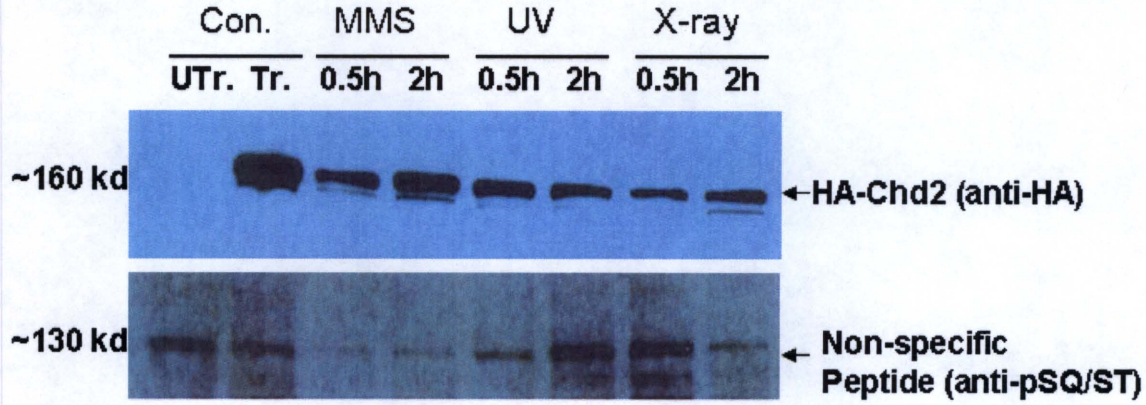

Figure 5. Analysis of SQ/ST phosphorylation patterns of HA-Chd2. Comparison of α-HA and α-ATM/ATR phosphorylated SQ/ST western blots is shown above. UTr., untransfected control; Tr., transfected untreated control.

Discussion

The Chd2 protein has 2 chromodomains and a helicase/ATPase domain, suggesting it has a role in chromatin remodeling, chromosomal stability, and DNA repair. Our lab previously created a knockout mouse model for Chd2 using gene trap technology. We found that mice heterozygous for Chd2 had a propensity to develop lymphomas, and nullizygous mutants suffered from neonatal lethality. Based on the previous observations, we hypothesized that Chd2 plays an important role in genomic stability maintenance.

In an effort to determine the biochemical functions of Chd2 that relate to genome stability maintenance we sought to determine if Chd2 was a substrate of the DNA damage inducible kinase, ATM. Sequence analysis of the mouse Chd2 protein also suggested ten potential SQ motifs for ATM phosphorylation. To investigate whether

Chd2 is a potential substrate of ATM, a recombinant Chd2 peptide (1395 amino acid Chd2 tagged with HA) was created and expressed in 293T and U2OS cells. HA-Chd2 transfected human cancer cells were exposed to three DNA damage-causing agents. Expression and localization of the recombinant peptide into the nucleus was confirmed by western blot and immunofluorescence, respectively.

The ATM phosphorylation experiment yielded unexpected results in that the bands from the anti-HA blot and the anti-ATM/ATR phosphorylated SQ/ST did not match. The HA blot expressed expected bands for HA-tagged Chd2 between 160 – 170 kD. The α-ATM/ATR phosphorylated SQ/ST blot revealed a lower molecular weight band. Even though the band does not represent Chd2, its mere presence indicates that something else within the cell is being phosphorylated by ATM or ATR, as the antibody is extremely specific to ATM/ATR-phosphorylated SQ/ST motifs. It is unclear as to what this unknown ATM substrate could be because ATM has numerous substrates, some of which are still unidentified. The α-ATM/ATR phosphorylated SQ/ST blot also showed evidence of phosphorylation of the non-specific protein in the untransfected control, suggesting an SQ or ST motif may have acted upon by constitutive ATM phosphorylation activity without DNA damage. The 293T cell line was originally chosen for its fast growth, high transfection efficiency, and ease of manipulation; however, after further research, it was found that 293T cells may not be the best cells for use in ATM phosphorylation experiments. The 293T cells are human embryonic kidney cells transformed by a rare adenovirus integration event that alters E1A and E1B genes, among several others [18]. Further literature searches found that 293 cells are highly susceptible to a variety of virus transformants including herpes simplex virus, parainfluenza virus,

and respiratory syncytial virus [19]. Furthermore, simian virus 40 (SV40) affects ATM functioning and SV40 expression can activate ATM and subsequently affect downstream ATM substrates and pathways [20]. These findings and our inconclusive results suggest that 293T is an unsuitable cell line for DNA damage response related studies. This is due to the fact that expression of SV40 in 293T cells makes it difficult to distinguish the differences between the effects of constitutive ATM activation by SV40 and the experimental DNA-damaging treatments.

