The tumor suppressor CDC73 interacts with the ring finger proteins RNF20 and RNF40 and is required for the maintenance of histone 2B monoubiquitination

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Received June 17, 2011; Revised October 12, 2011; Accepted October 18, 2011

Monoubiquitination of histone H2B is a dynamic post-translational histone modification associated with transcriptional elongation and the DNA damage response. To date, dysregulation of histone monoubiquitination has not been linked to pathogenic mutations in genes encoding proteins, or co-factors, catalyzing this modification. The tumor suppressor cell division cycle 73 (CDC73) is mutated and/or down-regulated in parathyroid carcinoma, renal, breast, gastric and colorectal tumors, as well as in the germline of patients with the familial disorder—hyperparathyroidism jaw tumor syndrome. Using CDC73 as bait in a yeast two-hybrid assay, we identified the ring finger proteins RNF20 and RNF40 as binding partners of this tumor suppressor. These polypeptides constitute a heterodimeric complex that functions as the E3 ubiquitin ligase for monoubiquitination of histone H2B at lysine 120 (H2B-K120). We show that RNF20 and RNF40 bind to discrete, but closely located, residues on CDC73. Monoubiquitinated H2B-K120 was significantly reduced after loss of nuclear CDC73, both in vitro upon down-regulation of CDC73, and in CDC73 mutant parathyroid tumors. A second histone modification, trimethylation of histone 3 at lysine 4 (H3-K4me3), remained unchanged in the presence of mutant or down-regulated CDC73, suggesting that H3-K4me3 is not always tightly linked to H2B-K120 monoubiquitination for transcription as previously described. This is the first report of pathogenic mutations affecting histone monoubiquitination. We conclude that CDC73 is required for the maintenance of H2B-K120 monoubiquitination and propose that reduction in levels of monoubiquitinated H2B-K120 is a major mechanism whereby mutations in CDC73 exert their tumorigenic effect.

INTRODUCTION

Post-translational modifications such as ubiquitination, methylation, acetylation and phosphorylation of core nucleosomal histone tails play key roles in transcriptional regulation by determining the availability of chromatin to protein effectors (1). Monoubiquitination of histone 2B at lysine 120 (H2B-K120) is a dynamic process associated with transcriptional elongation in undamaged cells (2). This histone modification is also induced after DNA damage (3) and has been shown to have a role in the maintenance of replication-dependent histone mRNA 3'-end processing (4). At the structural level, monoubiquitination of H2B-K120 interferes with compaction of chromatin, resulting in open chromatin fibers that display greater accessibility to transcription factors and their co-regulators (5). Some reports suggest that H2B-K120 monoubiquitination is preceded by and tightly linked to trimethylation of histone 3 at lysine 4 (H3-K4me3), another histone modification involved in transcriptional regulation (6, reviewed in 7); however, recent in vitro evidence suggests that this pattern may not always occur (3,8).
H2B-K120 monoubiquitination is mediated by the E3 ubiquitin ligase ring finger protein–20–ring finger protein 40 (RNF20–RNF40) complex. RNF20 and RNF40 exist as a heterodimeric complex containing two copies of each polypeptide (9). There is conflicting literature as to whether the complex as a whole functions as the E3 ubiquitin ligase mediating mono-ubiquitination of H2B-K120, as described recently by Moyal et al. (3), or whether RNF20 alone is the key functional unit (10). RNF20 itself is thought to behave as a tumor suppressor given that its promoter is hypermethylated in breast cancer (8,11). Furthermore, a somatic mutation in RNF20 has been reported in 1 of 36 colorectal cancers, although possible functional effects of this mutation were not investigated (12).

