行政院國家科學委員會專題研究計畫 期中進度報告

FACT 蛋白複合體在轉錄及 DNA 複製之調控及功能(1/5)
期中進度報告(精簡版)

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利用「與『基因轉錄延伸促進者—FACT複合體』有交互作用之蛋白」的系統性搜尋實驗中，我們找到了EDD：一具有HECT區位（domain）的細胞泛素E3接合酵素。當更進一部研究EDD的次複合體組成時發現其中包含著一個不太為人所之的蛋白，AND-1。AND-1同時具有氨基端的WD40及羧基端的HMG-box區位為一酸性的核質蛋白。現今的結果指出AND-1是一個與染色質和細胞核基質具相關性的因子。有趣的是，AND-1在細胞核中具有清晰的點狀分佈，此一分佈與S phase的進行有很密切的關係。此外，根據分析其交互蛋白後證實了AND-1可能與許多修剪複合體（spliceosome complex）或核糖核酸編輯酵素的關鍵因子有關。然而，AND-1在水溶性及核基質部份的交互蛋白絡顯不同，故而猜測細胞內不同區域之AND-1也許再調控或功能上各自扮演獨特的角色。利用AND-1為目標的小片段干擾核糖核酸（siRNA）來進一部研究就其功能上的特徵時指出，此蛋白可能與S到G_2的過渡時期有關。總和以上結果，AND-1可能為一具有多重功能的蛋白，並能將轉錄及後轉錄連同去氧核糖核酸合成的功能連結在一起。

Abstract

EDD, a HECT-domain E3 ubuquitin ligase, was identified to be associated with the transcription elongator – FACT complex (2)(3). Further characterization the subcomplex component of EDD revealed that it contains another novel protein, AND-1 (1). AND-1 (acidic nucleoplasmic DNA-binding protein) is an acidic, nucleoplasmic protein that contains a WD40 domain at amino-terminus and a DNA-binding HMG-box at carboxy-terminus. Current findings indicate that AND-1 is a chromatin and nuclear matrix-associated factor. Interestingly, AND-1 possesses a distinct punctate pattern of subnuclear localization, which closely correlates with S phase progression. Additionally, interactomic analysis demonstrated that AND-1 may associate with many key factors of the spliceosome complex or RNA-editing enzymes. However, the interacting protein profiles seem slightly different between the soluble and nuclear matrix fractions, suggesting that subcellular pools of AND-1 may possess distinct regulatory or functional roles. Further functional characterization using AND-1 targeting siRNA revealed a possible link of this protein to S-G_2 transition. Collectively, these observations imply that that AND-1 may be a multifunctional protein that links and coordinates transcriptional and post-transcriptional events, as well as DNA synthesis.

Materials and Methods

G1/S Phase Synchronization

NIH 3T3 cells were plated at low density and serum starved. After 48 hours, cells were stimulated with 1 μg/ml aphidicolin (Sigma) containing NIH 3T3 growth

medium. After 16-18 hours, cells were washed to remove the aphidicolin and overlaid with common NIH 3T3 growth medium then harvest in specific time course.

HeLa cells were seeded at low density. At 24 hours after seeded, cells were blocked with thymidine (Sigma) for the final concentration at 2 mM. After 16-18 hours incubation, gently washed three times with PBS, and then incubated with fresh medium. After 10 hours, the second block with 2 mM thymidine at least for 15 hours. After which the cells were released by extensive wash with PBS, added with fresh growth medium then harvest in specific time course.

Immunoprecipitation with soluble antibody

Whole cell extracts or nuclear extract were incubated with G-Sepharose beads (Sigma) for 1 hour rotating at 4°C and then the pre-cleared extracts were incubated with the G-Sepharose beads conjugating desired antibodies for 2-3 hours rotating at 4°C. Briefly spin down the immunocomplexes (3,000 rpm, 30 sec) and then washed with WCE buffer for three times and added 4X sample buffer to boiled.

