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## And-1 is required for the stability of histone acetyltransferase Gcn5

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### Abstract

Histone acetyltransferases (HATs) play a central role in the modification of chromatin as well as in pathogenesis of a broad set of diseases including cancers. Gcn5 is the first identified transcription-related histone acetyltransferase (HAT) that has been implicated in the regulation of diverse cellular functions. However, how Gcn5 proteins are regulated remains largely unknown. Here we show that And-1 (a HMG domain-containing protein) has remarkable capability to regulate the stability of Gcn5 proteins and thereby histone H3 acetylation. We find that And-1 forms a complex with both histone H3 and Gcn5. Downregulation of And-1 results in Gcn5 degradation, leading to the reduction of H3K9 and H3K56 acetylation. And-1 overexpression stabilizes Gcn5 through protein-protein interactions *in vivo*. Furthermore, And-1 expression is increased in cancer cells in a manner correlating with increased Gcn5 and H3K9Ac and H3K56Ac. Thus, our data reveal not only a functional link between Gcn5 and And-1 that is essential to regulate Gcn5 protein stability and histone H3 acetylation, but also a potential role of And-1 in cancer.

### Keywords

Ctf4/And-1; Gcn5; DNA replication; histone acetylation

### Introduction

Histone acetyltransferases (HATs) play a central role in the modification of chromatin to create a favorable environment for many important cellular processes (Shahbazian & Grunstein, 2007). Gcn5 (general control non-derepressible 5) is the first identified transcription-related HAT that has been implicated in the regulation of transcription as a subunit of several transcriptional coactivator complexes (Brownell et al., 1996; Kuo et al.,

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1996). In metazoans, Gcn5 exists in at least two HAT complexes including SAGA (Spt–Ada–Gcn5 acetyltransferase) and ATAC (Ada Two A containing) (Lee & Workman, 2007; Nagy & Tora, 2007). In addition to transcription regulation, Gcn5 is involved in diverse cellular processes such as DNA repair (Brand et al., 2001; Tamburini & Tyler, 2005), telomere maintenance (Atanassov et al., 2009), cell cycle progression (Orpinell et al., 2010; Paolinelli et al., 2009), and nucleosome assembly (Burgess et al., 2010). As a key catalytic component of the larger SAGA and ATAC complexes, Gcn5 preferentially acetylates lysines 9, 14, 27, and 56 of histone H3, and lysines 8 and 16 of H4 (Brownell et al., 1996; Burgess et al., 2010; Kuo et al., 1996; Tjeertes et al., 2009).

Although both human and yeast Gcn5 possess HAT activity, human Gcn5 is structurally different from yeast Gcn5 by possessing a PCAF homology domain at its N-terminal region (Nagy & Tora, 2007). This difference might explain why human Gcn5 can not complement the growth defect of Gcn5 depleted yeast cells (Wang et al., 1997). Gcn5 exhibits conserved functions on histone H3 acetylation, and yet Gcn5 acetylates H3K56 in mammalian cells but not in yeast cells (Burgess & Zhang, 2010; Tjeertes et al., 2009). Increased H3K56 acetylation and Gcn5 expression have been seen in tumors, indicating a potential role of Gcn5 in tumorigenesis (Armas-Pineda et al., 2007; Das C, 2009). Indeed, accumulated evidence suggests that Gcn5 is involved in the regulation of oncogenic genes. For instance, Gcn5 interacts with both Myc and E2F transcription factors and acts as a co-factor to regulate their target genes (Lang et al., 2001; Liu et al., 2003). More recent evidence shows that Gcn5 is required for the activation of PI3K/Akt survival pathway (Kikuchi et al., 2011). The lysine acetylation specificity of Gcn5 is regulated by its association with other regulatory subunits that are part of the SAGA and ADA complexes (Grant et al., 1999). However, how Gcn5 proteins are regulated remains largely unknown.

High mobility group (HMG) containing proteins represent the largest group of non-histone components that decompact the higher-order chromatin structure to facilitate the formation of nucleoprotein complexes with histones and transcription factors (Bustin, 1999; Grosschedl et al., 1994). And-1 (acidic nucleoplasmic DNA-binding protein) is a HMG domain-containing protein. The homolog of And-1 in *S. cerevisiae* is Ctf4 that was originally identified in a genetic screen for mutants affecting chromosome transmission fidelity (Kouprina, 1992), and later was shown to be required for sister chromatid cohesion (Hanna et al., 2001; Mayer et al., 2004; Petronczki et al., 2004). And-1 homolog in *S. pombe*, *mcl1*, is essential for viability, maintenance of genome integrity, DNA damage repair, and regulation of telomere replication (Tsutsui et al., 2005; Williams & McIntosh, 2002). We previously have shown that And-1 is required for chromatin loading of DNA polymerase alpha p180 in a *Xenopus* system and for the stability of pol alpha p180 in human cells (Zhu et al., 2007). Recent studies from other groups further indicate that And-1/Ctf4 is involved in the formation of Cdc45-Mcm2-GINS complex, stimulates the polymerase activities of DNA polymerases alpha and epsilon, and couples MCM2-7 to DNA polymerase alpha (Bermudez et al., 2009; Gambus et al., 2009; Im et al., 2009).

Using immunofluorescence, we and others have observed And-1 proteins in nuclei throughout interphase in formaldehyde-fixed cells, and co-localization of And-1 with Mcm10 or RPA were seen only when cells were pre-treated with extraction buffer to remove

non-detergent resistant And-1 proteins (Yoshizawa-Sugata & Masai, 2009; Zhu et al., 2007). This cellular localization pattern indicates that And-1 may play additional roles in the regulation of chromatin functions. Supporting this idea, CTF4 was genetically defined as part of the H3K56 acetylation pathway in yeast (Collins et al., 2007), and deletion of CTF4 suppresses the phenotype of cells lacking Hst3p and Hst4p, two histone deacetylases against acetylation of H3K56 (Celic et al., 2008). Nothing is known on the mechanism by which Ctf4p regulates H3K56 acetylation in yeast cells.

Here we report that And-1 acts as a Gcn5 co-factor to maintain its stability. We found that And-1 forms a complex with both histone H3 and Gcn5 and has the remarkable capability to regulate the stability of Gcn5. Loss of And-1 substantially reduces Gcn5 protein levels without affecting its mRNA expression, resulting in the decrease of H3K9 and H3K56 acetylation. And-1 overexpression stabilizes Gcn5 proteins through protein-protein interactions. Furthermore, we found that And-1 expression is increased in both tumors and tumorigenic cell lines in a manner correlating with increased levels of Gcn5 and H3K9Ac and H3K56Ac. We therefore propose that there is a functional link between Gcn5 and And-1 that regulates Gcn5 protein and histone H3 acetylation, and that And-1 could play an important role in cancer development by regulating Gcn5 and histone H3 acetylation.

