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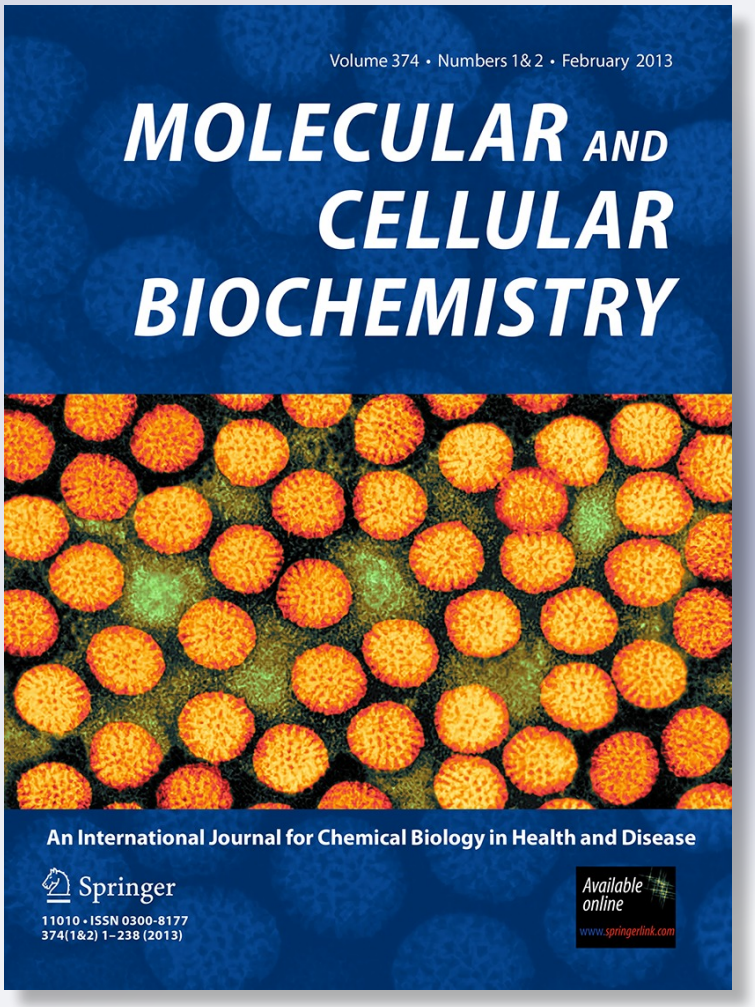

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INMAP, a novel truncated version of POLR3B, represses AP-1 and p53 transcriptional activity

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Abstract INMAP was first identified as an interphase nucleus and mitotic apparatus-associated protein that plays essential roles in the formation of the spindle and cell-cycle progression. Here, we report that INMAP might be conserved from prokaryotes to humans, is a truncated version of the RNA polymerase III subunit B POLR3B, and is up-regulated in several human cancer cell lines including HeLa, Bel-7402, HepG2 and BGC-823. Deletion analysis revealed that the 209–290 amino-acid region is necessary for the punctate distribution of INMAP in the nucleus. Furthermore, over-expression of INMAP inhibited the transcriptional activities of p53 and AP-1 in a dose-dependent manner. These results suggest that INMAP may function through the p53 and AP-1 pathways, thus providing a possible link of its activity with tumourigenesis. Integrating our data and those in previous studies, it can be concluded that INMAP plays dual functional roles in the coordination of mitotic kinetics with gene expression as well as in cell-fate determination and proliferation.

Keywords INMAP · POLR3B · p53 · AP-1

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Introduction

Three nuclear DNA-dependent RNA polymerases (Pol I, Pol II and Pol III) in eukaryotes share the task of transcribing the information contained in genes into RNA entities [1, 2]. Pol III transcribes a diverse group of small noncoding RNAs, including tRNAs, 5S rRNA, U6 snRNA, 7SL RNA and others [3, 4]. These RNAs participate in the regulation of transcription, splicing and translation. After transcription, Pol III molecules are either directly degraded or modified for participation in the regulation and execution of processes in the nucleus and the cytoplasm including transcription regulation, RNA processing, ribosome assembly, translation and others which ultimately leads to protein synthesis. Pol III is highly conserved from yeast to humans and is composed of 17 subunits. Its two largest subunits, Rpc1 (~160 kDa) and Polr3B (~130 kDa), provide a large surface area for the interaction with many of the other subunits [4]. Transcription by Pol III is precisely regulated in normal cells, but this regulation is lost during tumourigenesis [5]. Previous studies have revealed that Pol III transcription is negatively regulated in normal cells by tumour suppressor gene products (e.g. Rb, p53, BRCA1, Maf1 or PTEN) or other factors (e.g. MAF1 and EGCG), and its transcription is activated via multiple signal transduction cascades including the MAP kinase and the PI3 kinase pathways [5–8].