Results

In a previous proteomic characterizaiton of FACT-associated multiprotein complexes, we have identified a nuclear protein termed AND-1. AND-1 (acidic nucleoplasmic DNA-binding protein) is an acidic, nucleoplasmic protein which molecular weight is about 125 kDa. AND-1 contains a WD40 domain at amino-terminus and a DNA-binding HMG-box at carboxy-terminus. The amino terminus harbours the seven consecutive “WD40 repeats”, which are sequence motifs of about 40 amino acids that characteristic of a large group of regulatory proteins and postulated in phospho-residue binding. Within the carboxyl terminus, there is another domain called HMG-BOX. This 63-amino-acid-long box is typical of a family of DNA-binding proteins involved in the regulation of chromatin assembly, transcription and replication. The dual-domain of AND-1 reflects its dual-role either in protein-protein interaction and/or DNA-binding activity. It has been studied that AND-1 can bind intensely to DNA and is very abundant in cell. Despite the presence of these protein domains, the cellular role of AND-1 is still largely unknown.

Dynamic subnuclear localization of AND-1

As shown in Fig. 1 (A-L), immunostaining experiments illustrated that AND-1 localizes primarily in the nuclear compartment. Additionally, by the Western blot analysis on sub-nuclear fractionations, we detected a significant fraction of AND-1 in the nuclear matrix fraction (as shown in Fig.1M), which indicates that AND-1 can

also localize to this subnuclear compartment. As shown in Fig.1A, D, G and J, several different patterns of AND-1 could be observed. In the interphase cells (Fig.1A), the AND-1 signal appears homogenously in the nucleoplasm and co-localize with DNA. Interestingly, when cells entering M phase (Fig.1G), AND-1 seems diffused and does not co-localize with condensed chromatin. Moreover, upon exiting the M phase, during which chromatin becoming decondensed, AND-1 starts to reassociate with DNA since the signal is again co-localize with DAPI. Moreover, we identified some punctate patterns of AND-1 in a sub-population of the cells (Fig.1D and J). Such distinct speckled pattern was later found closely associated with HeLa (Figure 2) and NIH-3T3 (Figure 3) cells in the S phase (mid to late).

Identification of AND-1-Interacting Proteins

Due to the multiple regulatory domains present in AND-1 (WD40 and HMG-BOX), which suggests that AND-1's function may be mediated by directly interacting with DNA or associating with other protein factors, we next sought to identify protein factors that may interact with AND-1. With the AND-1-specific antibody, we could examine the AND-1-interacting proteins by performing the immunoprecipitation experiments. AND-1 immunoprecipitates from HeLa were resolved on 7.5% (Figure 4) and 15% (not shown) SDS-PAGEs. Specific bands were cut down and identified by LC/MS analysis. Surprisingly, there were many nuclear proteins identified and many of those are key components of the spliceosome. As summerized in Table 1, based on the mass spectrometric analysis, the bands around the size of 200 kDa represent some core proteins of the spliceosome, such as Prp8 and U5 snRNP. It is quite reasonable that AND-1 can interact with helicase, but the interaction of AND-1 and the spliceosome is intensely interesting. Furthermore, we also identified the hnRNP U and PSF (Polypyrimidine tract-binding protein-associated splicing factor) in the 120 and 100 kDa bands, respectively, which could provide stronger evidences for the association of AND-1 and spliceosome complex.

