The A-kinase-Anchoring Protein AKAP95 Is a Multivalent Protein with a Key Role in Chromatin Condensation at Mitosis

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Abstract. Protein kinase A (PKA) and the nuclear A-kinase–anchoring protein AKAP95 have previously been shown to localize in separate compartments in interphase but associate at mitosis. We demonstrate here a role for the mitotic AKAP95–PKA complex. In HeLa cells, AKAP95 is associated with the nuclear matrix in interphase and redistributes mostly into a chromatin fraction at mitosis. In a cytosolic extract derived from mitotic cells, AKAP95 recruits the RIIα regulatory subunit of PKA onto chromatin. Intranuclear immunoblocking of AKAP95 inhibits chromosome condensation at mitosis and in mitotic extract in a PKA-independent manner. Immunodepletion of AKAP95 from the extract or immunoblocking of AKAP95 at metaphase induces premature chromatin decondensation. Condensation is restored in vitro by a recombinant AKAP95 fragment comprising the 306-carboxy-terminal amino acids of the protein. Maintenance of condensed chromatin requires PKA binding to chromatin-associated AKAP95 and cAMP signaling through PKA. Chromatin-associated AKAP95 interacts with Eg7, the human homologue of Xenopus pEg7, a component of the 135 condensin complex. Moreover, immunoblocking nuclear AKAP95 inhibits the recruitment of Eg7 to chromatin in vitro. We propose that AKAP95 is a multivalent molecule that in addition to anchoring a cAMP–PKA–signaling complex onto chromosomes, plays a role in regulating chromosome structure at mitosis.

Key words: AKAP • cAMP • chromosome condensation • mitosis • PKA

Proper packaging of DNA into chromosomes is an essential process in preparation for mitosis. Chromosome condensation at mitosis requires DNA topoisomerase II (Adachi et al., 1991) and a family of proteins of highly conserved ATPases called SMCs (structural maintenance of chromosomes) (Hirano and Mitchison, 1994; Saitoh et al., 1994; Hirano et al., 1997). Evidence that SMCs promote chromosome condensation was provided by the purification of Xenopus 8S and 13S multiprotein complexes, termed condensins (Hirano et al., 1997), and the demonstration that two of these proteins are required for chromosome condensation and maintenance of condensed chromatin (Hirano and Mitchison, 1994). A nether component of the 13S condensin complex, pEg7, was also recently shown to be implicated in mitotic chromosome condensation in vitro (Cubizolles et al., 1998).

cAMP-dependent protein kinase A (PKA) has been proposed to be a negative regulator of mitosis. PKA activity oscillates in cycling Xenopus egg extracts (Grieco et al., 1994). Onset of mitosis correlates with a decrease in cAMP level and PKA activity, whereas cAMP level and PKA activity rise during metaphase to peak at early interphase (Grieco et al., 1996). Consistent with this finding, downregulation of PKA mediated by microinjection of the PKA inhibitor PKI was shown together with activation of cyclin-dependent kinase 1 (CDK1) to be required for mitotic nuclear envelope disassembly and chromosome condensation in cultured mammalian cells (Lamb et al., 1991). In contrast, PKA activation is necessary for nuclear reassembly upon exit from mitosis (Grieco et al., 1996). These results suggest a requirement for a downregulation of cAMP/PKA signaling for entry into mitosis, but they do not explain the gradual rise in PKA activity during mitosis. Biological effects of cAMP are mainly mediated by PKA types I and II in eukaryotic cells. The PKA type II holoenzyme complex consists of two catalytic (C) and two regulatory (RIα or RIβ) subunits, which modulate the...
catalytic activity of PKA by binding and inactivating C 
(Scott, 1991). PKA is activated by binding of two cAMP 
molecules to each R subunit that promotes release of the 
C subunits from the R–cAMP complex. A cative C 
subunits phosphorylate specific substrates and can be translocated 
to the nucleus, where they play a role in gene activation 
(R labowol et al., 1988).

The specificity of cellular and nuclear responses to 
cAMP is mediated by targeting of the R1I subunit of PKA 
to discrete subcellular loci through associations with 
A-kinase–anchoring proteins or AKAPs (Colledge and 
Scott, 1999). A 95-kD AKAP, designated AKAP95, has 
been cloned and characterized in the rat (Coghlan et al., 
1994) and human (Eide et al., 1998). A AKAP95 has been 
localized exclusively in the nucleus of interphase rat and human 
fibroblasts (Coghlan et al., 1994; Eide et al., 1998); 
however, as no RIIα has been detected in interphase nu-
clei (Eide et al., 1998), the role of A AKAP95 in the nucleus 
remains elusive. At mitosis, A AKAP95 interacts with RIIα 
apparently in the vicinity of the metaphase plate (Eide et al., 
1994) and human (Eide et al., 1998). AKAP95 has been lo-
cloned and characterized in the rat (Coghlan et al., 
1991); thus, Ht31 was used as a specific inhibitor of AKAP– 
RIIα complex at mitosis and provide a functional significance for in-
volving AKAP95 in the regulation of chromatin struc-
ture at mitosis and provide a functional significance for in-
creasing PKA activity during mitosis.

We demonstrate here in vivo and in vitro immuno-
blocking and rescue experiments the formation of an 
A AKAP95–PKA signaling complex onto mitotic chromo-
somes, and a role of A AKAP95 in chromatin condensation 
and maintenance of condensed chromosomes during mi-
tosis. The latter process also requires cAMP/PKA signaling 
and anchoring of PKA to chromatin by AKAP95. The 
data also suggest that one function of A AKAP95 is to pro-
mote the recruitment of components of the condens 
complex onto chromatin. The results argue towards a criti-
cal role of A AKAP95 in the regulation of chromatin struc-
ture at mitosis and provide a functional significance for in-
creasing PKA activity during mitosis.