Recessive mutations in *POLR3B* cause a rare hypomyelinating leukodystrophy in humans [9], and a deficiency of Pol III subunit C11, which mediates Pol III RNA cleavage activity and is important for the termination of transcription, disrupts zebrafish digestive development [10].

AP-1 (activator protein-1) is a heterodimeric transcription factor composed of proteins belonging to the c-Fos, c-Jun, and ATF (activating transcription factor) families. It directly binds to a DNA motif with the sequence 5′-TGAGTCA-3′ in promoters [11]. Both cells and mice deficient in AP-1 proteins exhibit abnormal cell proliferation, neoplastic transformation and apoptosis [12–16]. AP-1 proteins control cell proliferation and death through their ability to regulate the expression of cell-cycle regulators, such as cyclin D1, p53, $p21^{cip1/waf1}$, $p19^{ARF}$ and p16 [17–23]. Numerous studies have suggested that p53 is an important tumour suppressor protein [24, 25]. The *p53* gene is mutated in approximately half of human tumours [26–32]. Furthermore, p53 is a transcription factor whose activity gives rise to a variety of cellular outcomes, mainly cell-cycle arrest and apoptosis [33–36].

We previously cloned the gene encoding INMAP, an interphase nucleus and mitotic apparatus-associated protein, and found that it is highly homologous with the 3′-terminal sequence of *POLR3B* through bioinformatics analysis [37]. In addition, INMAP plays an essential role in spindle formation and cell-cycle progression [37]. However, the molecular mechanism by which INMAP is involved in cell-cycle progression and its relationship with POLR3B has not been clarified.

In this study, we demonstrated that INMAP is a novel truncated version of POLR3B, might be evolutionarily conserved from prokaryotes to humans, and is over-expressed in several cancer cell lines. We found that the 209–290 amino-acid (aa) region is responsible for the nuclear localisation of INMAP. Moreover, over-expression of INMAP in HEK293T cells repressed AP-1 and p53 transcriptional activity in a dose-dependent manner.

Materials and methods

Cell culture, reagents and antibodies

HeLa, HEK293T, A375, MCF-7, BGC823, HepG-2 and Bel-7402 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) (Gibco-BRL, USA) supplemented with 10 % (v/v) foetal bovine serum (Invitrogen, New Zealand) in a CO_2 (5 %) incubator at 37 °C. DAPI, penicillin and streptomycin were purchased from Sigma. Other reagents included the high-efficiency transfection reagent Vigofect (Vigoruse, Beijing, China), geneticin G418 (Merck, USA) as well as anti-FLAG, anti-tubulin and anti-GAPDH monoclonal antibodies (Proteintech Group, Inc., USA). The anti-INMAP monoclonal antibody was obtained as previously described [37]. Alkaline phosphatase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG antibodies were obtained from Vector Laboratories, Peterborough, UK. Oligonucleotides were synthesised by Sangon (Shanghai Sangon Biotechnology, Shanghai, China).

Plasmid construction

3XFLAG-*INMAP* was generated by PCR using pEGFP-C3-*INMAP* as a template and the following pair of primers: 5′ primer (GGAATTCCATGGGGCCCATGTTG) and 3′ primer (CGGGATCCTTCATTGTACTTGGACAG) containing restriction site for *Eco*R1 and *Bam*H1, respectively (underlined). The PCR fragment was then cloned into the p3XFLAG-CMV14 expression vector (Sigma, St. Louis, MO) [37]. The PCR reaction programme was 94 °C for 5 min followed by 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min for 30 cycles, with a final extension at 72 °C for 10 min.

For the synthesis of digoxigenin-labelled INMAP and POLR3B RNA probes, *INMAP* and *POLR3B* DNAs were obtained by PCR from plasmids p3Xflag-*INMAP* and pEGFP-C3-*POLR3B*, respectively. The PCR products were then cloned into the pGEM-T vector containing SP6 and T7 promoters (Promega, China).

pEGFP-C3-truncated *INMAP* plasmids were constructed by PCR using pEGFP-C3-*INMAP* as a template with *Eco*R1linked 5′ primers (Table S1 in supplement) and *Bam*H1-linked 3′ primers (Table S1 in supplement) and then cloned into the p3XFLAG-CMV14 expression vector.