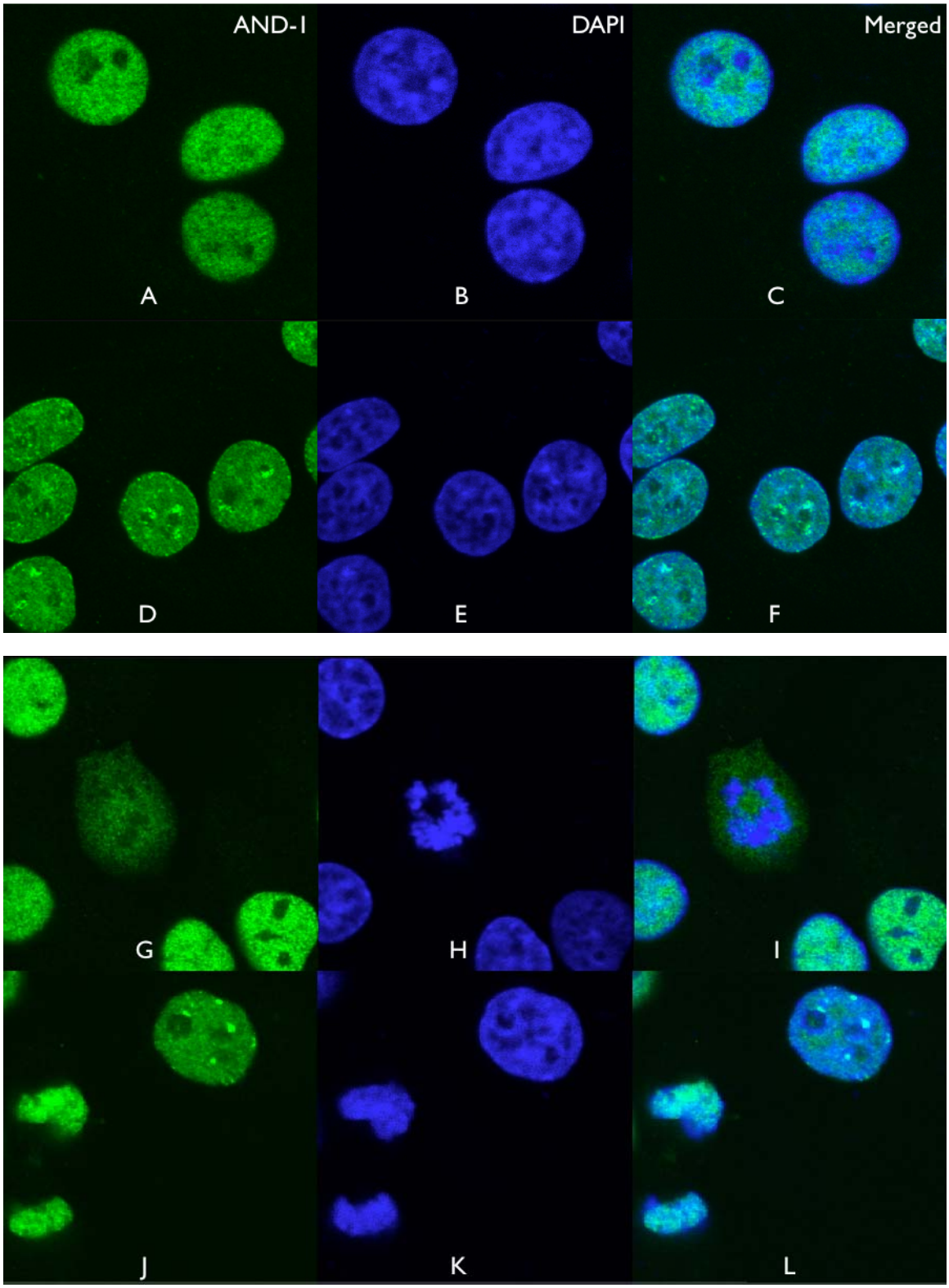
Since AND-1 was found primarily in the nuclear matrix fraction, we also aimed to identify the AND-1-associated proteins in this cellular context. However, solubilization of the nuclear matrix cannot be achieved under native and immunoprecipitation-compatible condition. Toward this end, we performed an intracellular crosslinking experiment (using crosslinker DSP) prior to cell extraction and immunoprecipitation. Subsequently, de-crosslinking of the isolated proteins was done by adding the denaturing electrophoretic sample buffer. Interestingly, as shown in Figure 5, we could identify many of the same protein factors identified in the soluble fraction (Figure 4 & Table 1). However, there were additional novel

components in the nuclear matrix-associated immunocomplexes. These include several RNA-editing enzymes such as RNA helicase A, adenosine deaminase, and DEAD box polypeptide 21. Structural protein matrin 3 and DNA topoisomerase II were also identified in this experiment. These findings are consistent with the previous finding of AND-1- immunoprecipitation, which suggested that the AND-1 may associate with many key factors of the spliceosome or RNA-editing enzymes. However, the interacting protein profiles seem slightly different between the soluble and nuclear matrix fractions, indicating that subcellular pools of AND-1 may possess distinct regulatory or functional roles.

