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## Ets-1 interacts through a similar binding interface with Ku70 and Poly (ADP-Ribose) Polymerase-1

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### ABSTRACT

The Ets-1 transcription factor plays an important role in various physiological and pathological processes. These diverse roles of Ets-1 are likely to depend on its interaction proteins. We have previously showed that Ets-1 interacted with DNA-dependent protein kinase (DNA-PK) complex including its regulatory subunits, Ku70 and Ku86 and with poly (ADP-ribose) polymerase-1 (PARP-1). In this study, the binding domains for the interaction between Ets-1 and these proteins were reported. We demonstrated that the interaction of Ets-1 with DNA-PK was mediated through the Ku70 subunit and was mapped to the C-terminal region of Ets-1 and the C-terminal part of Ku70 including SAP domain. The interactive domains between Ets-1 and PARP-1 have been mapped to the C-terminal region of Ets-1 and the BRCA1 carboxy-terminal (BRCT) domain of PARP-1. The results presented in this study may advance our understanding of the functional link between Ets-1 and its interaction partners, DNA-PK and PARP-1.

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Ets-1 is the founding member of the Ets family characterized by a well-conserved DNA binding domain (ETS domain). It regulates gene expression by binding to specific DNA elements, called ETS-binding sites, found in the promoters of its target genes [1]. By binding to these elements, Ets-1 activates the transcription of various genes involved in numerous cellular mechanisms, including angiogenesis, apoptosis, and tumour invasion [2]. Human full-length Ets-1 can be subdivided structurally and functionally into different domains. Two inhibitory domains flank and interact with the ETS domain and inhibit its DNA binding [3]. The N-terminal inhibitory domain belongs to the exon VII-encoded region, a target of calcium mediated-phosphorylation [4] and of caspase-3-mediated cleavage [5]. Ets-1 harbors also a transactivation domain in the middle portion of the protein [6] and a Pointed domain in the N-terminus of the protein which is involved in protein-protein interaction [7].

Ets-1 activity is modulated by interactions with a number of factors. Most proteins that directly contact Ets-1 bind to the ETS domain. Others associate with the exon VII-encoded region, the Pointed domain or the transactivation domain [2]. In a search for additional interaction partners that might regulate Ets-1 functions, we previously purified Ets-1-bound proteins using a streptavidin pull-down assay with biotinylated Ets-1, which we have developed [8]. With this strategy, we have demonstrated an interaction

between Ets-1 and two DNA repair proteins, DNA-PK [9] and PARP-1 [10]. DNA-PK is a nuclear serine/threonine protein kinase composed of a catalytic subunit, DNA-PKcs, and two regulatory subunits, Ku86 and Ku70. DNA-PK is implicated in a variety of cellular processes, including the repair of double-stranded DNA breaks, V(D)J recombination, telomere maintenance and transcriptional regulation [11]. The interaction of DNA-PK with Ets-1 causes its phosphorylation even in the absence of DNA. Although DNA was originally shown to be a potent activator of DNA-PK [12], we and others have indicated that protein interactions may be another, more important mechanism for the activation of DNA-PK kinase activity [9,13,14].

In addition to DNA-PK subunits, another protein was found in the Ets-1-interacting complex, corresponding to PARP-1 [10]. PARP-1 is an abundant and ubiquitous nuclear protein that catalyses poly(ADP-ribosylation) (PARylation) by using NAD<sup>+</sup> as substrate to synthesize branched poly (ADP-ribose) polymers on target proteins. PARP-1 plays diverse roles in many molecular and cellular processes, including DNA damage detection and repair, chromatin modification and transcriptional regulation [15,16]. Ets-1 activates, by direct interaction, the catalytic activity of PARP-1 and is then PARylated in a DNA-independent manner. Under catalytic inhibition of PARP-1, Ets-1 transcriptional activity is enhanced which correlates with Ets-1

proteins accumulation in cell nuclei and an increase in DNA damage that leads to cancer cell death [10].

In order to better understand the functional consequences of the interactions between Ets-1 and its protein partners, DNA-PK and PARP1, and essentially how Ets-1 can stimulate their activity, we set out to identify the DNA-PK subunit that physically interacts with Ets-1 and the regions of their interaction and then we characterize the domains of Ets-1 and PARP-1 required for their interaction. Collectively, these results establish the regions important for the Ets-1/DNA-PK and Ets-1/PARP-1 interactions, providing mechanistic insights into the better understanding the physiological roles of Ets-1 and the interacting factor.