Materials and Methods

Buffers, Reagents, and Antibodies

Nuclear isolation buffer (buffer N) consisted of 10 mM 
Hepes, pH 7.5, 2 mM MgCl2, 250 mM sucrose, 25 mM 
KCl, 1 mM DTT, 1 mM PMSF, and 10 μg/ml each of 
aprotinin, leupeptin, and pepstatin A. Cell lysis buffer 
consisted of 20 mM Hepes, pH 8.2, 5 mM MgCl2, 10 mM 
EDTA, 1 mM DTT, and 20 μg/ml cytochalasin B and protease inhibitors. A GST– 
AKAP95 fragment covering amino acids 387–692 and including the RII– 
A AKAP95 complexes at mitosis were described previously (Chaudhary and 
Courvalin, 1993; Collas et al., 1996; Buendia and Courvalin, 1997; M inc et al., 1999). A nR–human Eg7 
polyclonal antibodies were generated by immunizing rabbits with a pep-
tide comprising the last 15 amino acids of human Eg7 (K T T P I L R A S A R 
R H S) (Cubizolles et al., 1998).

Cell Synchronization and Microinjection

HeLa cells were grown in EMEM medium ( Gibco BRL) as described in 
Eide et al. (1998). For microinjections, cells were synchronized in S phase 
by a double thymidine (2.5 mM) block (K eyer et al., 1998). Cells were 
synchronized in M phase by subsequent exposure to 1 μM nocodazole for 
18 h (Eide et al., 1998). Mitotic indexes were typically ~95%.

For nuclear microinjections, HeLa cells were plated onto glass cover 
slips at 10 cells per 28-cm2 dish and synchronized in S phase by double 
thymidine block. Nuclei were microinjected with 25–50 pl of solution us-
ing a Narishige micromanipulator and picoinjector. Injection solution 
consisted of EMEM containing 10 μg/ml of a 150-kD FITC–dextran (Sigma 
Chemical Co.) to visualize the injections. Nuclei were injected with either 
2–5 pg affinity-purified anti–A AKAP95 polyclonal antibodies (at ~0.1 mg/ml), or 
~250 pg GST–A AKAP95–386 peptide together with anti–A AKAP95 anti-
bodies. Inhibitors, antagonists, or catalytic peptides were injected at concen-
trations indicated in Results. Successful nuclear injections were as-
essed by retention of the FITC–dextran within the nucleus 1 h after 
injection (see Results). A flter a resting period of 6 h, injected cells were 
synchronized in M phase by 15 h of 1 μM nocodazole. Mitotic nuclei were micro-
injected as described above while arrested in M phase with nocodazole. 
Injected cells remained in nocodazole for up to 2 h after injection during 
the period of observation.

Mitotic and Interphase Cell Extracts

Mitotic HeLa cells were washed once in ice-cold PBS, once in 20 vol of 
ice-cold lysis buffer, and packed at 800 g for 10 min. The cell pellet was re-
suspended in 1 vol of lysis buffer and incubated for 30 min on ice. Cells were 
homogenized by a 2–3 min sonication on ice and the lysate was cen-
trifuged at 10,000 g for 15 min at 4°C. The supernatant was cleared at 
20,000 g for 3 h at 4°C in a Beckman SW55 rotor. The clear supernatant 
(mitotic cytosolic extract) was aliquoted, frozen in liquid nitrogen, and 
stored at ~80°C. Protein concentration of the extract was usually ~15 mg/ 
ml. Interphase cytosolic extracts were prepared as above from unsynchron-
ized HeLa cells (95–98% in interphase) except that EDTA was omitted from 
the cell lysis buffer.

Preparation of Nuclei and Chromatin

Confluent unsynchronized HeLa cells were harvested, washed in PBS, 
sedimented at 400 g, and resuspended in 20 vol of ice-cold buffer N con-
taining 10 μg/ml cytochalasin B. A flter a 30-min incubation on ice, cells 
were homogenized on ice by 140–170 strokes of a tight fitting (B-type) 
glass pestle in a 7-ml homogenizer. Nuclei were used fresh 
judged by phase-contrast microscopy and labeling with 10 μg/ml of a 150-kD 
FITC–conjugated ConA (data not shown) (Collas, 1999). Nuclei were used fresh 
or frozen at ~80°C in buffer N containing 70% glycerol. High salt (2 M 
NaCl)–extracted nuclear matrices were prepared from purified nuclei as 
described previously (R eyes et al., 1997). M Atices were solubilized in 8 M 
urea/10 mM Tris-HCl, pH 8.0.

To prepare interphase chromatin, 108 HeLa nuclei suspended in 200 μl 
buffer N containing 1% Triton X-100 were incubated with 5 μl of micro-
cococal nuclease (Sigma Chemical Co.) at 37°C for 5 min. Digestion was 
terminated by adding EDTA to 5 mM and the mixture was centrifuged at 
10,000 g for 5 min. The supernatant (S1) was collected and the pellet was resuspended in 2 mM EDTA and incubated for 15 min at 4°C. A flter sedi-
mentation as above, the supernatant (S2) was combined with S1 to yield 
the chromatin fraction, proteins were precipitated with 10% TCA and dis-
solved in SDS sample buffer. T o solubilize mitotic condensed chromatin, 
mitotic cell lysates were centrifuged at 10,000 g for 10 min. The pellet was resuspended in 500 μl buffer and sedimented at 1,000 g for 20 min in a 
swingout rotor (E pendorf) through a 1-M sucrose cushion made in 
buffer N. Mitotic chromosomes were recovered from the pellet. This pel-
et was extracted with 1% Triton X-100 at 4°C for 30 min, sedimented at 
15,000 g for 15 min, and the detergent-insoluble fraction was digested with 
5 U/ml micrococcal nuclease at 37°C for 5 min. A flter sedimentation as
above, proteins of the supernatant (soluble chromatin) were precipitated in 10% TCA and dissolved in SDS-saline buffer.

**Loading of Nuclei with Anti-AKAP95 Antibodies**

Purified HeLa cell nuclei (2,000 nuclei/μl) were permeabilized in 500 μl buffer N containing 0.75 μg/ml lysosomechitin for 15 min at room temperature. Excess lysosomechitin was quenched by adding 1 ml of 3% BSA made in buffer N and incubating for 5 min on ice, before sedimenting the nuclei and washing once in buffer N. Nuclei were resuspended at 2,000 nuclei/μl in 100 μl buffer N containing affinity-purified anti-AKAP95 antibodies (1:40 dilution; 2.5 μg) or 2.5 μg of preimmune rabbit IgG. A 1-h incubation on ice with gentle agitation, nuclei were sedimented at 500 g for 20 min and held in buffer N on ice until use. A nuclease loading of nuclei was verified by immunofluorescence. AKAP95 was not proteolytically degraded during permeabilization and antibody loading procedures, and lysosomechitin treatment of nuclei did not affect nuclear envelope disassembly or chromatin condensation in mitotic extract (data not shown).