The process of H2B-K120 monoubiquitination involves association of the RNF20–RNF40 complex with the human PAF1 (RNA polymerase II-associated factor 1) transcriptional complex composed of LEO1, CTR9, SK18, RTF1, CDC73 (cell division cycle 73) and PAF1 (6). As part of this process, the PAF1 complex associates with RNA polymerase II on the chromatin of actively transcribed genes, participating in transcription-related events, including initiation, elongation and 3’-end processing (13–15). The requirement of individual PAF1 complex members in the process of H2B-K120 monoubiquitination is largely unknown; however, a recent study in vitro suggests that the PAF1 subunit of this complex is primarily responsible for interaction between the PAF1 complex and RNF20 (6).

CDC73 (also known as parafibromin) is the only PAF1 complex member reported to harbor pathogenic mutations, although loss of CTR9 and over-expression of PAF1 have been found in pancreatic cancer (reviewed in 16). It is a 531 amino acid, ubiquitously expressed tumor-suppressor protein localized to both the nucleus (14,17,18) and nucleolus (19,20). CDC73 is reported to function as a negative regulator of cell cycle progression (13) and to promote apoptosis (21). Furthermore, CDC73 has a role in the regulation of replication-dependent histones by regulation of the 3’ processing of histone mRNA (22).

Germline loss-of-function mutations in CDC73 (also known as HRPT2) lead to loss of nuclear CDC73 (23) and are associated with hyperparathyroidism jaw tumor syndrome (HPT-JT; OMIM 145001) (24–26, reviewed in 27). Many HPT-JT family members will develop a parathyroid tumor by the age of 40 years, 10–15% of these being malignant (28,29). Other manifestations of HPT-JT include fibro-osseous hamartomas and/or cystic kidney disease. Somatic CDC73 mutations have also been identified in sporadic parathyroid carcinomas (25,26). Missense CDC73 mutations have also been identified in sporadic parathyroid carcinomas (25,26). Missense CDC73 mutations have also been identified in sporadic parathyroid carcinomas (25,26). Missense CDC73 mutations have also been identified in sporadic parathyroid carcinomas (25,26). Missense CDC73 mutations have also been identified in sporadic parathyroid carcinomas (25,26). Missense CDC73 mutations have also been identified in sporadic parathyroid carcinomas (25,26). Missense CDC73 mutations have also been identified in sporadic parathyroid carcinomas (25,26). Missense CDC73 mutations have also been identified in sporadic parathyroid carcinomas (25,26).

To date, the mechanisms whereby loss of CDC73 promotes tumorigenesis have largely been investigated in vitro. We know that CDC73 is a member of the PAF1 complex (15), and have knowledge of some of its binding partners that include β-catenin, facilitating a role for CDC73 in the regulation of Wnt signaling (34), and actin-binding proteins that suggest a role for CDC73 outside of the nucleus (35). To further understand the emerging role of CDC73 in cancer, we sought to elucidate mechanisms of its tumor-suppressive function by identification of significant protein–protein interactions and the effects of disrupting these interactions both in vitro and in tumors. This study provides new insights into the regulation of H2B-K120 monoubiquitination and suggests that dysregulation of H2B-K120 monoubiquitination may be significant for the development of cancer.

RESULTS

CDC73 associates with the components of the E3 ubiquitin ligase RNF20–RNF40 complex

To elucidate mechanism(s) of the tumor-suppressive function of CDC73, we performed a high-stringency yeast two-hybrid (Y2H; Matchmaker 3, Clontech, Mountain View, CA, USA) screen of an adult human kidney cDNA library (Clontech) using CDC73 as bait. This screen generated 371 yeast clones, sequencing of which revealed 53 unique putative CDC73-binding partners. Putative binding partners requiring further validation were involved in histone modification, DNA repair, RNA processing, transcription, immune and redox regulation, cell signaling and metabolism, translation and the cytoskeleton. Given the nuclear and nucleolar localization of CDC73 and growing elucidation of its roles in transcription and RNA processing, potential binding partners involved in nuclear processes were prioritized for validation and further study. Two putative nuclear interactors identified at high frequency were the ring finger proteins RNF20 (7 unique clones identified a total of 19 times) and RNF40 (2 unique clones identified a total of 21 times) (Supplementary Material, Fig. S1). A number of clones of RNF20 or RNF40 lacked the N-terminal regions of these polypeptides, suggesting that these regions are not critical for binding with CDC73 (Supplementary Material, Fig. S1). Re-transformation of CDC73 and either full-length RNF20 or the C-terminus of RNF40, obtained in the original Y2H assay, back into yeast confirmed these interactions (Supplementary Material, Fig. S2).