Northern blot analysis

Total RNA extraction from HeLa cells was performed using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Digoxigenin-labelled anti-sense RNA probes for human *INMAP* (nucleotides 138–643) and human *POLR3B* (nucleotides 448–849) were synthesised using the DIG Northern Starter kit (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). In brief, 2 μg of total RNA was separated on a 1.5 % (w/v) denaturing agarose gel (MP-Agarose, Boehringer-Mannheim, Germany) in 1 × MOPS buffer containing 4 % (v/v) formaldehyde. After electrophoresis, the gels were immersed and gently shaken in 20 × SSC (3 M NaCl, 0.3 M citric acid, pH 7.0) for 30 min at room temperature. Capillary transfer of RNAs was conducted with 20 × SSC for 12 h to a positively charged nylon membrane (Boehringer-Mannheim, Germany), and RNAs were cross-linked to the membrane

by exposure to UV (254 nm, 125 mJ). The membranes were prehybridised in 0.05 mL/cm^2 DIG Easy Hyb solution (Boehringer-Mannheim, Germany) at 68 °C. For hybridisation, the solution was replaced with an equal amount of DIG Easy Hyb containing 100 ng/mL of labelled and denatured *INMAP* or *POLR3B* probes and incubated at 68 °C overnight [38]. Chemiluminescent detection was performed according to the procedure of the DIG Northern Starter kit.

Cell culture, transient transfection and subcellular localisation analyses

HeLa cells used in these studies were maintained and passaged according to previously described standard methods [39] and were transfected with pEGFP-C3-truncated *INMAP* plasmid DNAs using Vigofect according to the manufacturer's instructions. 48 h after transfection, the localisation of the fusion protein was visualised with an Olympus laser-scanning confocal microscope (Olympus Fluoview FV300, Japan) after labelling with DAPI for nuclear staining. HeLa cells transfected with the pEGFP-C3 vector were used as a control.

Reporter gene assays

HEK293T cells were cultured as described above and supplemented with 100 U/mL of penicillin and streptomycin. For luciferase assays, the recombinant vectors were transiently transfected into HEK293T cells using the high-efficiency transfection reagent Vigofect according to the manufacturer's instruction. 48 h later, the cells were harvested, and the luciferase activity assay was performed according to the protocols developed by Stratagene. Each experiment was performed in triplicate, and each assay was repeated at least three times. The means of the data from three individual transfected wells were calculated after normalisation for β-galactosidase.