**Nuclear Disassembly and Chromatin Condensation Assay**

A nuclear disassembly/chromatin condensation reaction consisted of 20 μl mitotic extract, 1 μl nuclear suspension (~2 × 10⁶ nuclei), and 0.6 μl of an ATP-generating system (1 mM ATP, 10 mM creatine phosphate, 25 μg/ml creatine kinase; Collas, 1998). The reaction was started by addition of the ATP-generating system and allowed to proceed at 30°C for up to 2 h. The extract supported nuclear envelope disassembly and chromatin condensation without apoptotic proteolysis of nuclear envelope proteins or DNA degradation (data not shown; see Lazebnik et al., 1993; Duband-Goulet et al., 1998). Chromatin condensation was monitored by staining with Hoeschst 33342. Chromatin was considered to condense when it acquired an irregular and compact morphology sometimes with distinct chromosomes or chromosome fragments. In some instances, extracts were preincubated with inhibitors for 30 min on ice before adding nuclei and the ATP-generating system. Percent chromatin condensation was calculated as the ratio of condensed chromatin masses per number of nuclei examined. For biochemical analyses, condensed chromatin was sedimented at 1,000 g through 1 M sucrose as described above and recovered from the pellet.

**Premature Chromatin Decondensation Assay**

HeLa chromatin condensed in mitotic extract was recovered by sedimentation at 1,000 g through 1 M sucrose, washed by resuspension and sedimentation in lysis buffer, and incubated for up to 2 h in fresh mitotic extract, either intact or immunodepleted of AKAP95 or RIIα. In some experiments, this extract contained antibodies or inhibitors. DNA was labeled with Hoeschst and examined as above. Premature chromatin decondensation (PCD) referred to swelling of the chromatin into ovoid or spherical objects, with no discernible chromosomes. Kinetics of PCD was evaluated by measurement of the proportion of chromatin masses undergoing decondensation at regular time intervals.

**Histone H1 Kinase Assay**

5 μl of HeLa cytosolic extract was mixed with 5 μl of a 2× histone kinase buffer (160 mM β-glycerophosphate, 20 mM EGTA, 30 mM MgCl₂, 2 mM DTT, 20 μg/ml each of aprotinin, leupeptin, and pepstatin A, and 100 mM PTK I). The kinase reaction was initiated by addition of 10 μl of a cocktail containing 2.5 mg/ml histone H1, 0.7 mM ATP, and 50 μCi of γ-[32P]ATP. The reaction was carried out at room temperature for 15 min and stopped by addition of 15 μl of 2× SDS loading buffer and boiling. Proteins were resolved by 15% SDS-PAGE, the gel was dried, and phosphorylation of histone H1 was visualized by autoradiography.

**Immunological Procedures**

Immunoblotting of nuclei, chromatin, and reaction supernatants was performed as described (Collas et al., 1996) using the following antibodies: rabbit anti-AKAP95 (1:250 dilution), rabbit anti-lamin B (1,000), anti-LBR (1:100), RIIα mAb (1:250), anti-Eg7 (1:500), and HRP-conjugated secondary antibodies. Blots were revealed with chloronaphthol/hydrogen peroxide (Collas et al., 1996) or by enhanced chemiluminescence (Amersh.). For immunoprecipitations, whole mitotic or interphase cells (as indicated) were sonicated twice for 2 min on ice in immunoprecipitation (IP) buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1% Triton X-100, and protease inhibitors), and the lysate was centrifuged at 15,000 g for 15 min. The supernatant was precleared with protein A/G agarose (1:25 dilution) at 4°C for 1 h. Immunoprecipitations were carried out with relevant antibodies (anti-RIIα mAb, anti-AKAP95 polyclonal, and anti-Eg7, each at a 1:50 dilution) from the supernatant at room temperature for 2.5 h, followed by incubation with protein A/G agarose (1:25 dilution) for 1.5 h and centrifugation at 4,000 g for 10 min. Immune complexes were washed three times in IP buffer and proteins were eluted in 2× SDS boiling sample buffer. RIIα was also immunoprecipitated from mitotic extract, or chromatin, or reaction supernatant fractions diluted 10× in IP buffer and sonicated (chromatin fraction only). AKA95 or RIIα was also immunodepleted from undiluted cytosolic extracts using affinity-purified anti-AKAP95 antibodies or anti-RIIα mAb at a 1:50 dilution. For immuno-}

**Results**

**Subcellular Distribution of Human AKAP95 during the Cell Cycle**

The subcellular localization of human AKAP95 was examined throughout the HeLa cell cycle. Immunofluorescence analysis using an affinity-purified anti-AKAP95 antibody confirmed that AKA95 was exclusively nuclear in interphase, colocalized with DNA, and was excluded from nucleoli (Fig. 1 A). As early as prometaphase and until telophase, AKA95 staining colocalized with chromosomes over a diffuse cytoplasmic labeling (Fig. 1 A). In late telophase/interphase, AKA95 labeling became excluded from the cytoplasm and restricted to the daughter nuclei (Fig. 1 A). Similar results were obtained with two newly developed anti-AKA95 mAbs, mA b36 and mA b47, and in human 293T fibroblasts with all three antibodies (data not shown).

Immunoblotting analysis of AKA95 revealed similar levels of AKA95 in whole interphase and mitotic cell lysates (Fig. 1 B). Fractionation of interphase and mitotic cells confirmed that AKA95 was exclusively nuclear in interphase (Fig. 1 C). At mitosis, ~75% of AKA95 co-fractionated with chromatin, whereas ~25% was detected in a 200,000-g supernatant fraction (Fig. 1 C, cytosol). No AKA95 was detected in membrane fractions of interphase or mitotic cells (Fig. 1 C). Furthermore, fractionation of purified HeLa nuclei into chromatin and high salt–extracted nuclear matrices revealed that ~80% of AKA95 partitioned with the matrix, whereas ~20% co-fractionated with chromatin (Fig. 1 D). Thus, AKA95 is restricted to the nuclei of interphase cells, where it associates primarily with the nuclear matrix. At mitosis, AKA95 is redistributed into a major chromatin-associated fraction and a minor soluble pool. Upon nuclear reassembly at the end of mitosis, AKA95 is sequestered into the daughter nuclei where it re-acquires an interphase distribution.