## Results

### And-1 interacts with both histone H3 and Gcn5

To investigate the role of And-1 in the regulation of H3K56 acetylation in mammalian cells, we first examined whether And-1 forms a complex with histone H3, and Gcn5, as well as p300/CBP, two HATs involved in H3K56 acetylation in human cells (Das C, 2009; Tjeertes et al., 2009). Strikingly, Flag-And-1 expressed in 293T cells co-precipitated with H3. This interaction was detected in full-length And-1 and truncation mutant And-1 (330–1129), but not in other And-1 mutants [And-1 (1–336), and And-1 (984–1129)] (Fig. 1A, 1B), suggesting that the SepB domain is critical for interaction. Consistent with these results, similar interaction patterns were observed by *in vitro* co-immunoprecipitation experiments (Fig. 1C). Importantly, immunoprecipitation of endogenous And-1 resulted in the co-precipitation of endogenous Gcn5 and *vice versa* (Fig. 1D). Unlike its interaction with H3, only full-length And-1 interacts with Gcn5 (Fig. 1E), suggesting that And-1 utilizes distinct regions to bind Gcn5 and H3. The interaction between And-1 and Gcn5 was also detected by *in vitro* co-immunoprecipitation experiments (Fig. 1F). Both p300 and CBP were not detected in And-1 precipitates (data not shown). Thus, And-1 forms a complex with both H3 and Gcn5.

### Cell cycle regulation of And-1 and Gcn5

To further study the function of interactions between And-1 and Gcn5, we analyzed how And-1 and Gcn5 associate with chromatin in the cell cycle using immunofluorescence. Both And-1 and Gcn5 bound to chromatin at telophase and remained associated with chromatin until prometaphase, at which chromosomes started to condense (Fig. 2A) (Bermudez et al.). Consistent with the fact that And-1 interacts with Gcn5 by co-immunoprecipitation experiments (Fig. 1D), the majority of Gcn5 co-localized with And-1 throughout the cell

cycle except in early telophase, at which And-1 re-associated with chromatin followed by Gcn5 (Fig. 2A). Notably, both And-1 and Gcn5 associated with chromatin at a specific cell cycle period during which chromatin exists in a decompacted state with high levels of histone acetylation (Shahbazian & Grunstein, 2007). This chromatin association pattern suggests that And-1 may play a positive role in histone acetylation in collaboration with Gcn5.

We next examined the cell cycle regulation of And-1 and Gcn5 proteins. To this end, we synchronized HCT116 cells in early S phase with a double-thymidine block, and released them back into the cell cycle. The cell cycle progression was monitored by FACS analysis as shown in Figure 2C. Although their chromatin association is cell cycle regulated (Fig. 2A), both And-1 and Gcn5 protein levels were not variable throughout the cell cycle (Fig. 2B). In yeast cells, acetylation of histone H3K56 is cell cycle regulated with a higher level H3K56Ac at S phase (Masumoto et al., 2005). However, the variation of acetylation of H3K9 and H3K56 was minimal throughout cell cycle in human cells, which is consistent from a recent observation (Tjeertes et al., 2009).

### And-1 depletion reduces Gcn5 expression

Given that And-1 forms a complex with H3 and Gcn5, we reasoned that And-1, like many other HMG proteins, may facilitate the assembly of nucleoprotein complexes including Gcn5 containing complexes. To test this idea, And-1 was depleted by siRNA in three types of human cells, HCT116, HeLa, and U2OS. Intriguingly, in all three tested cell types, Gcn5 proteins were substantially reduced after And-1 depletion by two independent siRNAs (Fig. 3A, data not shown). In agreement, exogenous Gcn5 proteins were also reduced in And-1 depleted cells (Fig. 3B). Gcn5 is part of multiple subunit complexes such as SAGA and ATAC (Cavusoglu et al., 2003; Lee & Workman, 2007; Wang et al., 2008). Hence, we asked whether And-1 also regulates other components of these complexes. Four components TAFII32P, USP22, SPT3, and ADA2 were examined and none of their protein levels was altered after And-1 depletion (Fig. 3C).

To rule out siRNA off-target effects, expression of exogenous And-1 from cDNA rescued Gcn5 levels in U2OS cells transfected with And-1 siRNA targeting 3' UTR mRNA sequence (Fig. 3D). Thus, decreased Gcn5 protein levels are specifically due to the loss of And-1 expression. Taken together, our results revealed that And-1 is required for Gcn5 expression.

### And-1 specifically stabilizes Gcn5 proteins

Decreased Gcn5 protein levels might reflect decreased transcription of Gcn5 gene upon And-1 loss. To test this possibility, we examined Gcn5 mRNA levels by quantitative reverse transcriptase PCR in And-1 depleted cells transfected with two independent siRNAs. We were unable to detect any reduction of Gcn5 mRNA levels in And-1 depleted cells by two independent siRNAs (Fig. 4A), suggesting that And-1 loss affects Gcn5 expression at a posttranscriptional step. To more precisely explore how And-1 stabilizes Gcn5, we measured the half-life of Gcn5 proteins. In control siRNA transfected cells, the half-life of

Gcn5 was 5 hours, while in cells depleted of And-1, Gcn5 half-life was reduced to ~2 hours (Fig. 4C).

To determine when Gcn5 is degraded during cell cycle in the absence of And-1, we examined the localization of Gcn5 proteins in the cell cycle by immunofluorescence. Gcn5 proteins were significantly reduced in And-1 depleted cells from interphase to metaphase (Fig. 4B). Since And-1 depletion causes a cell cycle arrest before metaphase (data not shown), we could not examine Gcn5 protein in anaphase and telophase cells after And-1 depletion. Given that And-1 co-localizes with Gcn5 in the cell cycle except early telophase (Fig. 2A), these data suggest that the And-1 is important for Gcn5 protein stability during the cell cycle.

### **Overexpression of And-1 stabilizes Gcn5 via protein-protein interactions**

Depletion of And-1 reduces Gcn5 protein levels, we therefore asked whether overexpression of And-1 could stabilize Gcn5 proteins. To test this idea, we overexpressed Flag-And-1 in U2OS cells. Strikingly, overexpression of And-1 increased endogenous Gcn5 protein levels (Fig. 5A). We next co-expressed Flag-Gcn5 together with titrated Flag-And-1 in U2OS or 293T cells. Consistently, exogenous Gcn5 protein levels were also increased in a manner correlating with increased Flag-And-1 expression (Fig. 5B, data not shown). Taken together, these results reveal that And-1 has the remarkable capability to specifically regulate the stability of Gcn5.

And-1 directly interacts with Gcn5 (Fig. 1F), suggesting that the interaction between And-1 and Gcn5 might be required to stabilize Gcn5. To test this hypothesis, we co-expressed Gcn5 together with Full-length And-1, or its mutants [And-1(1–336), And-1(330–1129), And-1(984–1129)]. Strikingly, expression of full-length And-1 but not its mutants was able to stabilize Flag-Gcn5 (Fig. 5C). Given that Gcn5 only interacts with full-length And-1, these results suggest that protein-protein interactions between And-1 and Gcn5 are critical for And-1 to stabilize Gcn5 proteins.

### **Downregulation of And-1 reduces the acetylation of histone H3 lysine 56 and 9**

In human cells, Gcn5 is required for acetylation of H3K9 and H3K56 (Tjeertes et al., 2009). Therefore, we tested whether And-1 depletion compromises the acetylation of H3K9 and H3K56. Indeed, both acetylation of H3K9 and H3K56 were reduced after And-1 depletion in HCT116 cells (Fig. 6A), as well as in HeLa and U2OS cells (data not shown). We previously reported that And-1 depletion causes a G2/M phase cell cycle arrest (Zhu et al., 2007), raising the possibility that reduced Gcn5 may reflect cell cycle distribution rather than a direct effect from depletion of And-1. However, we observed that acetylation of H3K9 and H3K56 were not variable throughout the cell cycle (Fig. 2B) (Tjeertes et al., 2009), indicating that the decrease of H3K9Ac and H3K56Ac is not due to cell cycle effects.