Physical interaction between RNF20 and CDC73 was validated in mammalian cells by co-immunoprecipitation of over-expressed HA-tagged RNF20 and Myc-tagged CDC73 in HEK293 cells. Myc-CDC73 was co-immunoprecipitated by an anti-HA antibody when overexpressed in the presence of HA-RNF20. In contrast, an IgG control antibody could not co-immunoprecipitate Myc-CDC73. Furthermore, in the absence of HA-RNF20 expression, an anti-HA or IgG control antibody could not co-immunoprecipitate Myc-CDC73 (Fig. 1A). These results were confirmed in a reverse immunoprecipitation in which an anti-Myc antibody was used to co-immunoprecipitate HA-RNF20 (Supplementary Material, Fig. S3A). Physical interaction between over-expressed Myc-CDC73 and HA-RNF40 was confirmed in similar experiments (Fig. 1B and Supplementary Material, Fig. S3B).

To further verify this interaction, co-immunoprecipitation of endogenous RNF20 and CDC73 in HEK293 cells was
performed. An anti-CDC73 antibody co-immunoprecipitated RNF20 from HEK293 cell lysates, whereas an IgG control antibody could not (Fig. 1C). Interaction between endogenous CDC73 and RNF40 was similarly confirmed (Fig. 1D). Taken together, our data obtained in both the Y2H assay and co-immunoprecipitation studies in mammalian cells demonstrate that CDC73 associates with both RNF20 and RNF40 and suggest that CDC73 may interact with these proteins when they form a complex to function as an H2B E3 ubiquitin ligase.

CDC73 residues 108 and 98–100 are required for the binding of RNF20 and RNF40, respectively

The Y2H assay was again employed to determine the regions of CDC73 required for binding to RNF20 and RNF40. CDC73 truncating mutations were introduced into pGBKT7-CDC73 to allow expression of CDC73 fragments fused to the Gal4-binding domain in yeast (Fig. 2A and C). Co-transformation of truncated CDC73 constructs with full-length RNF20 fused to the Gal4 activation domain and subsequent assessment of reporter gene activation revealed that CDC73 residues from 108 to 110 were required for RNF20 binding (Fig. 2A). Similarly, co-transformation of a C-terminal fragment of RNF40 and either full-length or truncated CDC73 revealed that residues 98–100 were required for RNF40 binding (Fig. 2C). To confirm these findings, different combinations of residues 108–110 and 98–100 were mutated to alanine in full-length CDC73 (Fig. 2B and D). Co-transformation of these constructs with RNF20 or RNF40 and assessment of reporter gene activation demonstrated that residue 108 of CDC73 was required for the association of CDC73 with RNF20 (Fig. 2B). Furthermore, even though truncated fragments containing the first 98 residues of CDC73 did not interact with RNF40 in the Y2H assay (Fig. 2C), mutation to alanine of all three residues from 98 to 100 was required to completely abolish RNF40 binding to full-length CDC73 (Fig. 2D).

Alignment of the protein sequence of human CDC73 with that of multiple species revealed residues 98–100, leucine–asparagine–glycine, and the isoleucine at residue 108 are highly conserved. The only change observed at position 108 across species was the presence of the similar amino acid methionine in Caenorhabditis elegans. Although the leucine at residue 98 was conserved in all species, the asparagine at residue 99 was replaced by the similar amino acid glutamine in C. elegans and by the neutral amino acid threonine in Arabidopsis thaliana. The glycine at position 100 remained consistent for all species with the exception of Oryza sativa, where it formed part of a larger sequence of missing residues (Supplementary Material, Fig. S4).