## Materials and methods

### Expression vector construction

The construction of bacterial expression vectors containing the cDNA sequences of human Ets-1 p51, Ets-1 p27 and N-terminal deletion mutant  $\Delta$ N331 have been previously described [17,18]. The construction of pTyb2 vector expressing human Ets-1 p51 fused to a biotin tag has been previously described [8]. pGEX-2TK vectors expressing human full-length Ku70 and Ku80 fused to a GST as well as deletion mutant constructions of GST-Ku70 were kindly provided by Professor S. P. Jackson (University of Cambridge, UK) [19]. pET32a-His-PARP-1<sub>24</sub> and pET32a-His-PARP-1<sub>89</sub> vectors, expressing the caspase cleavage products of PARP-1 with hexahistidine tag (His), were generously provided by Professor M. S. Satoh (Laval University, Canada) [20]. To produce BRCT domain of PARP-1 fused to a GST tag, the sequence coding amino acids 378–479 of PARP-1 was obtained by PCR amplification, using the following primers: 5'-CAGT AGGGATCCGCTGCTGTGAACTCCTCTGC-3' and 5'-GTCAACGAATTCGGACAAGATGTGCGCTAAG A-3'. The PCR product was then cloned between the *Bam*HI and *Eco*RI restriction sites in a pGEX-2TK (GE Healthcare®) prokaryotic expression vector. Constructs were checked for nucleotide sequence.

### Expression and purification of untagged and biotinylated recombinant Ets-1

Proteins Expression and purification of Ets-1, Ets-1- $\Delta$ N331 and Ets-1 p27 were carried out according to [17,18], by using the T7 Impact System (New England Biolabs®). Biotinylated Ets-1 protein was produced and purified as described previously [8], using the principle of the T7-Impact™ System adapted to induce biotinylation.

### Expression and purification of GST-fusion proteins and Histidine-tagged (His) proteins

The expression plasmids pGEX-2TK and pET32a-6His were transformed into *E. coli* (BL21-(DE3)-pLysS) cells. *E. coli* cells were grown in 50 mL Luria-Bertani (LB) medium containing 50  $\mu$ g/mL ampicillin and 0.5% glucose at 30°C under shaken until the cells reached a turbidity value of 1 at OD<sub>600</sub>. 50 ml LB medium containing 50  $\mu$ g/ml ampicillin, 0.5% glucose and 2.5  $\mu$ g/mL vitamin B1 was added and cells continued to grow at 30°C until they reached exponential phase. Cells were then harvested by centrifugation at 4000g for 10 min and resuspended in 200 ml of LB medium containing 50  $\mu$ g/mL ampicillin, 2.5  $\mu$ g/mL vitamin B1 and 0.3 mM isopropyl-b-D-thiogalactopyranoside (IPTG). The culture was incubated at 30°C and shaken for 16 hours. The culture was then harvested and the pellet was washed with phosphate-buffered saline (PBS) solution and suspended in 20 mL of lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, Complete™ protease inhibitor cocktail (Roche Molecular Biochemicals) and 20  $\mu$ g/mL DNase I (Roche)). Bacteria were lysed with EmulsiFlex-C3 (Avestin®) according to the manufacturer's instructions. GST and GST fusion proteins were affinity purified with glutathione-sepharose beads (GST-bind™ Resin, Novagen®) for 16 h at 4°C and under constant rotation. The column was washed and then stored at 4°C. For His-tagged proteins, they purified in sepharose-nickel beads (His Catch™, Bionline®) for 16 h at 4°C and under constant rotation. Beads were washed and eluted with 30 mM imidazol (Sigma-Aldrich®).

### GST pull-down assays

Glutathione-sepharose beads (20  $\mu$ L) coupled with the purified GST proteins were blocked by incubating for 30 min at 4°C in PBS with 17 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich®). Ets-1 (1  $\mu$ g), Ets-1 p27 (500 ng) or Ets-1- $\Delta$ N331 (250 ng) proteins were incubated with the prepared glutathione-agarose beads for 1 hour at 4°C under constant rotation. After washing the beads three times with buffer containing 50 mM Tris pH 8; 150 mM NaCl; 0,1 mM EDTA; 0,5% Igepal and 0,5% Triton X-100, proteins bound to the beads were eluted by boiling for 5 min in Laemmli buffer (50 mM Tris pH 6.8; 2% SDS; 5%  $\beta$ -mercaptoethanol; 10% glycerol; 0,1% bromophenol blue).