Gcn5 depletion reduces level of H3 acetylation at Cdk1 and Cyclin B1 promoter regions (Tjeertes et al., 2009), we hypothesized that And-1 downregulation might decrease the levels of H3 acetylation at Cdk1 and Cyclin B1 promoter regions. To test this idea, we performed a chromatin immunoprecipitation assay in cells treated with siRNAs against control or And-1. Although H3 proteins at these promoter regions were not reduced (Figs.6B & 6C), the

acetylation of H3K9 at these promoter regions was substantially reduced in And-1 depleted cells (Figs. 6B & 6C). Thus, we conclude that And-1 is involved in Gcn5-mediated acetylation of H3K9 and H3K56.

### **And-1 expression is increased in multiple tumors**

Increased Gcn5 protein levels have been seen in tumor cells (Armas-Pineda et al., 2007). Given that And-1 is required for the maintenance of Gcn5 stability, we hypothesized that And-1 protein levels are increased in tumor cells to maintain higher Gcn5 protein levels. To test this idea, we examined And-1 expression in both tumorigenic cells and tumors. And-1 expression was measured by immunohistochemistry in patient specimens and found to be significantly increased in colorectal tumors compared to adjacent normal tissue (Figs. 7A & 7B). Consistently, And-1 protein levels are also increased in cultured melanoma cells (A375) and lung carcinoma cells (H1299) compared to their normal counterpart skin fibroblast cells (D-1) and lung fibroblast cells (WI38) as indicated by western blot (Fig. 7C). Strikingly, Gcn5, H3K9Ac and H3K56Ac were significantly increased in A375 and H1299 cells compared to D-1 and WI38 cells (Fig. 7C). Thus, And-1 expression is increased in cancer cells in a manner correlating with increased Gcn5 and H3K9/56Ac. These data suggest that And-1 may play an important role in tumor cell growth *via* regulating Gcn5 and histone H3 acetylation.

## **Discussion**

### **The biological consequences of a functional link between And-1 and Gcn5 in cancer**

Our data reveal, for the first time, an unexpected functional link between And-1 and Gcn5 to regulate the stability of Gcn5 proteins and H3K9/56Ac. And-1 is involved in H3K9/56Ac by regulating Gcn5 stability via protein-protein interactions. And-1 co-localizes with Gcn5 during most of the cell cycle (Fig. 2A), at which And-1 depletion reduces Gcn5 protein levels. Thus, it appears that And-1 protects Gcn5 from degradation *via* protein-protein interactions during the cell cycle.

What is the biological function of And-1 in the stabilization of Gcn5? Downregulation of And-1 significantly reduces the Gcn5 protein levels, resulting in the decrease in acetylation of H3K6 and H3K56. Alteration of global levels of histone acetylation are associated with cancer (Seligson and Grunstein, 2005). It is likely that And-1 stabilizes Gcn5 to maintain a physiological level of H3K9/56Ac for cell proliferation in cancer cells. Indeed, several lines of evidence from this study and studies from other groups support this notion. First, And-1 is required for H3K9/56Ac by stabilizing Gcn5 in multiple cancer cells (Figs. 3–6). Second, increased H3K56Ac and Gcn5 expression have been seen in multiple types of cancer (Armas-Pineda et al., 2007; Das C, 2009) (Fig. 7). Third, And-1 expression is increased in both tumorigenic cells and tumors, correlating with increased Gcn5 and H3K9/56Ac (Fig. 7). What role does And-1 play in cancer cells? Gcn5 has been linked to oncogenic gene regulation as evidenced by involvement in the oncogene c-Myc and E2F-mediated gene transcription (Lang et al., 2001; Liu et al., 2003). Moreover, Gcn5 is required for the activation of PI3K/Akt cell survival pathway (Kikuchi et al., 2011). Thus, it is likely that And-1 maintains cancer cell proliferation and survival by regulating Gcn5 stability, and yet

we could not rule out the possibility that And-1 plays a direct role. The exact functional roles of And-1 in cancer remain to be further determined.

We previously reported that pol alpha p180 is also degraded in the absence of And-1. Thus, And-1 seems to be a protein stabilizer. How does And-1 maintain the stability of these proteins? And-1 is a HMG box-containing protein. HMG proteins represent the largest group of non-histone components that decompact the higher-order chromatin structure to facilitate the formation of nucleoprotein complexes on the chromosome (Bustin, 1999; Grosschedl et al., 1994). Thus, And-1 may facilitate the assembly of protein complexes involved in DNA replication or chromatin modification. To support this notion, And-1 was recently found to be required for assembly of Cdc45-Mcm2-7-GINS complexes on the chromatin for DNA replication in human cells (Im et al., 2009). Loss of And-1 may cause the disassembly of protein complexes, resulting in release of some subunits (e.g. Gcn5 and pol alpha p180) that may be targeted for degradation by ubiquitination pathway. Since both pol alpha p180 and Gcn5 are degraded in And-1 depleted human cells, we do not yet know whether And-1 affects Gcn5 and pol alpha p180 before or after their eviction from parental protein complexes. Another possibility is that And-1 stabilizes Gcn5 or pol alpha p180 simply via protein-protein interactions rather than affecting protein complexes. Three lines of evidence support this model. First, And-1 interacts with Gcn5 or pol alpha p180 (Zhu et al., 2007). Second, only full length And-1 that interacts with Gcn5 is able to stabilize Gcn5 and And-1 mutants that do not interact with Gcn5 fail to stabilize Gcn5 proteins (Fig. 4C). Third, And-1 depletion does not cause the degradation of other components of SAGA or ADA complexes, indicating that And-1 may stabilize the proteins that interact with And-1 directly.

This study may explain the mystery in yeast where CTF4 was genetically defined as part of the histone H3K56 acetylation pathway (Celic et al., 2008; Collins et al., 2007). However, we did not observe decreased expression of yeast Gcn5p in CTF4 deletion mutant (Data not shown), suggesting evolutionary differences between human and yeast. In agreement, unlike And-1, Ctf4p does not have a HMG domain, a key domain to regulate assembly of nucleoprotein complexes for chromatin remodeling (Bustin, 1999; Grosschedl et al., 1994). The lack of a HMG domain may explain why Ctf4p is not required for the stability of Gcn5 in yeast.

### **Role of And-1 as a coordinator of DNA replication and histone modification**

Our results reveal that And-1 forms a complex with both H3 and Gcn5, and is involved in histone H3 acetylation by regulating the stability of Gcn5. Thus, And-1 is involved in both DNA replication and histone acetylation. Intriguingly, histone acetylation has been linked to pre-RC assembly and regulation of DNA replication initiation. For example, early-firing origins are typically localized in genomic regions which are actively transcribed and contain hyperacetylated chromatin (Goren et al., 2008; Karnani et al., 2007; Lucas et al., 2007; Zhou et al., 2005). Thus, the physical and functional interactions between And-1 and Gcn5 suggest that And-1 may act as a linker to recruit Gcn5 or other histone acetyltransferases to replication origins for initiation of DNA replication. Supporting this notion, accumulating evidence indicates a possible role of Gcn5 in the initiation of DNA replication. For instance,

Gcn5 physically associates with replication origins (Espinosa et al., 2010), stimulates origin activity (Vogelauer et al., 2002), and promotes H3 association with CAF-1 via H3 acetylation (Burgess & Zhang, 2010). It will be interesting to determine how And-1 cooperates with Gcn5 in DNA replication via regulation of histone acetylation in human cells, a process which is still poorly understood.