CDC73 is required for H2B-K120 monoubiquitination in vitro

RNF20 and RNF40 have been reported to exist as a heterodimeric complex that contains two copies of each polypeptide (9). There is conflicting literature as to whether the complex as a whole functions as an E3 ubiquitin ligase mediating monoubiquitination of H2B-K120, as described recently by Moyal et al. (3), or whether RNF20 alone is the key functional unit as described by Kim et al. (10). Furthermore, down-regulation of either polypeptide has been shown to decrease levels of the other and depletion of either by siRNA reduces the level of monoubiquitinated H2B-K120 (3). For these reasons, we chose to down-regulate RNF20 by siRNA to establish the effects on H2B monoubiquitination in our in vitro system.

HEK293 cells were transiently transfected with siRNA directed against either RNF20 or CDC73 after which the levels of monoubiquitinated H2B-K120 were analyzed by immunoblot (Fig. 3A). As previously reported, down-regulation of RNF20 significantly reduced levels of H2B-K120 by ≏50%, consistent with its role as an E3 ubiquitin ligase that catalyzes this modification (Fig. 3A and B) (3,10). Down-regulation of CDC73 resulted in a similar level of reduction of H2B monoubiquitination, suggesting that CDC73 is also required for monoubiquitination of H2B-K120 (3). For these reasons, we chose to down-regulate RNF20 by siRNA to establish the effects on H2B-K120 monoubiquitination in our in vitro system.

Figure 1. The ring finger proteins RNF20 and RNF40 are CDC73-interacting proteins. Co-immunoprecipitation was performed on overexpressed CDC73 and on either RNF20 (A) or RNF40 (B) in HEK293 cells. Myc-tagged CDC73 was co-immunoprecipitated by an anti-HA antibody only when both RNF20 and CDC73, or both RNF40 and CDC73 were co-expressed (lane 5 of each panel). In HEK293 cells, endogenous RNF20 (C) or RNF40 (D) was co-immunoprecipitated by an anti-CDC73 antibody (lane 3 of each panel).
in which nuclear CDC73 expression was lost should show altered levels of H2B-K120 monoubiquitination. In order to test this hypothesis, 11 parathyroid tumors in which expression of nuclear CDC73 was lost or weak (4 of 11 with characterized predicted loss-of-function mutations, and 1 with a pathogenic missense mutation in \( \text{CDC73} \)), and 10 parathyroid adenomas with expression of wild-type nuclear CDC73, were examined by immunohistochemistry with antibodies against monoubiquitinated H2B-K120 and total H2B. Given previously reported links between H2B-K120 monoubiquitination and H3-K4me3, samples were also examined with antibodies against H3-K4me3.

Immunohistochemical staining was allocated a score between 0 and 3 (0 being the lowest, 3 the highest level of expression based on the intensity and number of stained nuclei). All 11 tumors with either a characterized \( \text{CDC73} \) mutation and/or loss of nuclear CDC73 exhibited low to absent immunostaining for monoubiquitinated H2B-K120, with scores of either 0 (6 of 11) or 1 (5 of 11) (Fig. 4A and B, Table 1). In contrast, the majority of parathyroid adenomas expressing wild-type nuclear CDC73, were examined by immunohistochemistry with antibodies against monoubiquitinated H2B-K120 and total H2B. Given previously reported links between H2B-K120 monoubiquitination and H3-K4me3, samples were also examined with antibodies against H3-K4me3.

CDC73 is not required for H3-K4me3 in vitro or in vivo

Although a number of studies have suggested that monoubiquitination of H2B-K120 is a pre-requisite for H3-K4me3 (6), reviewed in (7), recent reports have demonstrated that this is not always the case (3,8). Consistent with these studies, neither siRNA down-regulation of RNF20 nor CDC73 altered the levels of H3-K4me3 in vitro (Fig. 3A and C). Furthermore, no difference in the levels of H3-K4me3 was observed between tumors expressing nuclear CDC73 or those where expression was lost (Mann–Whitney \( U \) test, \( P > 0.05 \); Fig. 4A, D and E, Table 1).