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AKAP95 Associates with the RIIα Regulatory Subunit of PKA at Mitosis and in the Mitotic Extract

A association of AKAP95 with RIIα in interphase and mitosis was investigated in dual immunofluorescence and immunoprecipitation experiments. In interphase, AKAP95 labeling was clearly distinct from the cytoplasmic staining of RIIα (Fig. 2 A, right cell in all panels). At mitosis, RIIα exhibited a more uniform cytoplasmic labeling that mostly overlapped with that of AKAP95 (Fig. 2 A, left cell in all panels). Although both AKAP95 and RIIα were detected by immunoblotting in interphase whole cell lysates (Fig. 2 B, left), immunoprecipitation of RIIα from such lysates did not coprecipitate AKAP95 in interphase, but did coprecipitate AKAP95 at mitosis (Fig. 2 B, right). Immunoprecipitation of RIIα also coprecipitated AKAP95 from both a mitotic cytosolic fraction and from mitotic chromatin solubilized by micrococcal nuclease digestion (Fig. 2 C). This illustrates the assembly of both soluble and chromatin-associated AKAP95–RIIα complexes at mitosis.

The dynamics of formation of the chromatin-associated mitotic AKAP95–RIIα complex was investigated using a cytosolic extract prepared from mitotic HeLa cells. The extract supports disassembly of exogenous purified interphase HeLa nuclei, including nuclear envelope breakdown and chromatin condensation (see Materials and Methods). Immunofluorescence and immunoblotting analysis of input nuclei and condensed chromatin showed that AKAP95 was associated with the condensing chromatin (Fig. 3 A, α-AKAP95, and B, top panel). As in vivo, a minor portion of AKAP95 was also released into the cytosol during chromatin condensation (data not shown). Concomitantly, RIIα, undetected in input interphase nuclei, was recruited from the extract onto the chromatin surface where it colocalized and cofractionated with AKAP95 (Fig. 3 A, α-RIIα and merge, and B, bottom panel). Furthermore, immunoprecipitation of RIIα from condensed chromatin coprecipitated AKAP95 (Fig. 3 C), illustrating the formation of a chromatin-associated AKAP95–RIIα complex during chromatin condensation in vitro. It is noteworthy that association of AKAP95 with chromatin also occurred in mitotic extract immunodepleted of RIIα (data not shown), indicating that mitotic redistribution of AKAP95 is independent of RIIα.

A together, these results indicate that in mitotic extract, nuclear AKAP95 is redistributed and primarily associates with condensing chromosomes in an RIIα-independent manner. Concomitantly, AKAP95 recruits RIIα from a cytosolic pool onto chromatin. Thus, one function of chromatin-associated AKAP95 at mitosis is to target PKA, via RIIα, to condensing chromosomes.

Mitotic Chromosome Condensation Requires Functional AKAP95

The functional significance of the chromatin-associated AKAP95–RIIα complex was first investigated using the mitotic extract. To this end, we attempted to block the function of nuclear AKAP95 by introducing affinity-purified anti–AKAP95 antibodies (or control preimmune rabbit IgGs) into purified HeLa nuclei as described in Materials and Methods. A antibody-loaded nuclei (Fig. 4 A, 1 input) were exposed to mitotic extract and nuclear disassembly assessed after 2 h by DNA staining and immunofluorescence analysis using anti-lamin B and anti-LBR antibodies. Fig. 4 A shows that anti–AKAP95 antibodies, but not preimmune IgGs, inhibited chromatin condensation although nuclear envelope disassembly took place as judged by the absence of lamin B or LBR labeling. Inhibition of chromatin condensation occurred with most (~90%) chromatin masses examined (see Fig. 4 C, Poly) and was reproduced by mAb b36, whereas mAb b47 was little effective (Fig. 4 C). Inhibition of chromatin condensation was specific for anti–AKAP95 antibodies since it did not occur with the following: anti–AKAP95 antibodies introduced into nuclei with 0.1 mg/ml of the GST–AKAP95 A1–386 peptide (Fig. 4 B) used to generate the antibodies (Fig. 4 C, Poly + AKAP95, mAb b36 + AKAP95); and affinity-purified polyclonal antibodies against heterochromatin
proteins H P1α (Fig. 4 C), H P1β, or H P1γ (not shown) (M inc et al., 1999).

Preincubation of anti-A K A P95 antibodies in the extract or immunodepletion of soluble A K A P95 did not inhibit condensation (Fig. 4 C). This illustrates a role for nuclear rather than cytosolic A K A P95 in chromatin condensation. Additionally, loading nuclei with anti-A K A P95 antibodies did not block the recruitment of R I I α onto the chromatin, as judged by immunofluorescence and immunoblotting analyses of condensed chromatin (data not shown). This argues that inhibition of chromatin condensation with anti-A K A P95 antibodies does not result from inhibition of the PKA-anchoring activity of A K A P95.

To determine whether inhibiting nuclear A K A P95 function in vivo affected mitotic chromosome condensation, nuclei of H e l a cells synchronized in S phase by a double thymidine block were microinjected with ~2–5 pg of anti-A K A P95 polyclonal antibodies or mAb b36, each with or without 250 pg of competitor GST-A K A P95Δ1-386 peptide, or with preimmune IgGs. Successful nuclear injections were verified by coinjection of a 150-kD FITC-dextran and scored by retention of the dextran within the nuclei 1 h after injection (Fig. 5 A, a, green). Injected cells were released from the thymidine block, exposed to 1 µM nocodazole, and the proportion of cells arrested at mitosis was determined after 17 h. A fraction of injected cells was also analyzed by immunofluorescence using antibodies against L B R, a marker of the inner nuclear membrane. Fig. 5 shows that, after nocodazole treatment, cells injected with anti-A K A P95 antibodies did not undergo chromosome condensation, as shown by the absence of detectable metaphase chromosomes under bright field microscopy (Fig. 5 A, c and d) and after DNA staining in most cells (Fig. 5 C), and by mitotic indexes of 15–20% (Fig. 5 B). In contrast, preimmune IgGs or anti-A K A P95 antibodies injected with GST-A K A P95Δ1-386 did not pre-
Mitotic Chromosome Condensation Does Not Require Anchoring of PKA to AKAP95 nor PKA Activity