## Materials and Methods

### Cells, tissue culture media and FACS analysis

HCT116, HeLa, U2OS, D-1, A375 and 293T cells were grown in DMEM media supplemented with 10% FBS at 37°C in 5% CO<sub>2</sub> supply. Flow cytometry analysis was performed as described previously (Zhu et al., 2004).

### Antibodies and Immunofluorescence

The following antibodies were used: rabbit anti-H3K9Ac (9671), rabbit anti-H3K9Ac (4243), and rabbit anti-H3 (9715) were from Cell Signaling. Mouse anti-tubulin (T6199), anti-flag-M2 antibody, and mouse anti-GAPDH (G9545) were from Sigma. Anti-Gcn5 (sc-20698) (sc-130374), Anti-TAFII32P (Sc-1247),  $\beta$ -Actin, Cyclin A(sc-751), and p300 (sc-585) were from Santa Cruz. USP22 (ab4812), SPT3 (ab64956), CBP (AB2832), and ADA2 (ab57953) were from Abcam. And-1 antibody was raised against a synthetic peptide (SKKQKPLDFSTNQKLSAF) of And-1 (Open Biosystems). Yeast anti-Gcn5 and anti-RTT109 were gifts from Dr. Zhiguo Zhang.

Immunofluorescence was performed as previously described (Zhu et al., 2004).

### Immunohistochemistry

Fresh-frozen colon tumor biopsy specimens and normal-matched controls from patients were cut into 5- $\mu$ m sections and fixed onto silanized glass slides (Dako, Carpinteria, CA) by immersion in 4% formaldehyde for 15 minutes. Slides containing sections were washed by immersion in PBS-T (1X phosphate-buffered saline solution with 0.1% Tween-20) for five minutes each. Sections were permeabilized by covering each slice with a droplet of 0.1% Triton-X100 and incubated in a covered humidity box for 10 minutes at RT; followed by washing twice by immersion in PBS-T for five minutes. The sections were covered with droplets of blocking buffer (5% BSA in PBS) and incubated in a covered humidity box for 1 hr at RT to block non-specific antibody binding. Rabbit anti-human And1 primary antibody was diluted 1:500 in blocking buffer and applied to sections overnight at 4°C. Sections were washed twice in PBS-T, and fluorescent staining was detected using goat anti-rabbit IgG conjugated with Alexa 488 (Invitrogen, Carlsbad, CA), diluted 1:500 in PBS and incubated for 45 minutes at RT in the dark. Sections were subsequently washed three times with PBS-T, twice with PBS, and once with distilled water; followed by mounting using Prolong Gold Anti-fade reagent with DAPI (Invitrogen, Carlsbad, CA). Images were acquired using a Zeiss LSM510 confocal imaging system with 60 $\times$  1.4 differential interference contrast apochromat objective.



Identical acquisition settings were used for each pair of colon cancer and normal tissue samples. And1 expression was determined by normalizing the total intensity of the And1 staining in a field to the total DAPI staining in the same field. Results from 5 pairs of cancer and control specimens were analyzed by paired t-test ( $p < 0.05$ ).

### Plasmids

Flag-And-1(full), Flag-And-1 mutants (1–336), (330–1129), and (984–1129) were constructed as previously described (Zhu et al., 2007). Flag-Gcn5 plasmid was a gift from Dr. Ezra Burstein.

### Immunoprecipitation

The *in vivo* immunoprecipitations were performed as follows: Cells were lysed in lysis buffer (25 mM HEPES-KOH at pH 7.6, 150 mM KAc, 5 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub> EGTA, 10% glycerol, 0.1% Nonidet P-40, protease inhibitor, supplemented with 300 µg/ml Ethidium bromide, and 15kU/mL DNase I) for 20 min on ice, followed by sonication. After centrifugation, the resulting supernatants were mixed with antibody or pre-bleed for immunoprecipitation overnight (two hours for Flag-tagged protein IP), followed by incubation with protein G/A beads (Santa Cruz) for 1 h. Beads were then washed three times with lysis buffer. Associated proteins were eluted by incubating beads with SDS loading buffer for immunoblotting.

For *in vitro* immunoprecipitation, cells were lysed and immunoprecipitated as described above. Washed beads were incubated with histone core proteins (Active Motif) in lysis buffer for 1 hour at 4°C. Beads were then washed three times with lysis buffer. Associated proteins were eluted for immunoblotting as described above.

### Western blotting and histone isolation

To make total protein, cells were lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0), followed by sonication. Histone extraction was performed as described previously (Shechter et al., 2007).

### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP assay kit from Millipore following the supplied protocol. Immunoprecipitations were performed using anti-H3, anti-acetyl-H3K9, or control IgG antibodies. PCR was performed with the primers designed from the sequences of the Cyclin-B1 and Cdk1 promoters as previously described (Tjeertes et al., 2009).

### Real-time PCR

Total RNA was isolated with an RNeasy kit (Qiagen) from HCT116 subconfluent cell cultures according to the manufacturer's instruction. An on-column DNase digestion step was performed to remove DNA contamination. cDNA was generated from 200 ng of total RNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed using TaqMan gene

expression assays and TaqMan Fast Universal PCR Master Mix kits (Applied Biosystems) on Applied Biosystems 7500 Real-time PCR detection system. TaqMan GAPDH detection reagents (Applied Biosystems) were used as an internal control for quantitation. Quantitative real-time PCR was performed at 95 °C for 20 s followed by 40 cycles of denaturing at 95 °C for 3 s and annealing/extending at 60 °C for 30 s. Real-time PCR data were analyzed by Ct method with 7500 Fast System SDS software.

### RNA interference

siRNA oligonucleotides And-1-1, And-1-2 were as described previously (Zhu et al., 2007). The sequences for siRNA oligonucleotides targeting And-1 3'UTR are: GCAUGUACCCUAAGAAUAA, and GCAAAGUUAUGGAAAGUAU. siRNA transfections were performed with 100 nM siRNA oligonucleotide duplexes using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions.

### Protein half-life measurement

Cycloheximide (100µg/ml) was added to G12 or And-1 siRNA transfected HCT116 cells 40 hours after siRNA transfection. Cells were harvested at indicated time points after addition of cycloheximide for immunoblotting.