DISCUSSION

Our data demonstrate that the tumor suppressor CDC73 is required for the maintenance of H2B-K120 monoubiquitination both in cell line models and in vivo. Loss of nuclear CDC73 caused by pathogenic mutations in \( \text{CDC73} \) leads to loss of monoubiquitinated H2B-K120 in human tumor specimens. Although cancer-associated mutations have been reported to affect methylation of core histones (36,37), this is the first report of pathogenic mutations regulating monoubiquitination of a histone. Furthermore, these findings provide direct in vivo evidence to support earlier speculation that dysregulation of histone monoubiquitination, especially that of histone 2B, occurs in human tumors (8,38).

The RNF20-RNF40 complex is recognized as the E3 ubiquitin ligase that facilitates both transcription and DNA damage-associated monoubiquitination of H2B-K120 (3,9,10), although the mammalian SWI/SNF-A subunit...
BAF250/ARID1 has recently been reported to also function as an E3 ubiquitin ligase targeting monoubiquitination of H2B-K120 (39). Earlier work suggested that the PAF1 subunit is the primary component of the PAF1 complex that mediates the interaction between the PAF1 complex and RNF20 (6). A more recent study has suggested that the RNF20–RNF40 complex, in fact, associates with WAC (WW domain-containing adaptor with coiled-coil) and not directly with the hPAF1 complex, although the hPAF1 complex would appear to be important for the recruitment of RNF20–RNF40–WAC to actively transcribed chromatin (40). The dynamic nature of these key interactions, both in transcription and in the cellular response to DNA damage, is complex and remains to be fully elucidated. We have now shown that both RNF20 and RNF40 bind to CDC73 at nearby residues; the close proximity of these binding regions consistent with the close association of these polypeptides in the heterodimeric RNF20–RNF40 complex. Our data therefore provide evidence to suggest that CDC73 plays a role in establishing physical linkages between RNF20, RNF40 and the PAF1 complex that are important for the regulation of H2B-K120 monoubiquitination.

Although down-regulation of either RNF20 or CDC73 by siRNA led to a decrease in H2B-K120 monoubiquitination, no changes were observed in the levels of H3-K4me3 (Fig. 3A and C). Furthermore, no difference in the levels of H3-K4me3 was observed between tumors expressing nuclear CDC73 or those where expression was lost (Mann–Whitney U test, P > 0.05; Fig. 4A, D and E, Table 1). CDC73 has been reported to interact with a histone methyltransferase complex important for H3-K4me3 (14); however, our in vitro and in vivo data would suggest that loss of CDC73 is not sufficient to cause detectable alterations in the levels of H3-K4me3 (Figs 3A and C, 4A, D and E). H2B-K120 monoubiquitination and H3-K4me would therefore appear not to be tightly linked in the tumors and in vitro model studied. These results are consistent with a study in HeLa cells reporting that down-regulation of RNF20 led to a decrease in global H2B-K120 monoubiquitination, but the effect on H3-K4me3 was only minor (8). It is also consistent with the observation that after DNA damage of HeLa cells, no change was seen in H3-K4me3, despite induction of H2B-K120 monoubiquitination (3).

Both H2B-K120 monoubiquitination and CDC73 are functionally linked to transcriptional elongation. CDC73 associates with non-phosphorylated and both serine 2 (linked to established elongation) and serine 5 (linked to initiation and early elongation) phosphorylated forms of RNA polymerase II. Through this linkage to RNA polymerase II, CDC73, most likely as a PAF1 complex member, is able to associate with coding regions of mammalian genes throughout transcriptional initiation to elongation (13,14). The observation that H2B-K120 is largely confined to the transcribed region of mammalian genes is consistent with the linkage of H2B-K120 monoubiquitination.
to transcriptional elongation (2). In order for RNA polymerase II to synthesize mRNA precursors, it must overcome the nucleosome that presents a physical barrier to RNA polymerase II-mediated transcriptional elongation. One possible model explaining how this barrier might be overcome suggests that the histone chaperone FACT is first recruited to the chromatin template, followed by the PAF complex, the E3 ubiquitin ligase complex RNF20–RNF40 and its ubiquitin-conjugating enzyme UbcH6, whereby H2B-K120 monoubiquitination occurs. This is then postulated to result in displacement of the H2A/H2B dimer from the core nucleosome, allowing the passage of RNA polymerase II through the nucleosomal barrier (41). Our data support the possibility that the key role of CDC73 in this process is to bind to and recruit the E3 ubiquitin ligase complex RNF20–RNF40 in order for monoubiquitination of H2B-K120 to occur.