The recruitment of RIIα by AKAP95 onto condensing chromatin led us to determine whether the putative role of AKAP95 in chromatin condensation required association with RIIα and anchoring of PKA. To this end, the RII-anchoring inhibitor peptide Ht31 (500 nM) was preincubated in mitotic extract before adding nuclei. Chromatin condensed to the same extent with Ht31 and control Ht31-P peptides that do not compete binding of RII to AKAPs (data not shown). Thus, the role of AKAP95 in chromosome condensation in vitro is independent of its RII-anchoring function. Furthermore, neither chromatin condensation nor nuclear envelope disassembly was affected by inhibiting PKA activity in the extract with 1 μM of the PKA inhibitor PKI, or by downregulating cAMP signaling with 100 μM of the cAMP antagonists Rp-8-Br-cAMPS or Rp-8-CPT-cAMPS (data not shown). Thus, chromosome condensation in mitotic extract does not require cAMP signaling or PKA activity.

The effects of disrupting AKAP-RII anchoring and downregulating cAMP/PKA signaling on chromosome condensation at mitosis were investigated by microinjecting nuclei of interphase HeLa cells with 50 nM Ht31, 50 nM Ht31-P, 10 nM PKI, or 10 μM Rp-8-Br-cAMPS, and assessing mitotic indexes after nocodazole treatment as done previously after immunoblocking of AKAP95. As shown in Table I, neither reagent inhibited nuclear envelope breakdown (data not shown) nor chromosome condensation, as judged by mitotic indexes of 79–90%. Therefore, disruption of AKAP95-RII anchoring or cAMP-PKA inhibition does not impair mitotic chromosome condensation.

AKAP95 Function Is Required to Maintain Chromosomes Condensed during Mitosis

Although chromatin condensation occurred normally in the mitotic extract containing anti-AKAP95 antibodies, we consistently observed a phase of decondensation of the chromatin upon prolonged (>4 h) incubation in the extract (data not shown). This raised the hypothesis that inhibition of AKAP95 might affect the morphology of the condensed chromatin. To test this possibility, chromatin was allowed to condense for 2 h in mitotic extract, anti-AKAP95 antibodies were added (1:50 dilution) and chromatin morphology was examined by DNA staining after another 2 h of incubation. Chromatin decondensation was observed in extract containing anti-AKAP95 antibodies (Fig. 6 A, +α-AKAP95), but not in the extract containing preimmune IgGs or anti-AKAP95 antibodies together with 0.1 mg/ml of GST-AKAP95Δ1-386 competitor peptide (Fig. 6 A). Thus, immunoblocking AKAP95 in the extract prevented the chromatin from remaining in a condensed state. Incubation of the extract with 100 μM of the cAMP antagonist Rp-8-Br-cAMPS, and as-
densed form. To determine whether immunodepletion of AKAP95 had a similar effect, chromatin condensed in mitotic extract was purified by sedimentation and further incubated in a fresh extract immunodepleted of AKAP95. Chromatin exposed to AKAP95-depleted, but not mock-depleted, extract underwent decondensation within 2 h (Fig. 6 A), indicating that AKAP95 is implicated in maintaining chromatin in a condensed form. Moreover, the extent of chromatin decondensation after immunoblocking or immunodepletion of AKAP95 was more limited than decondensation of mitotic chromatin exposed to an interphase extract (Fig. 6 A, Interphase), suggesting the involvement of distinct decondensation pathways. Consequently, chromatin decondensation in mitotic extract was referred to as premature chromatin decondensation (PCD).

As PCD might be interpreted as a consequence of premature exit of the extract from the M phase, we tested this possibility by measuring the level of histone H1 kinase activity in the mitotic extract incubated for 2 h without or with anti-AKAP95 antibodies and in AKAP95-depleted extract. Fig. 6 B shows that elevated (mitotic) levels of H1 kinase activity were maintained in antibody-containing and immunodepleted extracts (compare with basal H1 kinase activity in interphase HeLa cell extract). This result indicates that immunoblocking or immunodepleting AKAP95 did not release the extract from the M phase, and lends further support to the hypothesis that PCD and interphase chromatin decondensation involve distinct processes.

Conclusive evidence that AKAP95 was required for maintenance of condensed chromatin in vitro was provided in a rescue experiment. Purified condensed chromatin was exposed to mitotic extract immunodepleted of AKAP95. Increasing concentrations of GST-A KAP95Δ1-386 were added and after 2 h proportions of PCD were determined by DNA staining. GST-A KAP95Δ1-386 dramatically inhibited PCD in a concentration-dependent manner (Fig. 6 C). We determined whether GST-A KAP95Δ1-386 was also able to promote recondensation...
Table I. Disruption of AKA95-RII Anchoring or cAMP/PKA
Inhibition Does Not Inhibit Mitotic Chromosome Condensation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mitotic index (Percent)</th>
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<tbody>
<tr>
<td>None (~25 pl FITC-dextran)</td>
<td>88 ± 8</td>
</tr>
<tr>
<td>α-AKA95 (polyclonal; ~2 pg)</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>α-AKA95 (~2 pg) + GST-AKA95Δ1-386 (250 pg)</td>
<td>80 ± 6</td>
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<tr>
<td>Ht31 (50 nM)</td>
<td>79 ± 8</td>
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<tr>
<td>Ht31-P (50 nM)</td>
<td>82 ± 6</td>
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<tr>
<td>PKI (10 nM)</td>
<td>85 ± 5</td>
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<tr>
<td>Rp-8-Br-cAMPS (10 μM)</td>
<td>90 ± 4</td>
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Nuclei of HeLa cells synchronized in interphase were injected with either 2–5 pg affinity-purified polyclonal anti-AKA95 antibodies, 2–5 pg anti-AKA95 antibodies with 250 pg GST-AKA95Δ1-386 peptide, 50 nM RII-anchoring inhibitor peptide Ht31, 50 nM control Ht31-P peptide, 10 nM PKI, or 10 μM cAMP antagonist Rp-8-Br-cAMPS. Injection volumes were 25–50 pl. Control nuclei were injected with 25–50 pl FITC-dextran only. Cells were released from the thymidine block and synchronized with 1 μM nocodazole to induce M phase arrest. After 17 h in nocodazole, mitotic indexes were determined. Data from 30–50 injected cells per treatment are reported. (Similar results were obtained regardless of the site of injection within the cells; data not shown).