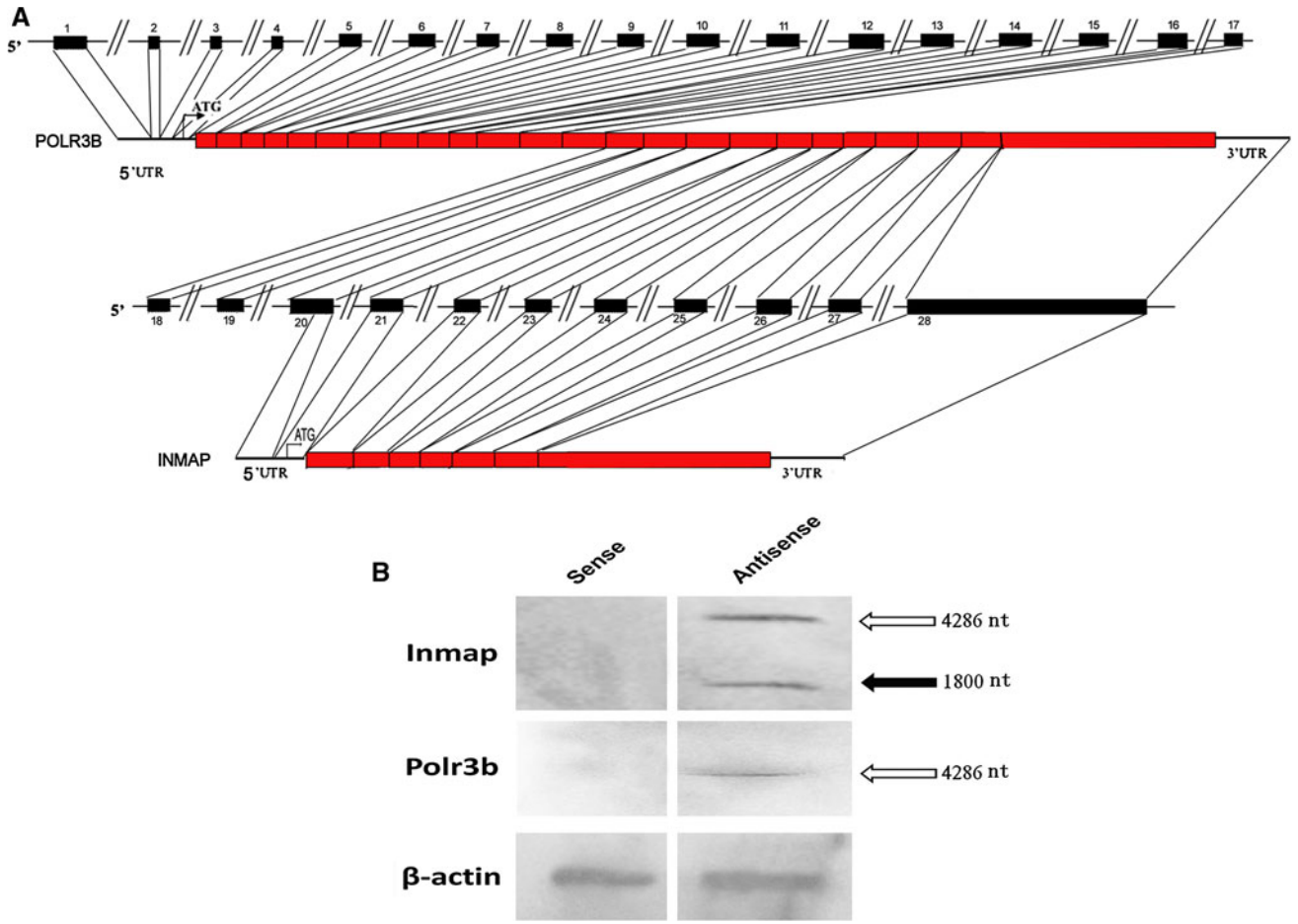

Fig. 1 Northern blot analysis of POLR3B and INMAP transcripts. **a** Genomic structure of *INMAP*. The exons are depicted by *black* boxes. The intron–exon organizations of *POLR3B* and *INMAP* are shown. ATG indicates the translation start site. **b** Northern blot analysis of *INMAP* and *POLR3B* mRNAs in HeLa cells

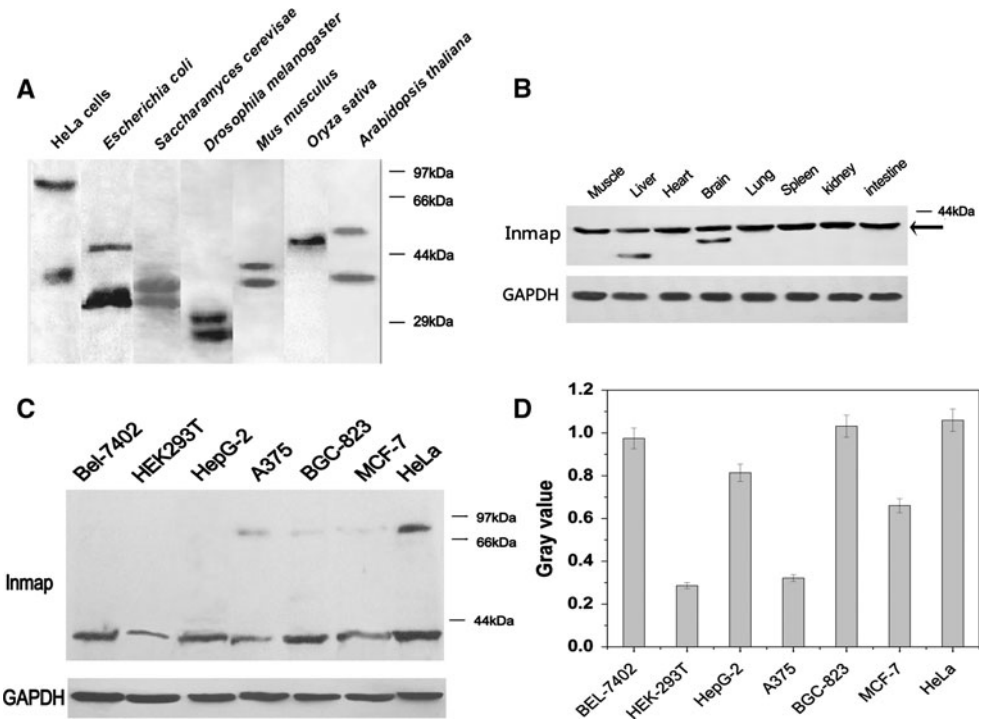

Fig. 2 An INMAP-specific monoclonal antibody detects strong signals in a wide range of organisms. **a** Western blot analysis of total protein extracts from various organisms as indicated using an INMAP-specific monoclonal antibody. **b** Western blot analysis of INMAP protein expression in multiple mouse tissues. GAPDH was used as a loading control. **c** Western blot analysis of INMAP protein expression in several cell lines. GAPDH was used as a loading control. **d** Band quantification of Fig. 2**c**