Both H2B-K120 monoubiquitination and CDC73 have been implicated in the maintenance of replication-dependent

Figure 4. H2B monoubiquitination is significantly reduced in parathyroid tumors with absent or weak nuclear CDC73. (A) Representative stained sections are shown of parathyroid tumors from four patients (patients 5 and 6 harboring germline CDC73 mutations, c.636delT and c.226>T respectively, demonstrate loss of nuclear CDC73; patients 17 and 19 display nuclear CDC73). Immunohistochemical scores are noted (+/− for nuclear CDC73; 0–3 for monoubiquitinated H2B-K120, total H2B and H3-K4me3). (B) Percentage values of CDC73 ‘+’ or ‘-’ tumors exhibiting each immunohistochemical score obtained for monoubiquitinated H2B-K120 are shown. (C) Comparison of the distribution of immunohistochemical scores between groups shows that in tumors where expression of nuclear CDC73 is lost, there are significantly reduced levels of monoubiquitinated H2B-K120 (Mann–Whitney U test, P < 0.01). (D) Percentage values of nuclear CDC73 ‘+’ or ‘-’ tumors exhibiting each immunohistochemical score obtained for H3-K4me3 are shown. (E) The distribution of immunohistochemical scores obtained for H3-K4me3 for the two groups was not significantly different (Mann–Whitney U test, P > 0.05).
histone mRNA 3'-end processing. These histones are the only transcripts known to be deficient in polyadenylation, instead terminating in a conserved stem–loop structure. Down-regulation of CDC73 in vitro leads to aberrant 3' processing of replication-dependent histone transcripts, resulting in more stable transcripts with poly(A) tails (22). An increase of replication-dependent histone transcripts, resulting in missense mutation.

It is possible that the presence of abnormal histone transcripts leads to alterations in chromatin structure that may influence the transcription of cancer-related genes.

Cancer is frequently described as a disease of aberrant transcription, and genetic or epigenetic events in cancer-associated genes may influence a range of co-valent histone modifications that play important roles in transcriptional regulation and the DNA damage response. It is possible that other components of H2B-associated transcriptional or ligase complexes involved in ubiquitination of this key histone may also harbor pathogenic mutations that dysregulate H2B-K120 monoubiquitination and disrupt transcription of cancer-associated genes.

**MATERIALS AND METHODS**

**Cell lines and patient samples**

Human embryonic kidney (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) were routinely cultured as previously described (42). Cell line identity was confirmed using short tandem repeat analysis by CellBank Australia (Westmead, NSW, Australia) using the AmpFl STR Identifiler PCR Amplification Kit (Applied Biosystems, Mulgrave, VIC, Australia). Twenty-one formalin-fixed paraffin-embedded parathyroid tumor samples were obtained from the Department of Anatomical Pathology, Royal North Shore Hospital under an ethics protocol approved by the Northern Sydney Central Coast Area Health Service Human Research Ethics Committee. The CDC73 mutations c.191C>T (L.64P) and c.1247delG have previously been reported by us (26,43). c.636delT and c.226C>T have been reported by others (reviewed in 27).