Future Work

There are several possible avenues for continuing with this work. As discussed, the ATM phosphorylation experiment with 293T cells was not successful. Currently, the ATM phosphorylation of Chd2 experiment is being repeated using U2OS cells, which are better suited for the experiment. U2OS cells are derived from bone tissue of an osteosarcoma patient [21]. They resemble epithelial cells and are free of adenoviruses, RSV, and most importantly, SV40, making them useful for DNA damage studies involving tumor suppressor genes [21]. Concurrently, our lab has developed a 1395 amino acid Chd2 clone with Tandem affinity purification (TAP) tags containing histidine and strep tags. We plan to repeat the ATM experiment with TAP-tagged Chd2 in U2OS cells. If the ATM phosphorylation experiments are successful, an *in vitro* kinase assay would be performed to determine whether Chd2 is phosphorylated by ATM/ATR. As a substrate, purified Chd2 will be used in conjunction with kinase obtained from U2OS cells and AT deficient cell lines. The results of this experiment would help determine if

Chd2 is indeed phosphorylated by ATM after DNA damage and provide mechanistic insights on the role of Chd2 in DNA damage response pathways.

References

1. Nelson DL, Cox MM. *Lehninger Principles of Biochemistry, Fourth Edition.* W. H. Freeman, 2004.
2. Armstrong JA, Emerson BM. Transcription of chromatin: these are complex times. *Curr Opin Genet Dev.*, 8(2):165-72; Apr 1998.
3. Kornberg RD, Lorch Y.Cell. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell.,* 6;98(3):285-94; Aug 1999.
4. Li B, Carey M, Workman JL. The role of chromatin during transcription. *Cell.*;128(4):707-19; 2007 Feb 23.
5. Bao Y, Shen X. Chromatin remodeling in DNA double-strand break repair. *Curr Opin Genet Dev.* 2007 Feb 21
6. Marfella CG, Imbalzano AN. The Chd family of chromatin remodelers. *Mutat Res.* 2007 Jan 21
7. Woodage T, Basrai MA, Baxevanis AD, Hieter P, Collins FS. Characterization of the CHD family of proteins. *Proc Natl Acad Sci USA.*;94(21):11472-7; Oct 1997.
8. Neely KE, Workman JL. The complexity of chromatin remodeling and its links to cancer. *Biochim Biophys Acta.*;1603(1):19-29; Oct 2002.
9. van Vugt JJ, Ranes M, Campsteijn C, Logie C. The ins and outs of ATP-dependent chromatin remodeling in budding yeast: Biophysical and proteomic perspectives. *Biochim Biophys Acta.*;1769(3):153-71; Mar 2007.
10. Simic R, Lindstrom DL, Tran HG, Roinick KL, Costa PJ, Johnson AD, Hartzog GA, Arndt KM. Chromatin remodeling protein Chd1 interacts with transcription

elongation factors and localizes to transcribed genes. *EMBO J.*;22(8):1846-56; Apr 2003.

11. Schurmann A, Sokolowski R, Haas M, Wolfes H. Characterization of direct readout contacts of the Myb DNA-binding domain. *Blood Cells Mol Dis.* (2):464-9; 2001.
12. Jackson J, Ramsay G, Sharkov NV, Lium E, Katzen AL. The role of transcriptional activation in the function of the Drosophila myb gene. *Blood Cells Mol Dis.* (2):446-55; 2001.
13. Marfella CG, Ohkawa Y, Coles AH, Garlick DS, Jones SN, Imbalzano AN. J Mutation of the SNF2 family member Chd2 affects mouse development and survival. *Cell Physiol.*;209(1):162-71; Oct 2006.
14. Kim ST, Lim DS, Canman CE, Kastan MB. Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem.*;274(53):37538-43; Dec 1999.
15. McKinnon PJ. ATM and ataxia telangiectasia. *EMBO Rep.*;5(8):772-6; Aug 2004.
16. Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer.*;3(3):155-68. Mar 2003.
17. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet.*;27(3):247-54. Mar 2001.
18. Flint SJ, Enquist LW, Racaniello VR, and Skalka AM. *Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses.* 2003.

19. Brown M, Petric M. Evaluation of cell line 293 for virus isolation in routine viral diagnosis. *J Clin Microbiol.*;23(4):704-8. 1986 Apr.

20. Shi Y, Dodson GE, Shaikh S, Rundell K, Tibbetts RS. Ataxia-telangiectasia-mutated (ATM) is a T-antigen kinase that controls SV40 viral replication in vivo. *J Biol Chem.*;280(48):40195-200. 2005 Dec 2.

21. http://www.microscopyu.com/moviegallery/livecellimaging/u2/index.html