Western blot analysis

Protein concentrations of cultured cells and mouse tissues were determined using the Bradford protein assay with bovine serum albumin (BSA) as the standard. Protein extracts were separated in 12 % SDS–polyacrylamide electrophoresis (SDS-PAGE) gels and electrotransferred onto nitrocellulose membranes. The membranes were blocked in 5 % fat-free milk and processed by incubation with primary and HRP-conjugated secondary antibodies. Protein bands were visualised using ECL and Kodak BioMix films.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Analysis of variance was performed using *t*-tests.

Results

INMAP, a novel truncated version of POLR3B

We previously reported that *INMAP* gene is highly homologous to the 3′-terminal sequence of *POLR3B* [37]. Figure 1a shows the proposed genomic organization of *INMAP* gene. Comparison of *POLR3B* genomic sequence and the cDNA sequence of *INMAP* revealed that the *INMAP* cDNA begins at a site within the 20th exon of *POLR3B*. All exon/intron boundaries followed the classical GT/AG rule (data not shown). Translation from an ATG codon in the 21st exon of *INMAP* generates a protein of 343 amino acids corresponding to the region between amino acids 791 and 1,133 of POLR3B. Human genomic BLAST shows that *INMAP* is mapped to chromosome 12 (in the region of *POLR3B* at 12q23.3). As the use of an alternative promoter within the coding sequence of p53 has been shown to yield a truncated protein Δ113p53 [40], we wanted to determine whether two transcripts exist that encode POLR3B and INMAP, respectively. To this end, we conducted Northern blot analysis of mRNAs prepared from HeLa cells using digoxigenin-labelled *INMAP* probe. As shown in Fig. 1b, we indeed detected two distinct bands with estimated sizes of 4,286 and 1,800 bases, sufficiently large to encode POLR3B and INMAP, respectively. In comparison, the digoxigenin-labelled *POLR3B* probe complementary to the 5′-terminal sequence of *POLR3B* detected only the upper band. Thus, the results suggest that the *INMAP* transcript is a novel truncated version of *POLR3B* probably transcribed from a promoter within the coding sequence of *POLR3B*.

Evolutionary conservation of INMAP

We have previously reported that INMAP is expressed in HeLa cells, murine erythroleukemia cells and the budding yeast [37]. Here, we performed Western blotting analysis of total proteins from HeLa cells, *Escherichia coli, Saccharomyces cerevisiae, Drosophila melanogaster, Mus musculus, Oryza sativa* and *Arabidopsis thaliana* with an INMAP-specific monoclonal antibody [37]. The result revealed the