Possible link of AND-1 to cell cycle progression

Next, toward elucidating the cellular roles of AND-1 and further examining its link to cell cycle, we undertook a vector-based small interfering RNA approach to abrogate endogenous AND-1 expression in 293T cells. Cell cycle profiles of the control and the siRNA knockdown cells were analyzed by FACS (Figure 6). Interestingly, the AND-1^{RNAi} cells exhibited markedly delayed G_2/M phase, suggesting a potential role of this protein in modulating the S-G_2 or G_2-M transition.

Results



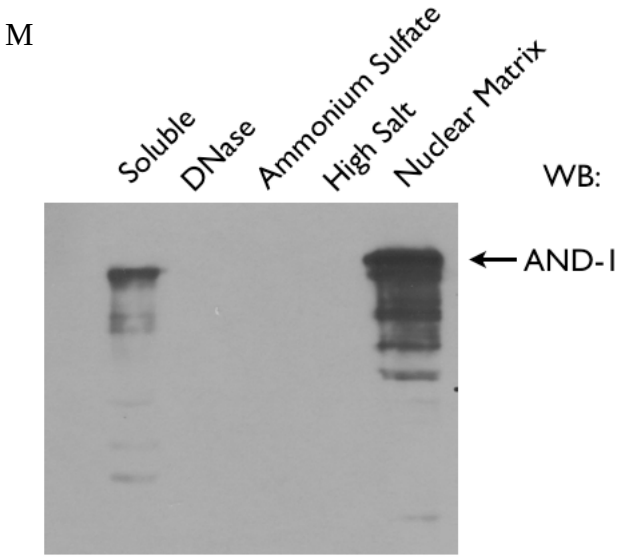


Figure 1. Subcellular localization of AND-1: distinct nuclear foci and nuclear matrix association.

Anti-AND-1 polyclonal antibody was used to examine the subcellular localization of AND-1. HeLa cells were fixed on coverslips and subjected to the immunofluorescence staining by anti-AND-1 antibody. (A, D, G and J) Staining of AND-1 (green). (B, E, H and K) Staining of DAPI (blue). (C, F, I and L) Merged images. Subnuclear fractionation was also done to further pinpoint the distribution of AND-1.