### Acknowledgements

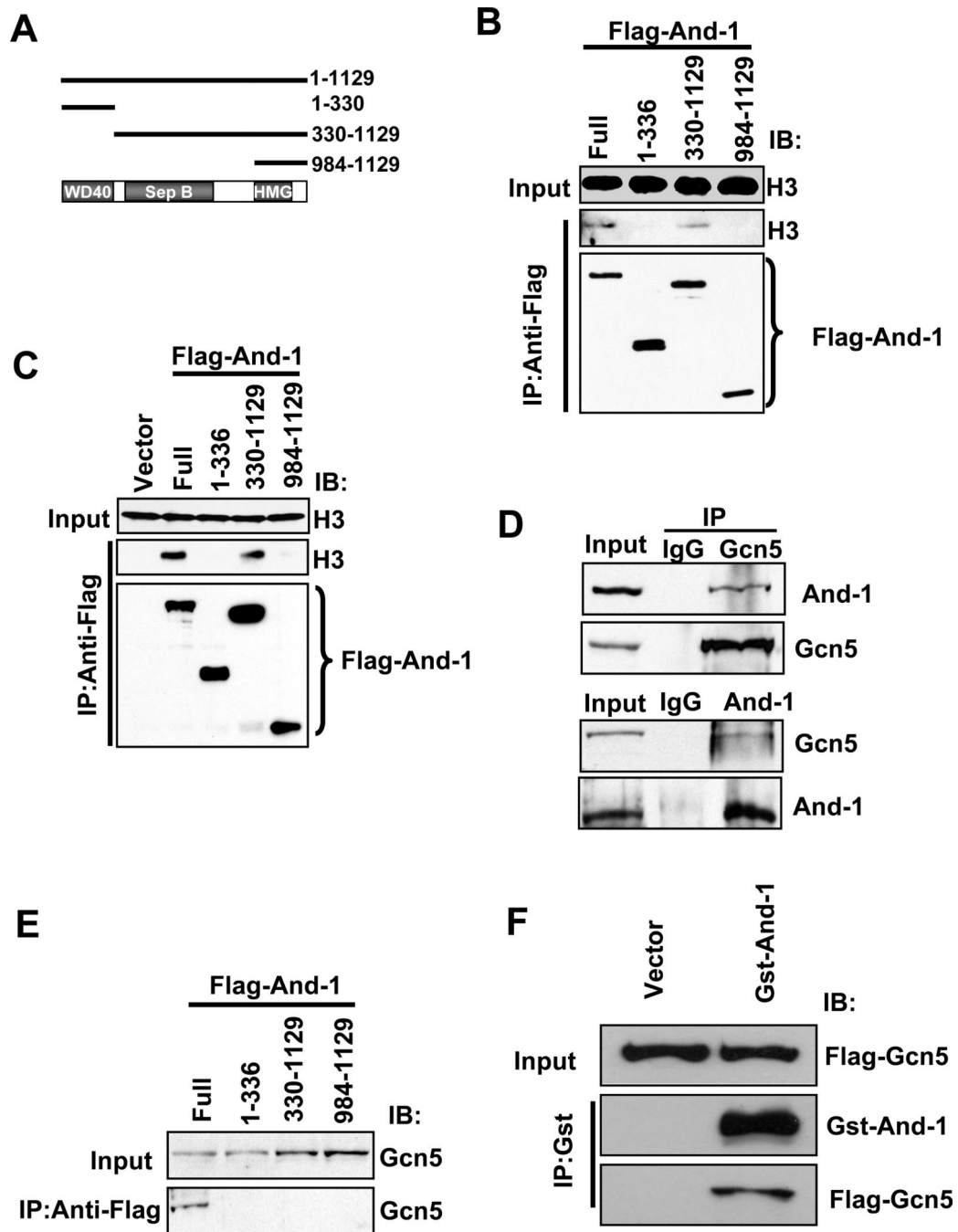
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**Figure 1. And-1 is complexed to histone H3 and Gcn5**

(A) Schematic of the And-1 protein domains and deletions used for protein interactions in Fig. 1. (B) And-1 interacts with histone H3 *in vivo*. 293T cells transfected with indicated Flag-And-1 mutants were lysed and immunoprecipitated with anti-Flag, resolved on SDS-PAGE, and immunoblotted with indicated antibodies. (C) And-1 interacts with histone H3 *in vitro*. Flag tagged full length And-1 as well as its mutants was expressed in 293T cells and immunopurified Flag-And-1 was mixed with histone core particles. After incubation and extensive washing, Flag-And-1 immunoprecipitates were resolved on SDS-PAGE, and

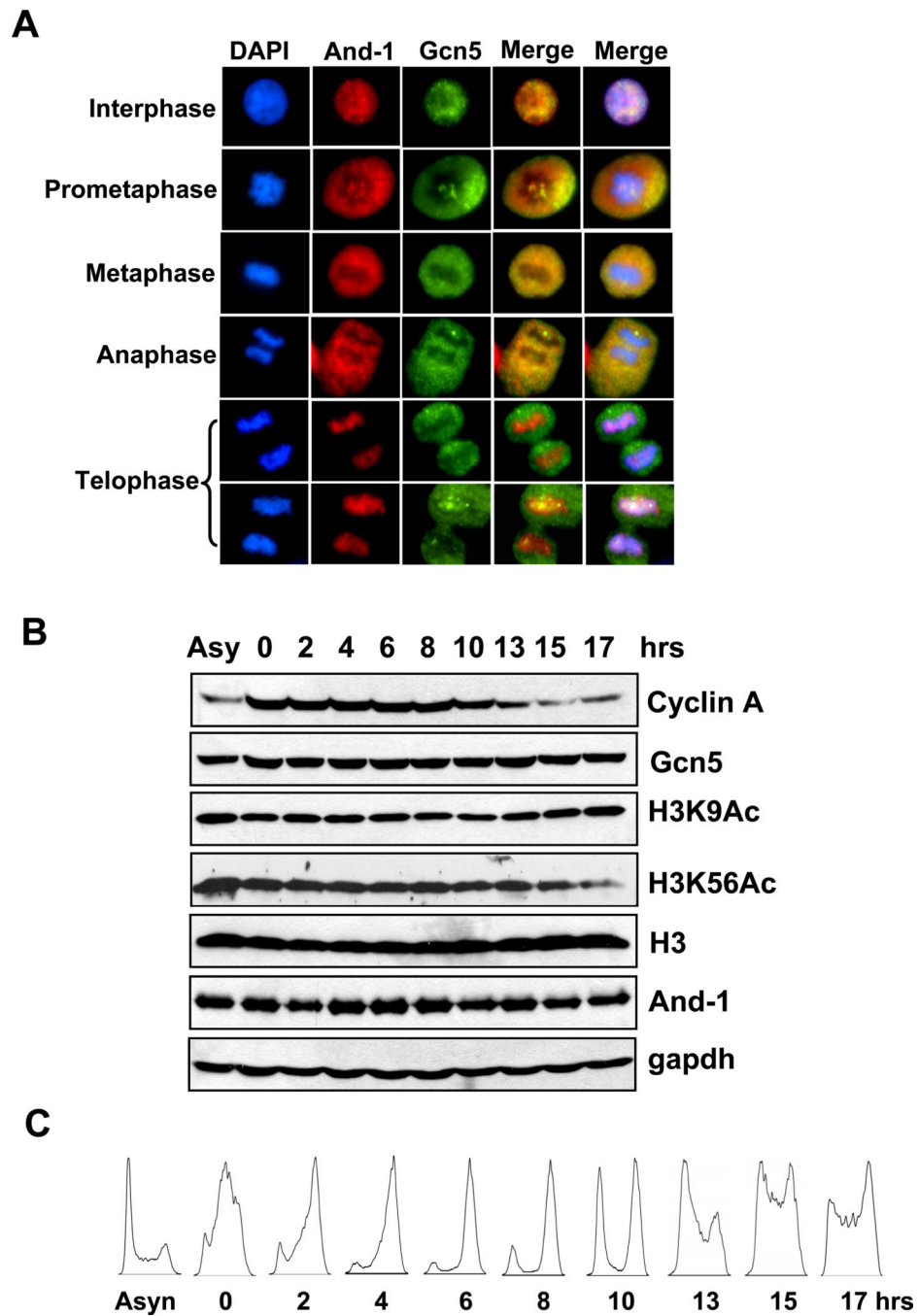
immunoblotted with indicated antibodies. (D) Gcn5 interacts with And-1. HCT116 cell lysates immunoprecipitated with IgG, anti-And-1, or anti-Gcn5 and immunoblotted with anti-And-1 or anti-Gcn5 as indicated. (E) Gcn5 interacts with full length And-1. 293T cells transfected with Flag-And-1 and treated as in (B). (F) Gcn5 interacts with And-1 *in vitro*. Immunopurified Gst-And-1 and Flag-Gcn5 from 293T cells were mixed and incubated for one hour. After washing, Gst-And-1 precipitates were then resolved on SDS-PAGE, and immunoblotted with indicated antibodies.