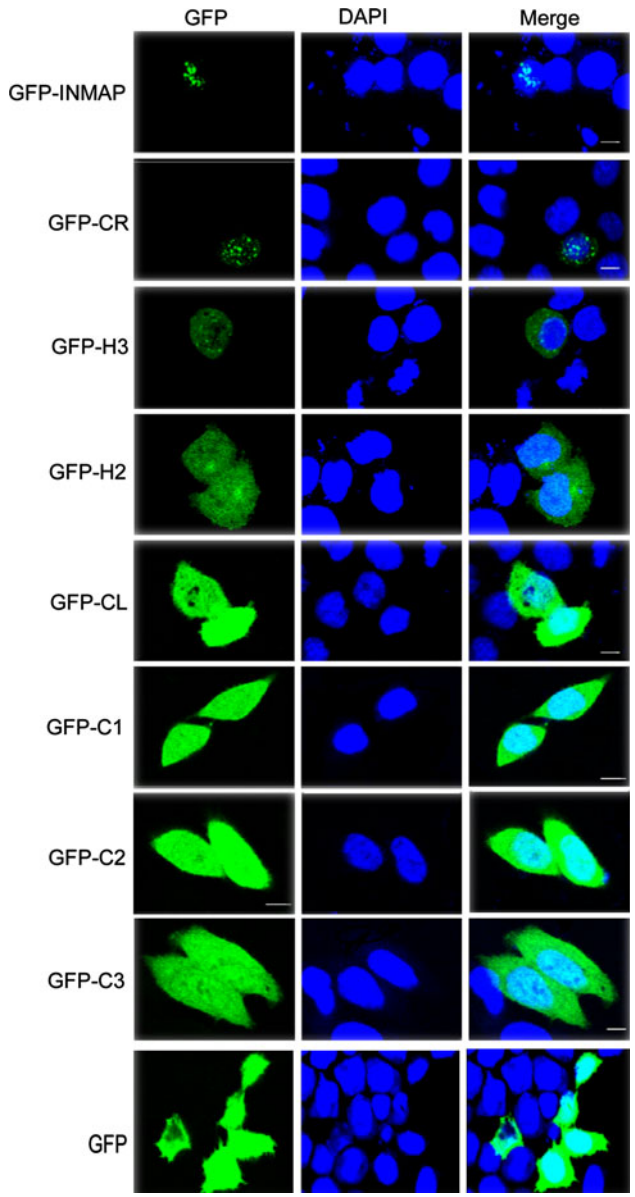

Fig. 3 Subcellular localisation of INMAP and various truncated versions of INMAP. Constructs expressing INMAP or various truncated versions of INMAP with a GFP tag were individually transfected into HeLa cells. The cells were counterstained with DAPI to label the DNA and examined by confocal microscopy

presence of immunoreactive polypeptides in all the seven species with molecular weights ranging from ~27 to 50 kDa (Fig. 2a). As shown, two bands were detected in all the species except *O. sativa*. In addition, the result suggests that INMAP might be evolutionarily conserved. Furthermore, Western blot analysis of eight tissues from adult mice detected a ~40 kDa band, matching the predicted molecular weight of INMAP, in skeletal muscle, heart, lung, spleen, kidney, intestine, liver and brain tissues, and an additional smaller band in the liver and brain (Fig. 2b). We also found that INMAP is expressed in the normal cell line HEK293T with a single ~40 kDa band as well as in the liver cancer cell lines HepG-2 and Bel-7402 (Fig. 2c). As shown previously, the HeLa cell protein extract showed two distinct bands at ~40 and 80 kDa, respectively. The same two bands were also observed in A375, BGC-823 and MCF-7 cancer cell lines. The distinct appereance of the 80 kDa INMAP variant in cancer cells suggests a possible link to tumourigenesis. Furthermore, we found significantly elevated cellular levels of INMAP in HepG-2, Bel-7402, BGC-823, HeLa and MCF-7 cancer cell lines compared with normal cell lines after normalisation to GAPDH as a loading control (Fig. 2d), suggesting that up-regulation of INMAP may contribute to carcinogenesis.

Cellular localisation of INMAP in HeLa cells

By Western blot analysis of nuclear and cytoplasm fractions of cells, we have found that INMAP is primarily a nuclear protein during the interphase (Fig. S1). And our previous deletion analysis revealed that the C-terminal part of INMAP is responsible for its nuclear localisation [37]. Here, we constructed a series of C-terminally truncated versions of INMAP based on bioinformatics analysis of the domain organization (Fig. S2A). Using PCR with oligonucleotide primers (Table S1) and pEGFP-C3-INMAP template, we created plasmids to express seven fragments of *INMAP* (named H3, H2, C1, C2, C3, CR and CL) corresponding to peptides 209–294, 209–270, 101–200, 181–265, 244–343, 101–265 and 181–343 aa, respectively. Each of the fragments was ligated into the expression vector pEGFP-C3 for expression as a GFP fusion protein. The recombinant vectors were then transiently transfected into HeLa cells. As shown in Fig. 3, only the cells expressing either CR or H3 exhibited the typical dots in the interphase nucleus. By contrast, the other five fragments were expressed with diffuse GFP signals throughout both the nucleus and the cytoplasm similar to the localization pattern in the control cells expressing GFP alone. Together, the results indicate that the H3 domain, which is relatively shorter and contains three α-helices (Fig. S2), carries the signals required for the specific localisation of INMAP in the nucleus. It is necessary to be indicated that H2 in fact displays dotted signal but is concealed by diffuse signal, that is to say, inappropriate length may produce poor signal–noise ratio (Fig. S2B). Does it mean that a third α-helix, as in H3 or CR, inhances more specificity? Deeper investigation is required.