Table I. Disruption of AKA95-RII Anchoring or cAMP/PKA
Inhibition Does Not Inhibit Mitotic Chromosome Condensation

Anchoring of RIIα to AKA95 and PKA Activity Are Required for Maintenance of Condensed Chromatin during Mitosis

Whether AKA95-RII association and cAMP signaling of prematurely decondensed chromatin. Purified condensed chromatin was allowed to undergo PCD for 2 h in AKA95-depleted mitotic extract. Subsequent addition of GST-AKA95Δ1-386 to the extract restored chromatin condensation also in a concentration-dependent manner (Fig. 6 D). Thus, GST-AKA95Δ1-386 was capable of inhibiting PCD and recondensing prematurely decondensed chromatin in AKA95-depleted extract, indicating that AKA95 is required for maintaining chromatin in a condensed form. Furthermore, since anti-AKA95 antibodies do not block binding of RIIα to AKA95, the data also argue that AKA95 has an intrinsic function in the maintenance of condensed chromatin.

The relevance of these in vitro observations on chromosome structure during mitosis was investigated. HeLa cells arrested in the M phase with 1 μM nocodazole were injected with affinity-purified anti-AKA95 antibodies and, after 1 h, chromatin morphology was assessed by phase-contrast and DNA staining while cells remained in nocodazole. Anti-AKA95 antibodies readily induced decondensation of chromosomes that could no longer be distinguished by phase-contrast (Fig. 7, α-AKA95, arrow points to the metaphase chromosomes of a mitotic cell that was not injected). Furthermore, no nuclear envelope was detected, arguing that decondensation was reminiscent of PCD observed previously in vitro and distinct from interphase nuclear reformation. In contrast, coinjection of anti-AKA95 antibodies with 250 pg GST-AKA95Δ1-386 did not alter the state of chromosome condensation (Fig. 7, α-AKA95 + pept.). These results indicate that chromosome decondensation is not an artefactual consequence of exposure of cells to nocodazole. Rather, they show that as in vitro, immunoblocking AKA95 at mitosis induces PCD, indicating that functional AKA95 is needed for maintaining chromosomes condensed throughout mitosis.

Figure 6. AKA95 is required for the maintenance of condensed chromatin in mitotic extract. (A) Immunoblocking or immunodepletion of AKA95 promotes PCD. Morphology of in vitro–condensed chromatin exposed for 2 h to mitotic extract containing either affinity-purified anti-AKA95 antibodies (+α-AKA95), preimmune IgGs (+IgG), or anti-AKA95 antibodies with 0.1 mg/ml GST-AKA95Δ1-386 competitor peptide (+α-AKA95 + GST-AKA95Δ1-386). Alternatively, chromatin was also exposed to mitotic extract immunodepleted and mock-depleted of AKA95 (AKA95-depl. and Mock-depl., respectively). Chromatin was also incubated in interphase extract for 1 h (Interphase). Bar, 10 μm. (B) Histone H1 kinase activity of either mitotic extracts after 2 h of incubation at 30°C without (M) or with anti-AKA95 polyclonal antibodies (M, +α-AKA95), mitotic extract immunodepleted of AKA95 (M, AKA95-depl.), or cell lysis buffer used to prepare the mitotic extracts (Buffer). (C) Inhibition of PCD with GST-AKA95Δ1-386. Purified condensed chromatin was incubated for 2 h in mitotic extract immunodepleted of AKA95 and containing increasing concentrations of GST-AKA95Δ1-386. Proportions of PCD were determined after DNA staining with Hoechst. (D) Restoration of condensation of prematurely decondensed chromatin with GST-AKA95Δ1-386. Purified condensed chromatin was allowed to undergo PCD for 2 h in mitotic extract immunodepleted of AKA95. Increasing concentrations of GST-AKA95Δ1-386 were added, and proportions of recondensed chromatin units determined after another hour of incubation. Through PKA were required for maintenance of condensed chromatin during M phase was examined. Purified condensed chromatin was added to a mitotic extract preincubated with 500 nM Ht31 (or control Ht31-P) peptides to...
disrupt AKAP95–RII interactions (data not shown) and PCD was assessed after 1 h by DNA staining. Fig. 8 A shows that while chromatin remained condensed with Ht31-P, Ht31 induced PCD, indicating a requirement for AKAP95–RII interaction to maintain chromatin condensed. Whether PKA activity and cAMP signaling were involved in this process was determined using PKA inhibitor PKI (1 µM) and the cAMP antagonist Rp-8-Br-cAMPS (100 µM). Both reagents induced PCD, whereas control activation of PKA with 1 µM cAMP had no effect (Fig. 8 A). Adding free C subunits together with anti–AKAP95 antibodies with C also promoted PCD (Fig. 8 A), suggesting that only PKA bound to chromatin-associated AKAP95 was implicated in maintaining condensed chromatin. Together, these results indicate that maintenance of condensed chromatin in mitotic extract requires functional AKAP95, cAMP signaling events mediated by PKA and anchoring of PKA (via RIIα) to chromosomes by AKAP95.

The requirement for RIIα binding to AKAP95 and PKA activity for maintenance of condensed chromatin was tested further by assessing chromatin morphology after immunodepleting RIIα from the extract. Up to 60% of condensed chromatin masses incubated in the mitotic extract depleted of RIIα underwent PCD over a 2-h period (Fig. 8 B). Moreover, immunoblotting analysis of chromatin at the beginning (0 h) and at the end (120 min) of incubation in an RIIα-depleted extract showed that whereas input chromatin harbored RIIα, most RIIα was solubilized by 120 min (Fig. 8 C, bottom). In contrast, no obvious change in the amount of AKAP95 bound to chromatin was detected under these conditions (Fig. 8 C, top). Together with our previous observation that RIIα is recruited from the cytosol to the chromatin via AKAP95, these results reflect a dissociation of RIIα from the chromatin and suggest that, as with AKAP95, keeping RIIα on condensed chromatin involves an equilibrium between soluble and chromatin-associated pools of PKA.