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**Figure 2. Cell cycle localization of And-1 and Gcn5**

(A) Cell cycle immunofluorescence image of HCT116 cells showing that the majority of Gcn5 co-localizes with And-1 in the cell cycle except in telophase during which And-1 re-associates with chromatin earlier than Gcn5. (B) Cell cycle analysis of Gcn5, acetylation of histone H3 and And-1 proteins. HCT116 cells were synchronized at S phase by a double thymidine procedure and subsequently released into the cell cycle. Samples were taken at indicated time points after release and subjected to western blot analyses of whole cell

extracts and probed with indicated antibodies. Asy, Asynchronous cells. (C) FACS analysis of cells harvested as in (B).

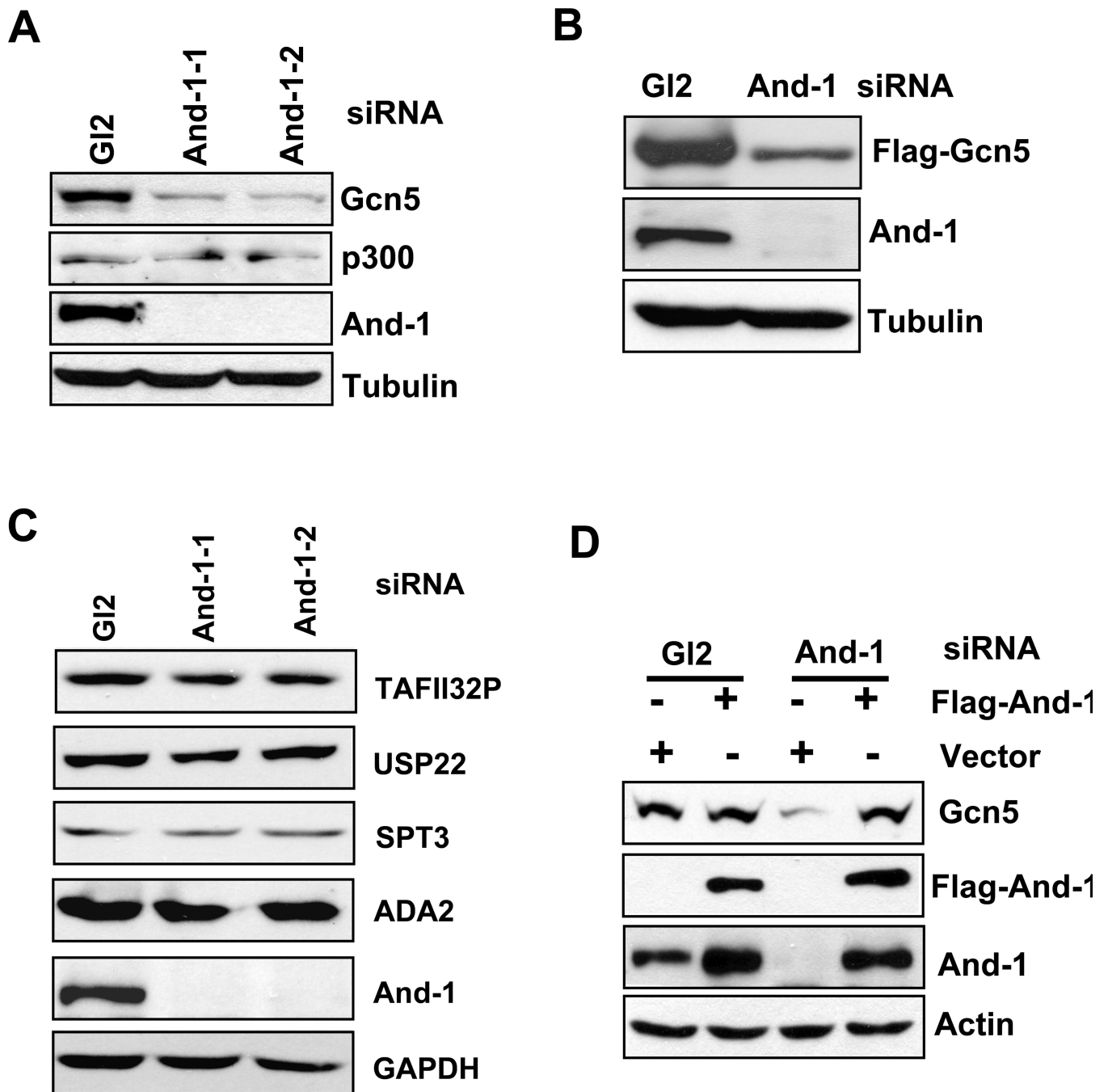
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### Figure 3. And-1 depletion reduces Gcn5 expression

(A) And-1 downregulation decreased endogenous Gcn5 expression. HCT116 cells transfected with siRNA control GI2 or two independent siRNAs were harvested, resolved on SDS-PAGE, and immunoblotted with indicated antibodies. (B) And-1 downregulation decreased exogenous Gcn5 protein levels. Flag-Gcn5 was co-transfected with siRNA control GI2 or And-1 in 293T cells. Cells were harvested 48 hours after transfection, resolved on SDS-PAGE, and immunoblotted with indicated antibodies. (C) Downregulation of And-1 does not affect expression of other components of hSAGA and ATAC. HCT116 cells treated

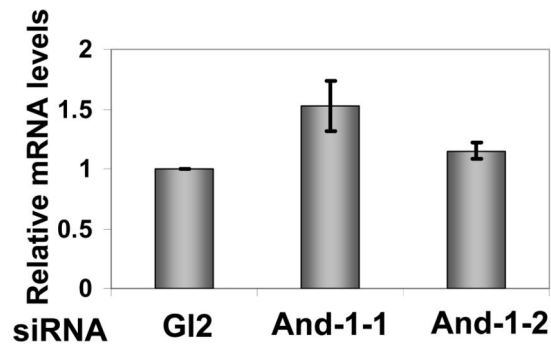
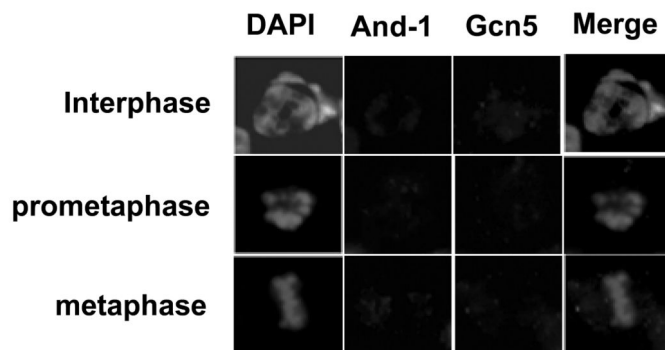
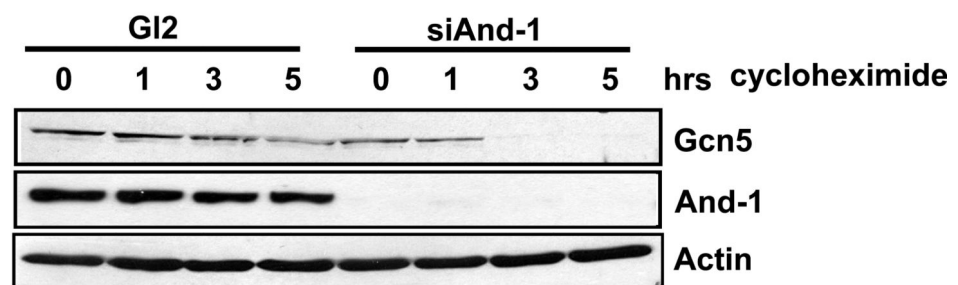
as in (A) were harvested and immunoblotted with indicated antibodies. (D) Decreased Gen5 proteins can be rescued by expression of And-1 from cDNA. U2OS cells were transfected with G12 siRNA or And-1 siRNA targeting its 3'UTR mRNA sequence alone, or co-transfected with vector plasmid or And-1 plasmid. Cells were harvested 48 hours after transfection and western blotted for indicated proteins.