Over-expression of INMAP inhibits p53 and AP-1 transcriptional activities in HEK293T cells

As abnormal RNA Pol III activity is a feature of cancer cells in which p53 is inactive [41, 42], we wanted to determine whether over-expression of INMAP affects p53 regulated

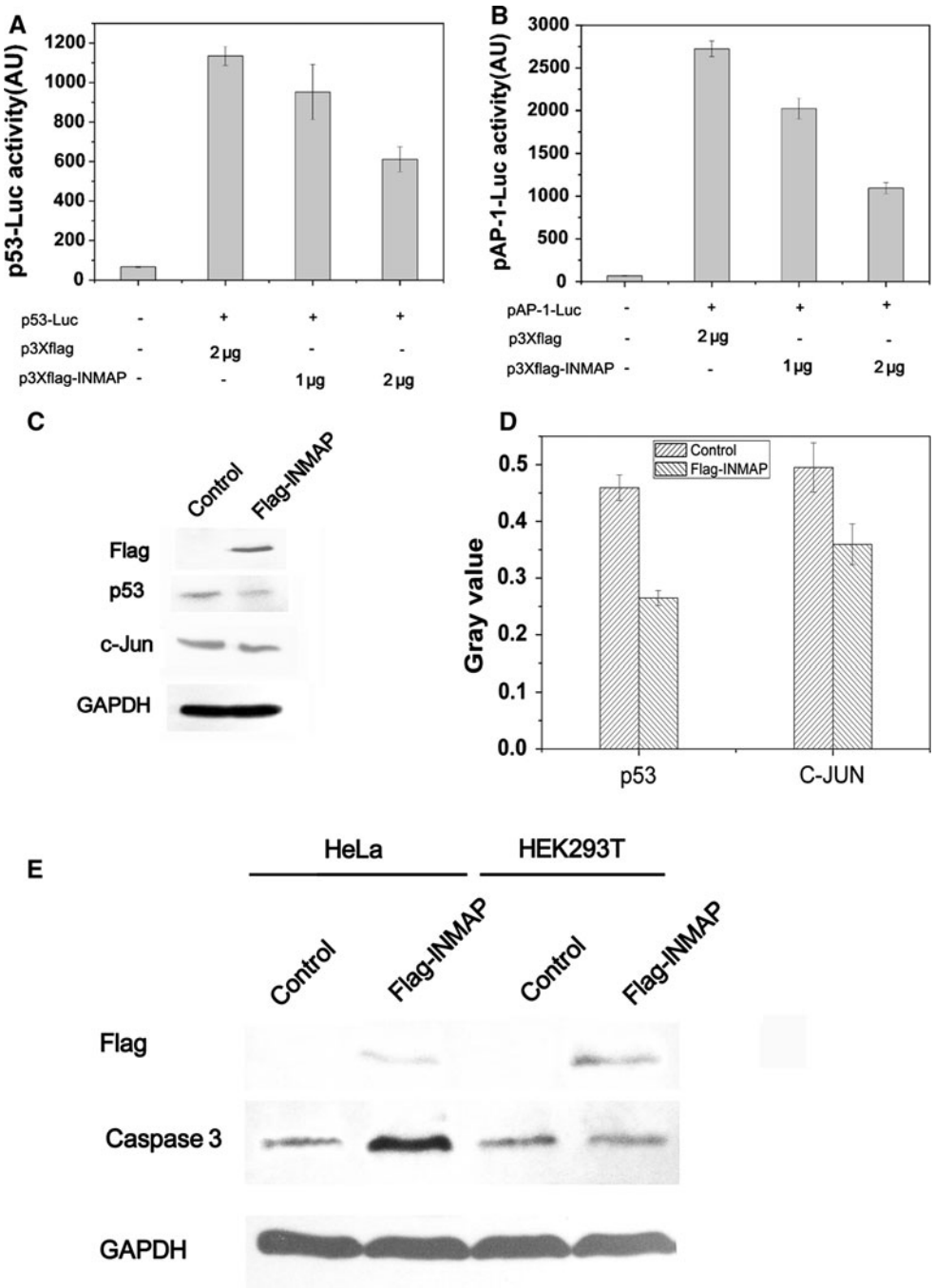

Fig. 4 Over-expression of INMAP suppressed the transcriptional activities of p53 and AP-1. (A, B) 48 h after transient transfection, the luciferase activity assay was performed according to the manufacturer's instruction. The data are the means of three repeats in a single transfection experiment after normalisation for β-galactosidase activity. **a** p53-Luc was co-transfected without or with 3XFLAG-INMAP fusion protein in varying doses (1, 2 μg). **b** pAP-1-Luc was co-transfected without or with 3XFLAG-INMAP fusion protein in varying doses (1, 2 μg). **c** Western blot of cell lysates of transfected HEK293T cells. The membranes were probed with anti-FLAG antibody, anti-p53 antibody and anti-c-Jun antibody, respectively. GAPDH was used as a loading control. **d** Band quantification of Fig. 4**c**. **e** Western blot of cell lysates of transfected HeLa and HEK293T cells. The membranes were probed with anti-FLAG antibody and anti-caspase-3 antibody, respectively. GAPDH was used as a loading control. All experiments were performed in triplicate and repeated three times. The data presented are representative

genes. First, we performed pathway-specific reporter gene assays to measure the modulation of p53 by INMAP in HEK293T cells. A fixed amount of p53-Luc and varying amounts of p3XFLAG-CMV–INMAP were co-transfected into HEK293T cells, and the total amount of DNA was equalised by adding the vector p3XFLAG-CMV. Figure 4a

and Fig. S3A show that INMAP suppressed the dose-dependent activation of the p53 reporter gene ($P < 0.05$). As the transcription factor AP-1 is known to regulate p53 expression [21–27], we next tested the effect of INMAP on the activity of AP-1 and found that the expression of INMAP significantly inhibited AP-1 activity in a dose-dependent manner (Fig. 4b and Fig. S3B). In addition, Western blot analysis showed that over-expression of INMAP caused down-regulation of p53 and c-Jun proteins in HEK293T cells (Fig. 4c, d). As shown in Fig. 4e, caspases-3, a preapoptotic signalling cascade protein, is up-regulated in HeLa cells with over-expression of INMAP. While, over-expression of INMAP in HEK293T cells does not influence on expression of caspase-3. Together, our data demonstrate that over-expression of INMAP inhibited the transcriptional activities of p53 and AP-1.

Discussion