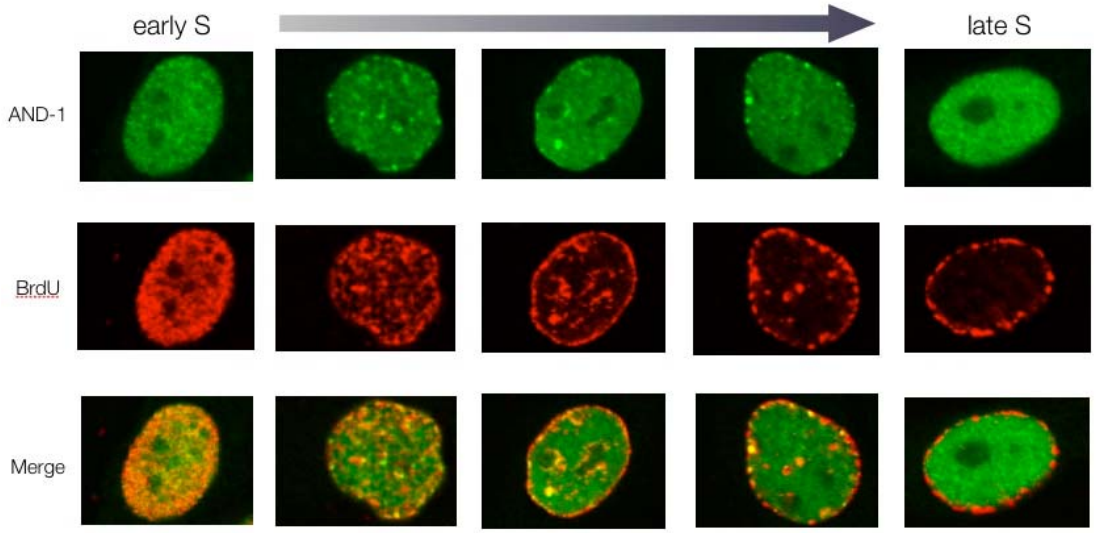
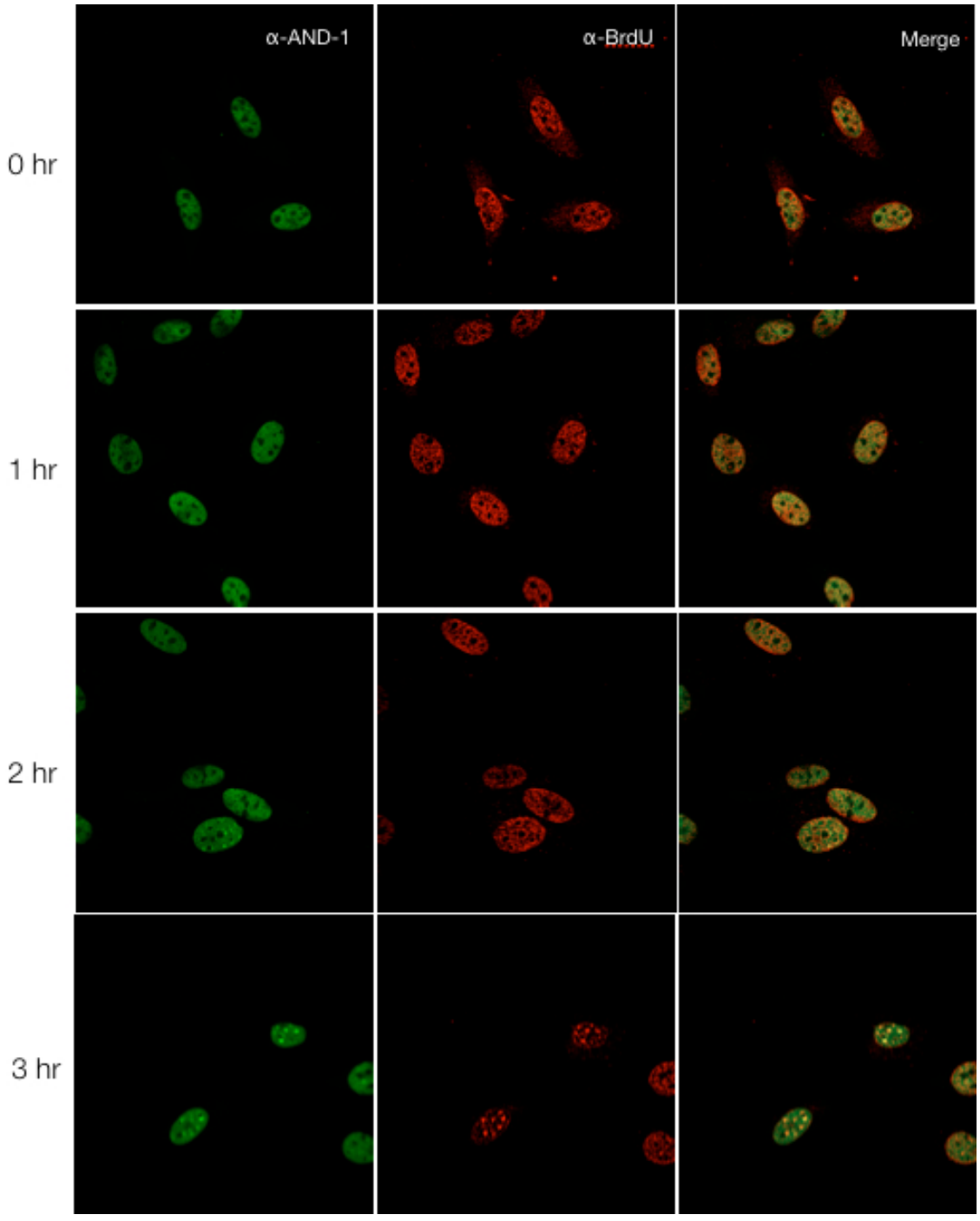


Figure 2. AND-1 proteins display distinct speckled pattern through early to mid-late S phase.

HeLa cells were grown on coverslip, treated with BrdU for 20 minutes before fixation and subsequently processed for immunofluorescence staining and imaging. (Top)

Staining of AND-1 (green). (Mid) Staining of BrdU (red). (Buttom) Merged images.