### Sterptavidin pull-down assays

Streptavidin sepharose™ beads (Amersham Biosciences, GE Healthcare, Velizy-Villacoublay, France) (30 mL) were washed with PBS and blocked

by incubating for 1 h at 4°C in PBS with 1 mg/mL BSA. Biotinylated full-length Ets-1 protein (5 mg) was incubated with the prepared streptavidin sepharose<sup>TM</sup> beads for 30 min at 4°C under constant rotation. The Ets-1 loaded beads were then incubated for 1 h at 4°C with 1 µg of recombinant PARP-1 protein (Trevigen, Gaithersburg, MD, USA), 800 ng of 6His-PAPR-1<sub>89</sub> or 220 ng of 6His-PARP-1<sub>24</sub>. After extensive washing, bound proteins were eluted by boiling for 5 min in Laemmli buffer and analysed by Western blot.

### Western blot analyses

Western blot analyses were performed according to [5]. Primary antibodies used were C-20 anti-Ets-1, H-250 anti-PARP-1 and H-15 anti-poly-His from Santa Cruz Biotechnology (Heidelberg, Germany).

## Results

### *Ets-1 interacts with Ku heterodimer via the C-terminal end containing the SAP domain of Ku70*

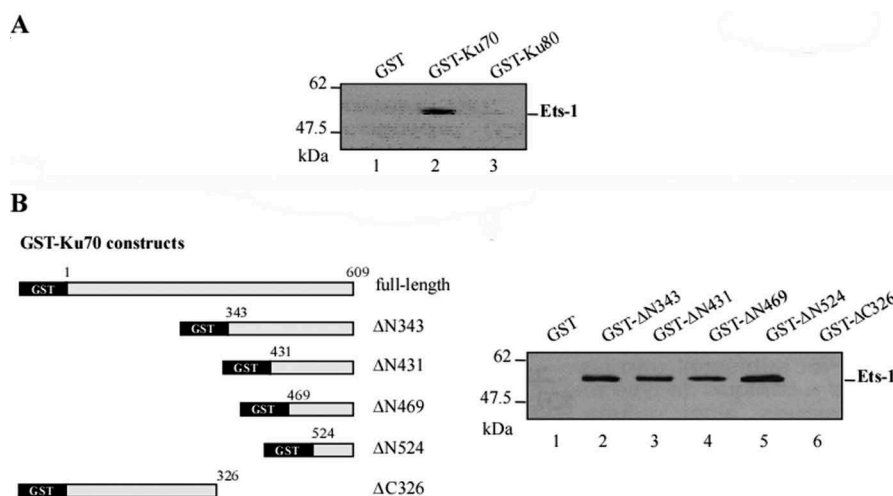
Based on our previous study identifying DNA-PK components as novel proteins that associate in a complex with Ets-1 *in vitro* and in cells [9], and knowing that regulatory subunits Ku70 and Ku86 often interact with proteins that regulate DNA-PK kinase activity [13,14], *in vitro* GST pull-down assay was carried out to compare the potential direct interactions of Ku subunits with Ets-1. GST, GST-Ku70 and GST-Ku86 fusion proteins were expressed in *E. coli* and purified on glutathione-sepharose beads. As a

binding partner, recombinant Ets-1 was expressed and purified using the T7 Impact System. Interestingly, as shown in Figure 1(a), Ets-1 interacted with Ku70 (lane 2), but failed to interact with Ku86 (lane 3). These data confirm that Ets-1 associates with the DNA-PK through direct interaction with the Ku70 subunit.

The interaction domains of Ets-1 and Ku70 were further mapped with a set of deletion mutants of Ku70 fused to GST (ΔN343, ΔN431, ΔN469, ΔN524 and ΔC326) (Figure 1(b), left panel) using GST-pull down assay. The full length Ets-1 interacted with all Ku70 N-terminal deletions that retained the C-terminal from amino acid 524–609 (Figure 1(b); right panel; lanes 2–5), but failed to interact with Ku70 that lacked the C-terminal beyond amino acid 326 (Figure 1(b); right panel; lane 6), indicating that the C-terminal end of Ku70 is required for Ets-1 interaction. Thus, the smallest peptide of Ku70 shown to interact with Ets-1 was amino acids 524–609 located at the C-terminal part including SAP domain (574–609), a well-known DNA-binding motif (named after the three proteins that contain SAF-A/B, Acinus and PIAS motif).