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**A****B****C****Figure 4. And-1 specifically stabilizes Gcn5 proteins**

(A) And-1 depletion does not affect Gcn5 mRNA. mRNA level of Gcn5 tested by real-time RT-PCR in HCT116 cells transfected with siRNAs against GI2 or And-1. Experiments were performed two times. mRNA level data were normalized to GAPDH, and GI2 transfected group values were set as 1. Error bars represent SD from the mean. (B) Cell cycle immunofluorescence image of HCT116 cells transfected with siRNA And-1 showing Gcn5 proteins are decreased in the absence of And-1 during cell cycle. (C) Gcn5 protein half-life is reduced in And-1 depleted cells. HCT116 cells transfected with siRNA GI2 or And-1

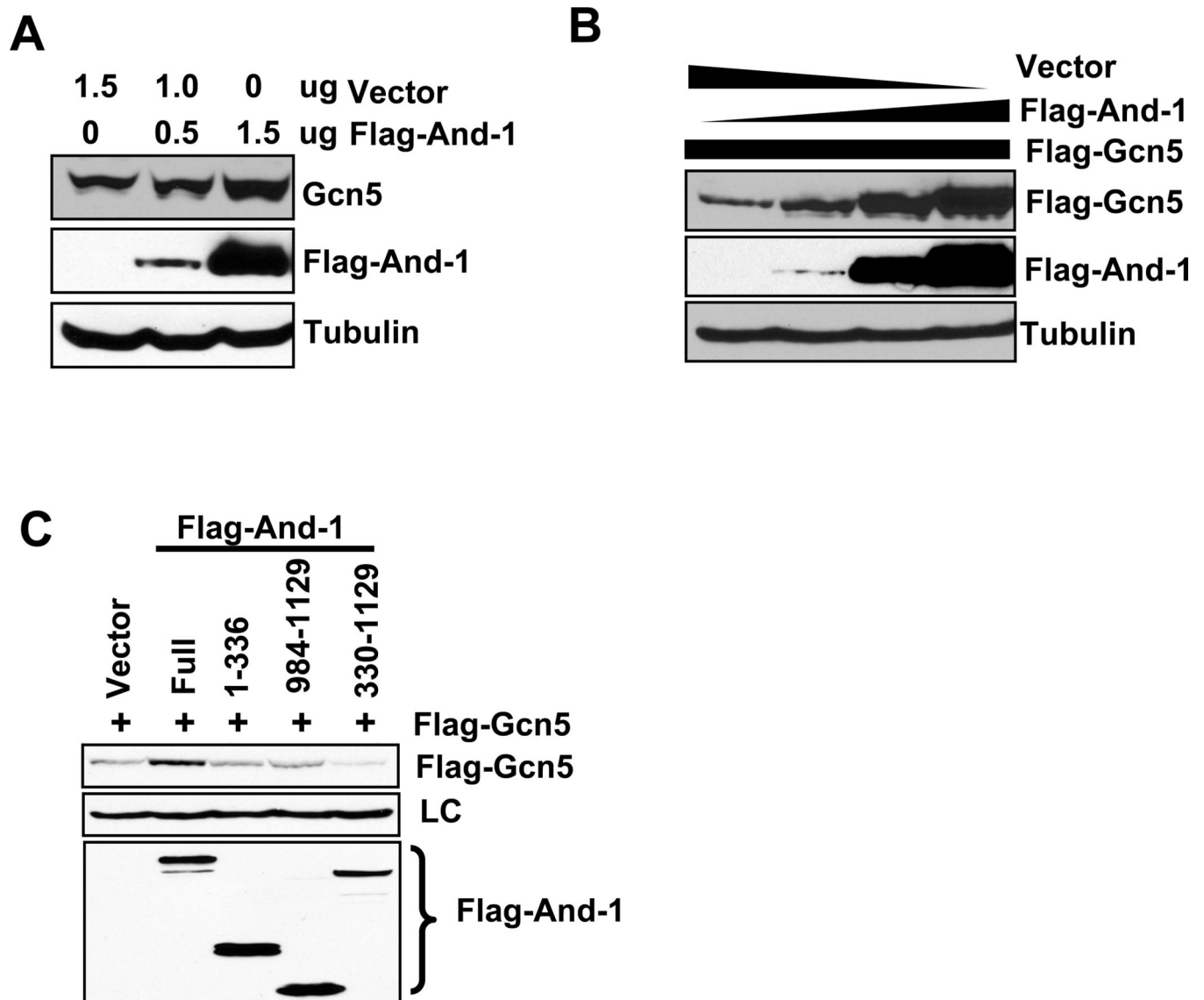
were treated with cycloheximide at 40 hours after transfection. Cells were harvested at indicated time points, and immunoblotted for indicated proteins.

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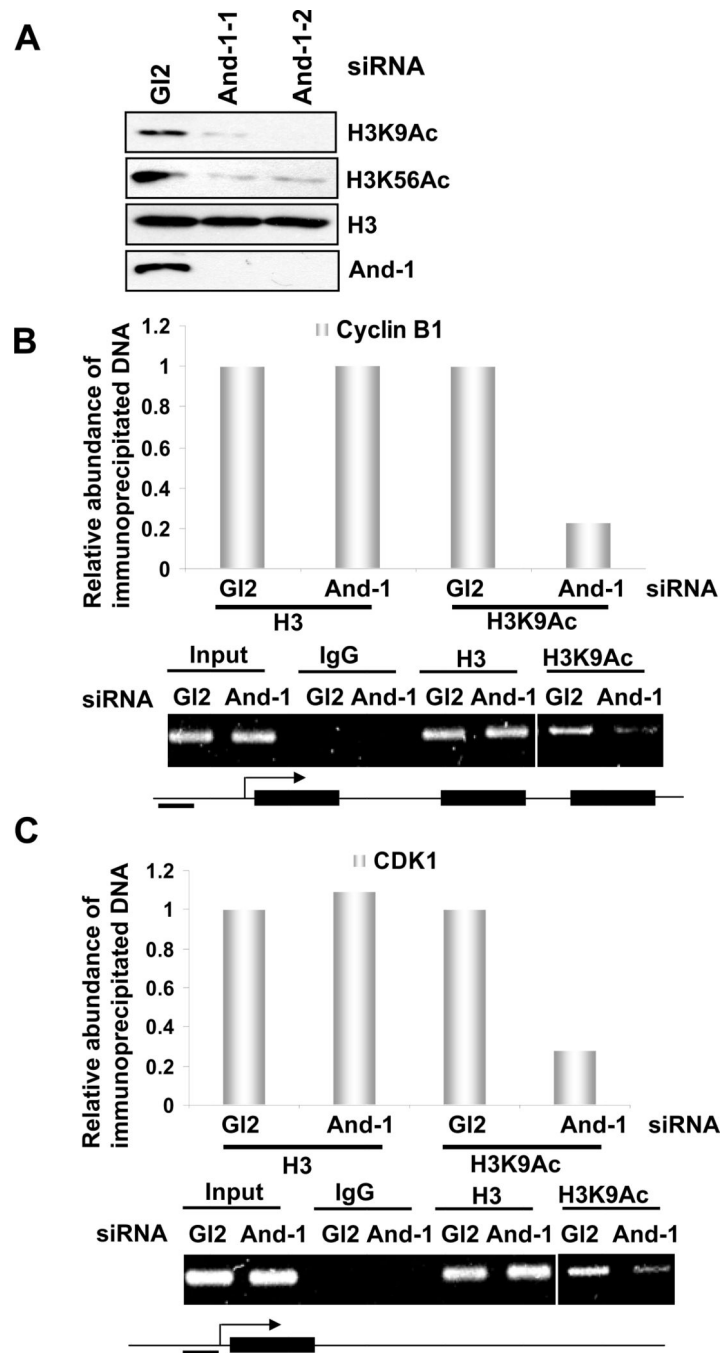
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**Figure 5. And-1 overexpression stabilizes Gcn5**