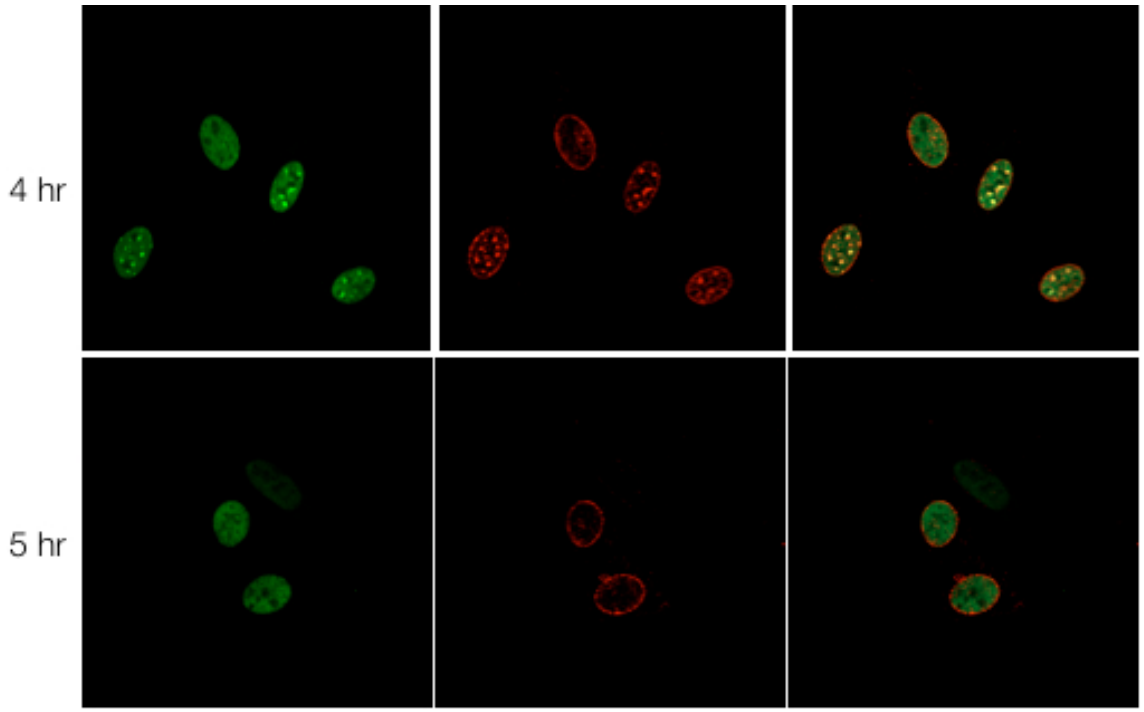


Figure 3. For synchronization, the speckled pattern of AND-1 also incorporate with BrdU through S phase in NIH 3T3 cell.

NIH 3T3 cells on coverslips were synchronized at G1/S stage, and then released in serum-rich medium for 0-5 hours. At specific time point, cells were pulsed with BrdU (red) for 20 minutes before being fixed for immunofluorescence analysis. Staining of AND-1 is also shown (green).