**Plasmid constructs and site-directed mutagenesis**

Full-length human CDC73 was PCR-amplified from a previously generated construct, pEGFP-C1-HRPT2 (17). CDC73 cDNA was cloned using restriction sites engineered into each primer pair into the yeast expression vector pGBK7 (Clontech) to generate pGBK7-CDC73, or the mammalian expression vector pCMV-Myc (Clontech) to create pCMV-Myc-CDC73. Full-length human RNF20 cDNA was PCR-amplified from an adult human kidney cDNA library vector (pACT2) and cloned into the Sall and NotI restriction sites in frame with the HA-tag of the mammalian expression vector pCMV-HA to generate pCMV-HA-RNF20. Full-length human RNF40 cDNA was PCR-amplified from HEK293 cDNA and cloned into the EcoRI and Sall restriction sites of a modified version of pCMV-HA that contained the pEGFP-C1 multiple cloning site to generate pCMV-HA-RNF40. CDC73 mutants were generated using the QuikChange II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) as previously described (17). All constructs

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*Loss of nuclear CDC73 is indicated by ‘×’, wild-type expression pattern of CDC73 is indicated by ‘✓’ and weak staining is noted for sample 8 that harbored a missense mutation.

*Patients were from the same family; ‘n.a.’ indicates not analyzed; ‘wild-type’ indicates a germline CDC73 mutation was not identified, the probable cause of loss of nuclear CDC73 being somatic mutation.
were verified by sequence analysis (Supamac, The University of Sydney, Australia). All primers used for cloning and mutagenesis are listed in Supplementary Material, Tables S1 and S2.

**CDC73 Y2H screen**

The Matchmaker 3 System (Clontech) was used to perform a high-stringency Y2H screen of full-length CDC73. Reporter gene activation was not observed upon transformation of the yeast strain AH109 with pGBK7-CDC73, suggesting that CDC73 alone did not induce Gal4-mediated transcription in this yeast strain. pGBK7-CDC73 and an adult human kidney cDNA library (Clontech) generated in the Gal4 activation domain yeast expression vector pACT2 were simultaneously transformed into AH109 cells and plated on high-stringency dropout media (SD-Trp/-Leu/-His/-Ade/ X-α-Gal). Plasmid DNA was isolated from resultant colonies and retransformed into the JM109 Escherichia coli bacterial strain. Selection was performed on ampicillin to isolate colonies containing the pACT2 vector only. DNA extracted from bacterial clones was sequenced using a T7 primer (Supamac).

In order to map the RNF20- and RNF40-binding sites within CDC73 using the Y2H assay, full-length RNF20 and the C-terminus of RNF40 in pACT2 (both obtained from the Y2H screen above, Supplementary Material, Fig. S1) were individually transformed with wild-type or mutant pGBK7-CDC73 (Supplementary Material, Table S2) into AH109 yeast cells. Transformations were plated on high-stringency dropout media (SD-/Trp-/Leu-/His/-Ade/X-α-Gal) and resultant colonies restreaked onto the same medium. Growth of multiple colonies on dropout media was considered positive for interaction.

**Co-immunoprecipitation studies**

For co-immunoprecipitation of endogenously expressed proteins, T175 culture dishes containing confluent monolayers of HEK293 cells were used. For co-immunoprecipitation of overexpressed CDC73, RNF20 and RNF40, HEK293 cells were seeded (3 × 10^5 cells/well) in six-well culture dishes and 24 h later co-transfected with 1 μg of pCMV-HA-RNF20 or pCMV-HA-RNF40 and pCMV-Myc-CDC73. Forty-eight hours later, cells were trypsinized, pelleted and resuspended in lysis buffer (300 μl of 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% TX100, 10 mM β-mercaptoethanol) was added to beads and incubated at 95°C for 5 min. Extracts were removed from beads and separated on SDS–PAGE and analyzed by western blots using HA and Myc-tagged antibodies as previously described (17). Anti-CDC73 and anti-RNF20 antibodies were purchased from Bethyl Laboratories (Montgomery, TX, USA), and the anti-RNF40 antibody was from Abcam (Sapphire Bioscience Pty Ltd, Waterloo, NSW, Australia).