(A) And-1 overexpression increases endogenous Gcn5 protein levels. U2OS cells co-transfected with Flag-And-1 or vector were harvested 48 hours after transfection. Cells were resolved on SDS-PAGE, and immunoblotted with indicated antibodies. (B) And-1 overexpression increases exogenous Gcn5 protein levels. U2OS cells were co-expressed with Flag-Gcn5 and titrated amounts of vector or Flag-And1 plasmids as indicated. Cells were harvested 48 hours after transfection and treated as in (A). (C) Full length Flag-And-1 stabilizes Gcn5 proteins. And-1 and its mutants were transfected together with Flag-Gcn5 plasmids in U2OS cells. Cells were harvested 48 hours after transfection and treated as in (A).



**Figure 6. Acetylation of H3K9 and H3K56 is decreased in And-1 downregulated cells**  
 (A) And-1 depletion reduces the acetylation of H3K9 and H3K56. HCT116 cells transfected with indicated siRNAs were harvested, resolved on SDS-PAGE, and immunoblotted with indicated antibodies. (B) The acetylation of H3K9 at promoters of Cyclin B1 is reduced in And-1 depleted cells. HCT116 cells were transfected with indicated siRNAs before analyses by ChIP. The PCR-amplified products were analyzed by agarose gel (middle). The amount of DNA was quantitated by Scion imaging software (top). The amount of DNA in GI2 siRNA transfected cells was taken as 100%. Schematic representation of Cyclin B1 genes

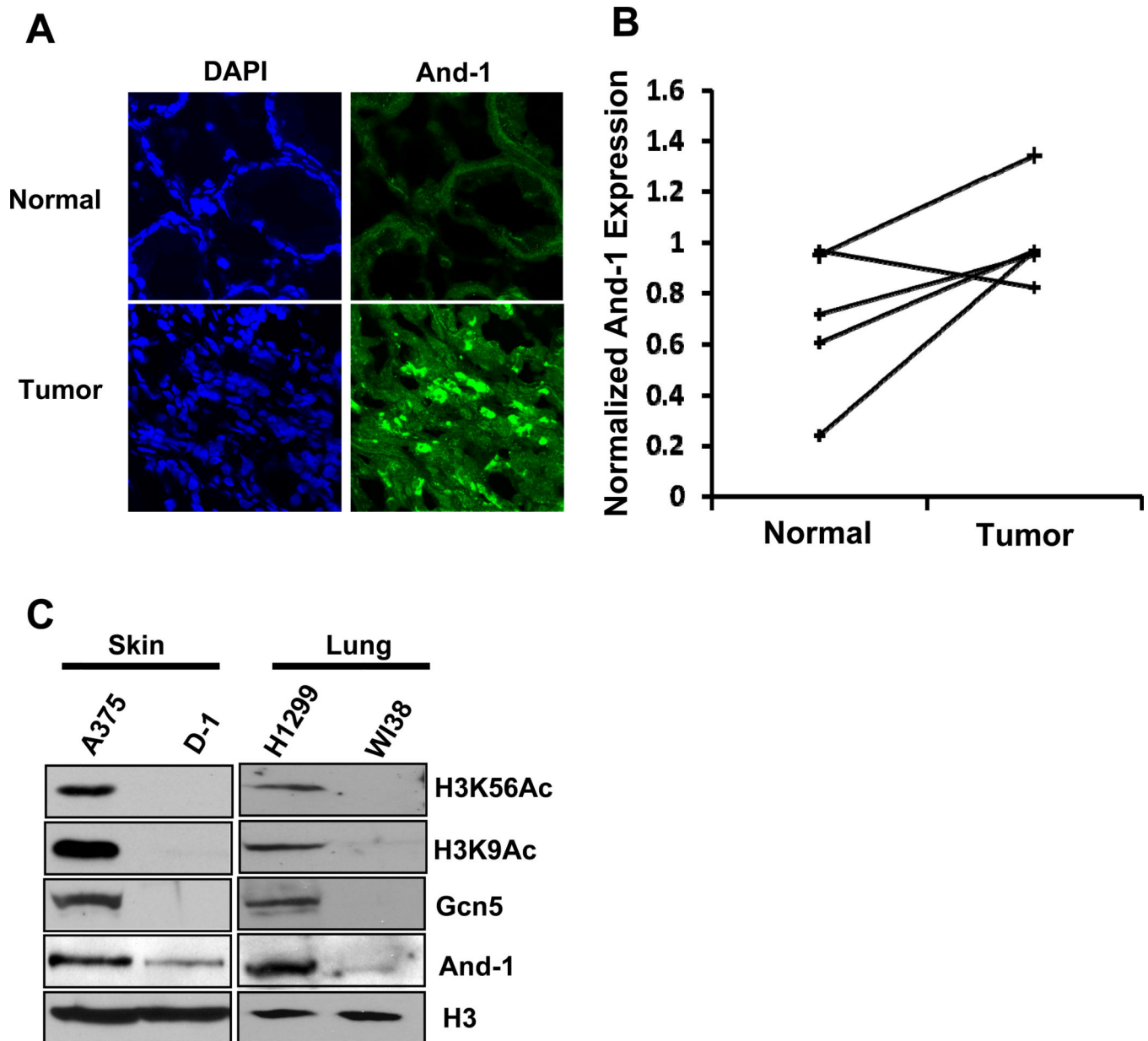
showing positions analyzed by ChIP (bottom). (C) The acetylation of H3K9 at promoters of Cdk1 is reduced in And-1 depleted cells. Assay was performed as in (B).

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**Figure 7. And-1 expression in tumors and tumorigenic cell lines**

(A) And-1 is more highly expressed in colorectal carcinomas compared to paired normal colon tissue. A representative fluorescent immunohistochemical image is shown. (B) Plots of And-1 expression in five paired colon tumor and normal tissues from individual patients. And-1 expression was increased in 4 of the 5 patients examined. Overall expression of And-1 was significantly higher in tumors compared to paired normal tissues by paired t-test ( $p < 0.05$ ). (C) And-1 expression is increased in tumorigenic cell lines compared to normal cells. A375 (melanoma) and D-1 (normal skin fibroblast) cells were harvested for immunoblotting for indicated proteins.