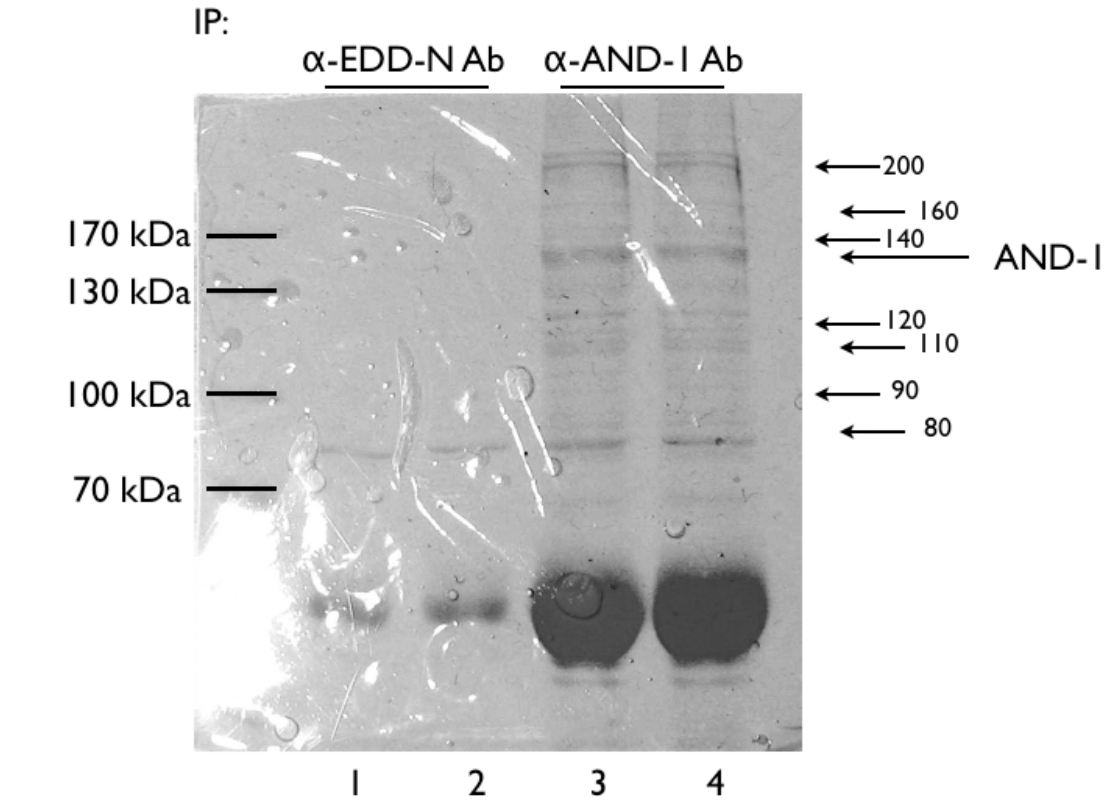


Figure 4. Identification of the AND-1-associated protein complexes by LC/MS
The AND-1-associated immunocomplexes were isolated from HeLa whole cell lysates by the α-AND-1 antibody (conjugated with protein G-Sepharose beads). Samples were then separated by 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1 and 2: Negative controls (duplicate) were performed by α-EDD-N antibody. Lane 3 and 4: Immunoprecipitation with α-AND-1 antibody. Distinct protein bands, as indicated on the right side, were subjected to LC/MS analysis. Identity of the associated proteins is summarized in Table 1.

Table 1. Putative AND-1-associated proteins

Size	Identified Protein
200 kDa	Prp8 (U5 snRNP-specific protein (220 kD)) U5 snRNP-specific protein (U5-200K, KIAA0788) DEAD-like helicase
170 kDa	multifunctional aminoacyl-tRNA synthase
130 kDa	AND-1
120 kDa	Gemin4 SART-1 protein

	hnRNP U
100 kDa	Nucleolin Cdc27 DEAD box polypeptide 20 (Gemin3)/23 (Prp28, U5-100K) SFPQ protein (PSF)
90 kDa	Bip
70 kDa	hsp70
60 kDa	Lamin B receptor variant (Fragment)
55 kDa	Seryl-tRNA synthase
40 kDa	Nucleophosmin 60S ribosomal protein L6 and L5 eIF3 S1 protein SMN1 hnRNP A1 (Helix-destabilizing protein)
32 kDa	Proteasome activator complex subunit 3 pre-mRNA splicing factor SF2, P32
15 kDa	Histone H2A/2B, H3