The requirements for AKAP–RII interaction and cAMP/PKA signaling events to maintain chromosomes condensed at mitosis were investigated by microinjecting Phase–arrested HeLa cells with either 50 nM Ht31 or Ht31-P, 10 nM PKI, 10 µM Rp-8-Br-cAMPS, 1 µM cAMP, 15 ng/µL recombinant catalytic subunit of PKA (C), or C plus anti-AKAP95 antibodies. Proportions (percent ± SD) of PCD were determined by DNA staining after 90 min. (B) Chromatin condensed in mitotic extract was purified and exposed to fresh mitotic extract immunodepleted of RIIα. Proportions (percent ± SD) of PCD were determined by DNA staining of sample aliquots at regular intervals. (C) Chromatin fractions at the start (Input) and at the end (120 min) of incubation in RIIα-depleted mitotic extract were sedimented and proteins were immunoblotted using anti-AKAP95 and anti-RIIα antibodies.

**Immunoblocking of AKAP95 Inhibits Recruitment of Eg7 to Chromatin in Mitotic Extract**

Several proteins have been shown to be required for mitotic chromosome condensation, including pEg7 (Cubizolles et al., 1998), a component of the 13S condensin complex (Hirano et al., 1997). To determine whether AKAP95 was part of the condensin complex or interacted with the complex, immunoprecipitations were carried out first from lysates of interphase or mitotic HeLa cells. Immunoblotting analysis of human Eg7 using a polyclonal antibody...
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against the 15-carboxy-terminal amino acids of human Eg7 revealed that interphase and mitotic cells harbored similar levels of Eg7 (Fig. 10 A). Immunoprecipitation data showed that AKAP95 and Eg7 did not coprecipitate from interphase cell lysates, indicating that these proteins do not interact at this stage of the cell cycle (Fig. 10 B). In contrast, in mitotic cell lysates, AKAP95 and Eg7 coprecipitated regardless of the antibody used (Fig. 10 C), suggesting that AKAP95 and Eg7 interact at mitosis. Since AKAP95 was found to be associated with chromatin at mitosis (Fig. 1 C), we tested the possibility that the AKAP95–Eg7 interaction was mediated by DNA. To this end, mitotic chromatin was isolated, solubilized with micrococcal nuclease, and the DNA was digested with 400 μg/ml DNase I in the presence of protease inhibitors. Immunoprecipitation of AKAP95 or Eg7 from the DNase-treated fraction revealed that both proteins also coprecipitated (Fig. 10 D), suggesting that the mitotic AKAP95–Eg7 interaction is not mediated by DNA and is probably direct.

pEg7, the Xenopus homologue of human Eg7, has been previously shown to be recruited onto condensing chromosomes at mitosis (Cubizolles et al., 1998). Results from our laboratory have shown that chromatin condensation in the mitotic extract is accompanied by association of Eg7 with chromatin (Landsverk, H., K.L. Guellec, and P. Colinas, unpublished observation). Since pEg7 is required for chromosome condensation, we determined whether immunoblocking nuclear AKAP95 would affect the recruitment of Eg7 to chromatin in vitro. Nuclei loaded with pre-immune IgGs (−α-AKAP95) or anti-AKAP95 antibodies (+α-AKAP95) were allowed to condense in mitotic extract. After 2 h, chromatin masses (Ch) were sedimented and immunoblotted using anti-Eg7 antibodies.

Discussion

This paper provides evidence that AKAP95 is a multivalent protein with a role in chromosome condensation at mitosis. AKAP95 acts as an anchor for a PKA-signaling complex onto mitotic chromosomes, which is required for maintenance of chromosomes in a condensed form throughout mitosis. AKAP95 also appears essential for the recruitment onto chromosomes of Eg7, a component
of the condensin complex. The data also provide insights on the significance of the rise in cAMP level and PKA activity during mitosis.

Several lines of evidence illustrate a function of AKA95 in chromosome condensation and maintenance of chromosomes in a condensed form during mitosis. First, immunoblocking intranuclear AKA95 inhibits chromatid condensation in vitro and in vivo. Second, immunoblocking AKA95 during mitosis or in vitro, or immunodepletion of soluble AKA95 from the mitotic extract, induces PCD. Inhibition of chromatid condensation with anti-AKA95 antibodies is unlikely to result from a nonspecific steric effect of the antibodies since antibodies against all variants of the heterochromatin protein H1 (Minc et al., 1999) are ineffective. Clearly, AKA95 is implicated in chromatid condensation before PKA associates with chromatin and interacts with AKA95. This is consistent with the established downregulation of PKA required for mitotic nuclear envelope breakdown (Lamb et al., 1991) and with the absence of PKA in interphase nuclei (Eide et al., 1998). Thus, in principle, AKA95 and RII cannot interact until the nuclear envelope has broken down. However, once nuclear envelope disassembly has occurred, the role of AKA95 in maintaining condensed chromosomes requires cAMP/PKA signaling and AKA95–PKA interaction.

A third observation supporting the involvement of AKA95 in regulating chromosome structure at mitosis is the finding that a recombinant AKA95 fragment (GST-AKA95Δ1-386) prevents PCD in AKA95-depleted extract, and restores condensation of prematurely decondensed chromatin, both in a dose-dependent manner. Thus, the domain(s) of AKA95 required for this activity and for interaction of AKA95 with chromatin reside(s) in the carboxy-terminal 306 amino acids (amino acids 387–692) of the protein. As expected from the RII binding requirement for maintenance of condensed chromatin, this AKA95 fragment also contains the RII binding motif (Eide et al., 1998).

The consequences of immunoblocking AKA95 may be accounted for by the hypothesis that AKA95 function may be disrupted by antibodies only when they bind AKA95 that is not associated with chromatin. Indeed, the antibodies block condensation when incorporated into interphase nuclei before association of AKA95 with chromatin (Landsverk, H., and P. Collas, unpublished results). Thus, it is possible that anti-AKA95 antibodies incubated in mitotic cytosol fail to inhibit chromatid condensation because by the time the nuclear envelope breaks down, nuclear AKA95 has already associated with chromosomes. Similarly, induction of PCD in cytosol immunodepleted of AKA95 suggests that the establishment of an equilibrium between chromosome-associated and soluble AKA95 is critical for maintenance of condensation. By binding AKA95 when it dissociates from chromosomes as well as soluble AKA95, the antibodies may sharply decrease the on-rate of AKA95 to chromosomes, causing decondensation of the chromatin.