**siRNA-induced down-regulation of CDC73 or RNF20 and subsequent analysis of monoubiquitinated H2B-K120**

HEK293 cells (3 × 10^5 cells/well) were seeded into six-well culture dishes and 24 h later transfected with 100 nm siRNA directed against CDC73 (target sequence: AAAGCGTCAA CATCGGCAAGTA, or a second target sequence: AAAC AAGGTTGTAACGAGAA) or RNF20 (target sequence: ACGGGTGAATTCCAAAGGTTA) using 12 μl of HiPerfect (Qiagen, Doncaster, VIC, Australia) according to the manufacturer's instructions. A non-silencing negative control siRNA, AllStars Negative Control (Qiagen), was included in all experiments. After 48 h, cells were washed with cold PBS and lysed (200 μl of 20 mM NaPO4, 0.5 mM NaCl, 20 mM imidazole, 8 M urea, 0.5% Triton, 20 mM Tris, pH 8, 0.5 mM DTT, 0.5 mM iodacetamide). Cells were removed from dishes by scraping and protein-loading buffer was added (100 μl of 6% w/v SDS, 40% w/v sucrose, 20 mM Tris, pH 6.8, 0.15% w/v bromophenol blue). Extracts were sonicated (2 × 30 s) and incubated at 95°C for 5 min before being separated on SDS–PAGE and analyzed by immunoblotting using an anti-monoubiquitinated H2B-K120 antibody (Medimabs, Montreal, Canada) as previously described (2).

**Immunohistochemistry**

Immunohistochemistry for total H2B, monoubiquitinated H2B-K120 and H3-K4me3 was performed on formalin-fixed paraffin-embedded tissue sectioned at 4 μm onto positively charged slides (Superfrost plus, Menzel-Glaser, Germany). For total H2B, a mouse monoclonal antibody (Abcam Cat 624484, Clone 52484) was used at a dilution of 1 in 500. For H2B-K120, a mouse monoclonal antibody (Medimabs, Montreal, Canada, Cat MM-0029) was used at a dilution of 1:200. For H3-K4me3, a rabbit monoclonal antibody (Cell Signaling Technology) was used at a dilution of 1 in 200. All slides were processed with an automated staining system, the Leica Microsystems BondmaX autostainer (Leica Microsystems, Mount Waverley, VIC, Australia), used according to the manufacturer’s protocol and with the manufacturer’s retrieval solutions. For total H2B and H3-K4me3, heat-induced epitope retrieval was performed at 97°C for 30 min in the manufacturer’s alkaline retrieval solution ER2 (VBS part no.: AR9640). For H2B-K120, heat-induced epitope retrieval was performed at 97°C for 30 min in the manufacturer’s acidic retrieval solution ER1 (VBS part no. AR9961). A biotin-free detection system was employed (VBS part no. DS 9713). Scoring was performed...
in a blinded fashion with no knowledge of mutational status by the pathologist A.J.G., and verified, also in a blinded fashion, by M.A.H., K.-A.D. and D.J.M.

**Statistical analysis**
Data analyses were performed using the SPSS software 19.0 (SPSS Australasia Pty Ltd, Chatswood, NSW, Australia). Data from immunoblotting are expressed as mean ± SEM from at least three independent experiments. Statistical significance for immunoblot analysis was determined by the one sample t-test. Statistical significance for immunohistochemical analysis was determined by the Mann–Whitney U test. For all cases, \( P < 0.05 \) was considered statistically significant.

**SUPPLEMENTARY MATERIAL**
Supplementary Material is available at HMG online.

**ACKNOWLEDGEMENTS**
D.J.M. is the recipient of an ARC Future Fellowship, CINSW Career Development Fellowship and National Health and Medical Research Council Senior Research Fellowship (Honorary).

**Conflict of Interest statement.** The authors have no potential conflicts of interest to disclose.

**FUNDING**
This work was supported by the Australian Research Council (ARC) (DP1093195), the Cure Cancer Australia Foundation in partnership with Cancer Australia, and the Cancer Institute New South Wales (CINSW), Australia.

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