AP-1 and p53 are tumour suppressors that may induce cell-cycle arrest or apoptosis in response to environmental duress [43–45]. Cancers generally evade this defence by inactivating p53 [46]. p53 can activate the c-Jun NH2-terminal kinase (JNK) pathway [47], and AP-1 has been linked to the modulation of p53 pathways to explain the role of AP-1 in cell survival [48]. Recently, it has been reported that MKK7, the upstream activator of JNK, functions as a major tumour suppressor in lung and mammary cancers in mouse through the kinases JNK1 and JNK2, and this signalling pathway directly combines oncogenic and genotoxic stress to the stability of p53 [49]. Besides, the growth arrest DNA damage-inducible gene 45β (GADD45β) which binds tightly to MKK7 forms a homodimeric complex and it does not disrupt the effect of MKK7 [50, 51]. Abnormal RNA Pol III activity is a feature of cancer cells in which *p53* is inactive [41, 42]. Thus, functional interaction between p53 and RNA Pol III may play an important role in the control of cell growth and, perhaps, tumour formation. Our results suggest that INMAP, as a sort of 'abnormal RNA Pol III subunit', may participate in the transcriptional regulation of certain genes. Our unpublished finding indicated that transfection with the anti-sense INMAP resulted in cell death, which may be due to the inactivation of the mRNAs for both POLR3B and INMAP. Perhaps more work should be performed to distinguish and discern the actually functional role of both POLR3B and INMAP protein on cell-cycle or tumour formation. In addition, our observation of the up-regulation of INMAP in several cancer cell lines suggests a possible role for INMAP in cancer. This hypothesis is consistent with the dose-dependent suppression of the transcriptional activity of AP-1 and p53 by INMAP over-expression in HEK293T cells (Fig. 4). Furthermore, we found that the cellular levels of p53 and c-Jun proteins were lower in cells that over-expressed INMAP than in control cells. Finally, we have found that the different function of INMAP on cell apoptosis may be related with dissimilar cells. Therefore, INMAP may play a role in the signal transduction through AP-1 and p53 which may ultimately affect the cell-cycle progression, leading to tumourigenesis or apoptosis.

We have previously reported the association of INMAP with α-tubulin, γ-tubulin and NuMA, suggesting a role for INMAP in the formation and stablization of the mitotic spindle [37]. The inhibition of p53 by INMAP over-expression is consistent with the reports that abnormal POLR3B is a feature of cancer cells in which *p53* is inactive [41, 42], conforming to the association of the AP-1-p53 pathway with carcinogenesis [52, 53].

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