Since it associates with chromatin early during mitotic nuclear disassembly, AKA95 could conceivably either directly affect chromatin structure, or facilitate the recruitment of factors controlling mitotic chromosome condensation (Hirano and Mitchison, 1994; Hirano et al., 1997). Sequence analysis of human AKA95 (Eide et al., 1998) reveals that AKA95 is unlikely to belong to the family of SM C proteins, factors shown to affect chromosome structure at mitosis and during development (Strunnikov, 1998). Unlike SM C proteins, AKA95 displays no coiled-coil domains, no amino-terminal ATP-binding sites and no DNA box, a signature motif of SM Cs (Strunnikov et al., 1993). Likewise, AKA95 displays no consensus site for topoiso merase II (A dachi et al., 1991); thus, unlike SM Cs or topoiso merase II, AKA95 is probably unlikely to directly impose a conformational change on chromatin inducing condensation. Rather, our data suggest that a function of AKA95 is to allow the recruitment of components of the condensin complex. First, immunoblocking of AKA95 inhibits association of Eg7 with chromatin in vitro. Eg7 is the human homologue of X. e nus pEg7, a protein associated with the 13S condensin complex (Hirano and Mitchison, 1994; Cubizolles et al., 1998), suggesting that anti-AKA95 antibodies prevent binding of the condensin complex onto chromosomes. Second, AKA95 can be coprecipitated with Eg7 from solubilized, DNase-digested mitotic chromatin, suggestive of an interaction between AKA95 and Eg7 that is not mediated by DNA. Collectively, these findings raise the attractive possibility that one function of AKA95 is to facilitate recruitment and/or anchoring of condensins to chromatin at mitosis.

The multivalent nature of AKA95 is not only evidenced by its role in chromatid condensation, but also by the observation that AKA95 maintains a crucial PKA-anchoring function during mitosis. Whereas AKA95 and RII are found in a different compartment in interphase, a chromosome-associated AKA95–PKA signaling complex is established at mitosis. Induction of PCD after immunodepletion of RIIα from mitotic cytosol reflects an equilibrium between chromatin-associated (via AKA95) and soluble RIIα, and a requirement for a soluble pool of RIIα to maintain the chromatin fraction of the AKA95–RIIα complex saturated and functional. Disruption of PKA anchoring with H31 peptides induces PCD in vitro and in vivo, indicating that the AKA95–PKA interaction is essential for the maintenance of condensed chromatin. Several physiological functions implicating AKA95-anchored PKA have been reported using similar disruption approaches (Colledge and Scott, 1999). Formation of the AKA95–PKA complex early during chromosome condensation may be critical to mediate the increasing cAMP signal as mitosis progresses (Grieco et al., 1996). It is conceivable that maintenance of a condensed chromatin structure results from PKA-dependent phosphorylation of chromatin substrates such as histones (van Hooser et al., 1998; Wei et al., 1999) or other DNA-related structural regulators such as topoiso merases (Roberge et al., 1990). Our results provide some significance for the rising level of cAMP and increasing PKA activity during mitosis (Grieco et al., 1996). Implications of PKA downregulation in entry into mitosis have been addressed previously (Lamb et al., 1991). It has been reported that cAMP levels and PKA (type II) activity are significant (Costa et al., 1976) or even rise (Grieco et al., 1996) during mitosis. We propose that rising cAMP levels during mitosis activate
that AKAP95 is likely to be a multivalent targeting molecule identified. Nevertheless, our results extend the concept that AKAP95 is required to ensure proper chromosome condensation and nuclear reassembly during mitosis (Grieco et al., 1996). This scenario implies the induction of antagonistic effects of cAMP/PKA signaling on chromosome structure at mitosis that are temporally distinct. It also implicates the existence of a molecular switch upstream of the cAMP/PKA pathway, that controls the dual effect of cAMP/PKA activity at mitosis. A likely candidate might be M phrase-promoting factor, as a threshold level of this activity has been shown to be required to initiate activation of the cAMP/PKA pathway during mitosis (Grieco et al., 1996). The dual effect of PKA during mitosis and at the exit of mitosis could also be due to the redistribution of AKAP95, which dissociates from PKA at mitosis exit.

Although the structural determinants mediating AKA-PKA interactions in general are being characterized (Colledge and Scott, 1999), whether additional processes modulate these associations is unknown. Circumstantial evidence suggests the involvement of posttranslational modification of RII in such regulation. First, RIIα is primarily localized in the centrosome–Golgi area (K ey er et al., 1998) in interphase HeLa cells, presumably via a centrosomal AKA-P (Witczak et al., 1999). Release of RIIα from centrosomes at mitosis correlates with CDK1-mediated phosphorylation of RIIα (K ey er et al., 1998). Second, CDK1-mediated phosphorylation of RIIα in vitro is sufficient to induce partial solubilization of RIIα from a Triton X-100 insoluble pool (K ey er et al., 1998). Third, association of AKAP95 with RIIα is mitosis-specific. Finally, decondensation of in vitro-condensed chromatin in an interphase extract is associated with release of RIIα from chromatin-associated AKAP95 (Collas, data not shown). This argues that binding of RIIα to AKAP95 at mitosis is not a mere consequence of both proteins being in the same subcellular compartment; rather, a modification of either protein seems to affect their interaction. Together with the data of K ey er et al. (1998), our results argue for a cell cycle-regulated redistribution of RIIα, suggesting an additional mechanism regulating PKA type II subcellular localization.

A new emerging feature of AKAPs is their ability to anchor entire signaling complexes other than PKA to specific substrates. To date, AKAP79, AKAP220, gravin, yotiao/AKA P450/CG-NAP, and ezrin have been shown to act as polyvalent anchoring proteins for signaling units involving PKA or protein kinase C together with protein phosphatases (Colledge and Scott, 1999). An additional binding partner of AKAP95 is AKAP that has yet to be identified. Nevertheless, our results extend the concept that AKAP95 is likely to be a multivalent targeting molecule anchoring signaling complexes as well as chromatin remodeling factors in a cell cycle-regulated manner. We are excited to determine whether additional AKAPs also harbor specific intrinsic cellular functions.

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