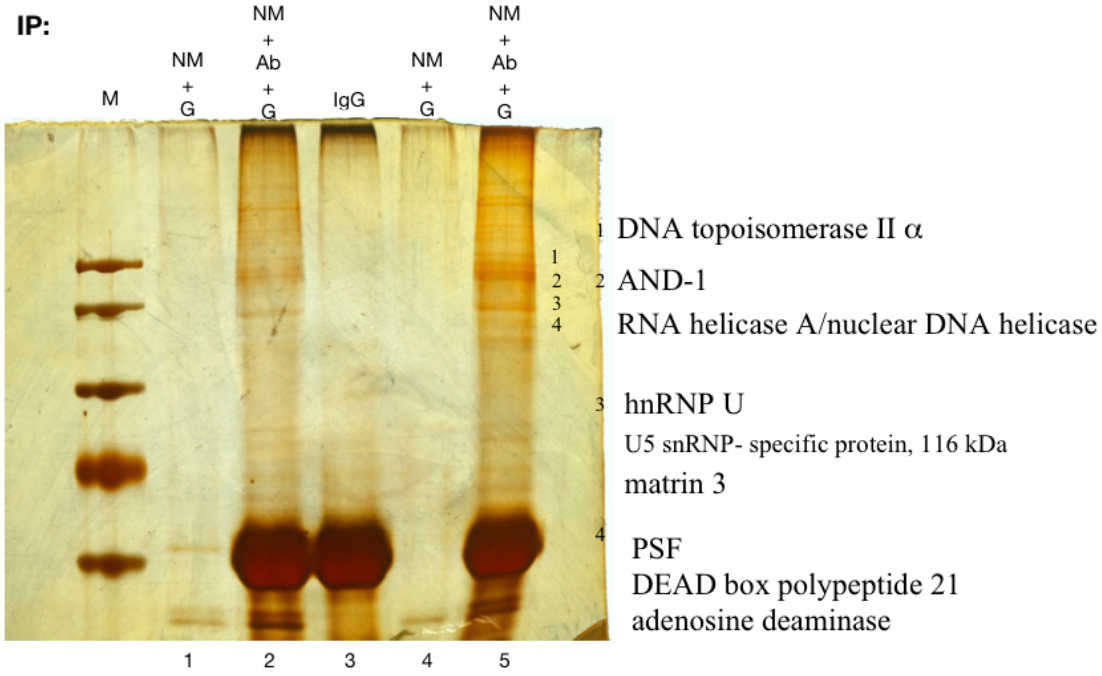


Figure 5. Identification of AND-1-associated proteins in the DSP treated nuclear matrix fraction by LC/MS.

Asynchronized (lane 1 and 2) and double thymidine-blocked (lane 4 and 5) HeLa cells were treated with 2.5 mM fresh DSP cross-linking reagent before being harvested. The solubilized nuclear matrix fraction was incubated with protein G-Sepharose

beads-conjugated α-AND-1 polyclonal antibodies. Samples were then separated by 7.5% SDS-PAGE and silver stained. Lane 2 and 5: protein G beads with α-AND-1 antibody only. Distinct protein bands, as indicated from number 1 to 4, were subjected to LC/MS analysis. Identity of the associated proteins is showed on the right side.

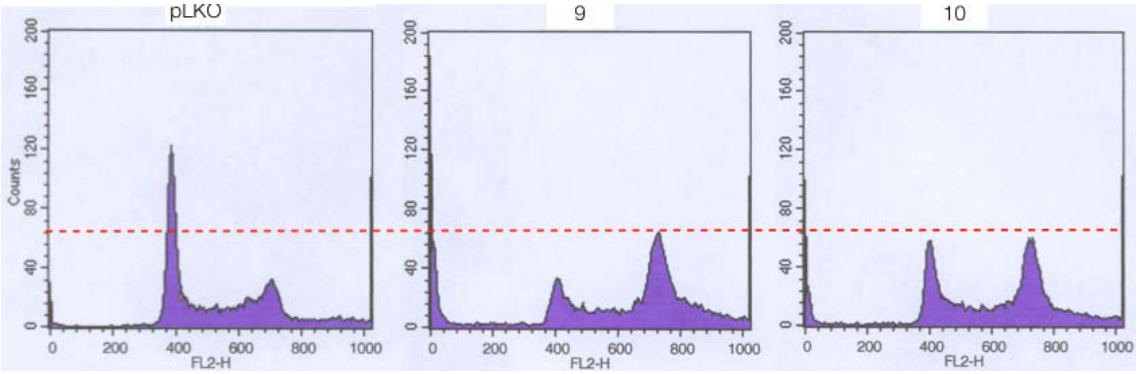


Figure 6. Transient transfection of AND-1 shRNA in 293T cells, endogeneous AND-1 knockdown promote cells arrest in G2/M stage.

293T cells were transfected with a luciferase control shRNA or AND-1-targeting shRNA construct. Approximately 48 hours post-transfection cells were fixed in 70% EtOH and subsequently subjected to PI staining. Cell cycle progression was analyzed (based on DNA content) by FACS. Comparing the control cells and AND-1 knockdown cells (two different shRNA constructs, shown in the two panels on the right), AND-1 seems to be involved in modulating G_2 phase progression.

Reference

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