### *A C-terminal region of Ets-1 interacts with the C-terminal end containing the SAP domain of Ku70*

Our previous work showed that besides the full-length form of Ets-1, its two alternatively spliced isoforms, Ets-1 p42 and Ets-1 p27, interact also with DNA-PK [9]. Given that the C-terminal region, consisting of the ETS domain and the C-terminal



**Figure 1.** Ets-1 full-length interacts with Ku heterodimer through the C-terminal SAP domain of K70. (A) GST-Ku70, GST-Ku86 or GST alone as a negative control were expressed in *E. coli* and purified with Glutathione-Sepharose beads. Purified proteins (on beads) were incubated with full-length untagged Ets-1 (1 µg), which was bacterially expressed using the T7 Impact System. After 1h, the beads were washed, and the bound fractions were separated by SDS-PAGE and subjected to Western blot analysis using a C-20 anti-Ets-1 antibody. (B) Schematic diagrams depict the different GST-Ku70 constructs used in the domain-mapping experiments. GST-Ku70 derivatives were produced, as described in section (A) above, and then incubated with Ets-1 protein. The bound proteins were separated by SDS-PAGE and subjected to Western blot analysis using a C-20 anti-Ets-1 antibody.



inhibitory domain, is a common region shared between the three Ets-1 isoforms [18,21], we suggest that Ku70 interacts with this region of Ets-1 protein. To test that, C-terminal deletion mutant  $\Delta$ N331 (Figure 2, top panel) was produced in *E. coli* and subjected to GST pull-down assay using GST-Ku70  $\Delta$ N469, GST-Ku70  $\Delta$ N524 and GST-Ku70  $\Delta$ C326. As shown in Figure 2, Ets-1  $\Delta$ 331 was pulled down by the interaction with GST-Ku70  $\Delta$ N469 and GST-Ku70  $\Delta$ N524 fusion proteins (Figure 2; lanes 2 and 3), whereas no interaction was noted with GST-Ku70  $\Delta$ C326 (Figure 2; lane 4). This result coincides with the GST pull-down assay with full-length Ets-1 data indicating that the binding site of Ets-1 to the Ku70 SAP domain was at the C-terminal part including the ETS domain.

### A c-terminal region of Ets-1 interacts with PARP-1 via the BRCT domain

We previously demonstrated that PARP-1 specifically and directly interacts with Ets-1 [10]. To identify the regions of PARP-1 that interact with Ets-1, we used two polyhistidine-tagged deletion mutants of PARP-1 corresponding to its cleavage products under apoptotic conditions: a 24 kDa N-terminal fragment with zinc finger (ZF) motifs 1 and 2 (ZF1 and ZF2) (PARP-1<sub>24</sub>) and an 89 kDa C-terminal fragment with ZF3 motif, an automodification region with BRCT domain, and a catalytic domain (PARP-1<sub>89</sub>) (Figure 3(a)). These recombinant proteins and the untagged full-length form were incubated with biotinylated Ets-1 immobilized on streptavidin beads. We confirmed that full-length PARP-1 interacted with Ets-1 (Figure 3(b-a), lane 2) but not with empty beads (Figure 3(b-a), lane 1).

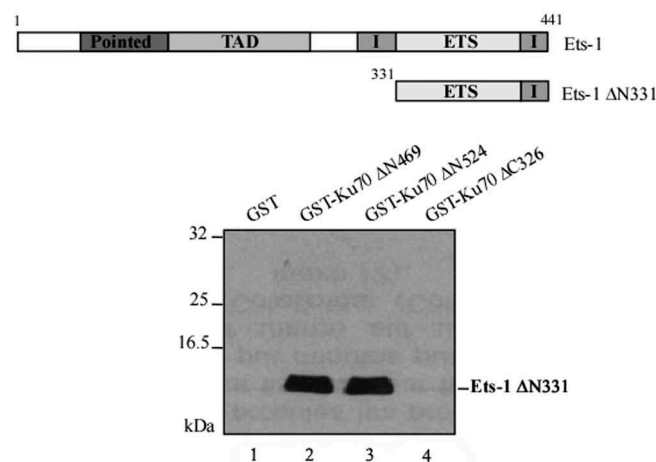
Furthermore, we found that Ets-1 interacted with PARP-1<sub>89</sub> (Figure 3(b-b), lane 2) but not with PARP-1<sub>24</sub> (Figure 3(b-b), lane 4). These results indicate that only C-terminal fragment is required for binding.

Given that BRCT domain is known to be an important protein-protein interaction module [22,23], we hypothesized that this domain might be important for the interaction between PARP-1 and Ets-1. To test that, BRCT domain is generated fused to GST (Figure 3(a)) and then subjected to GST pull-down assay. The result showed that BRCT domain of PARP-1 bound the Ets-1 protein (Figure 3(c-a)). A possible contribution from the other domains of PARP-1<sub>89</sub>, namely ZF3 motif and catalytic domain, was not examined and thus cannot be ruled out.

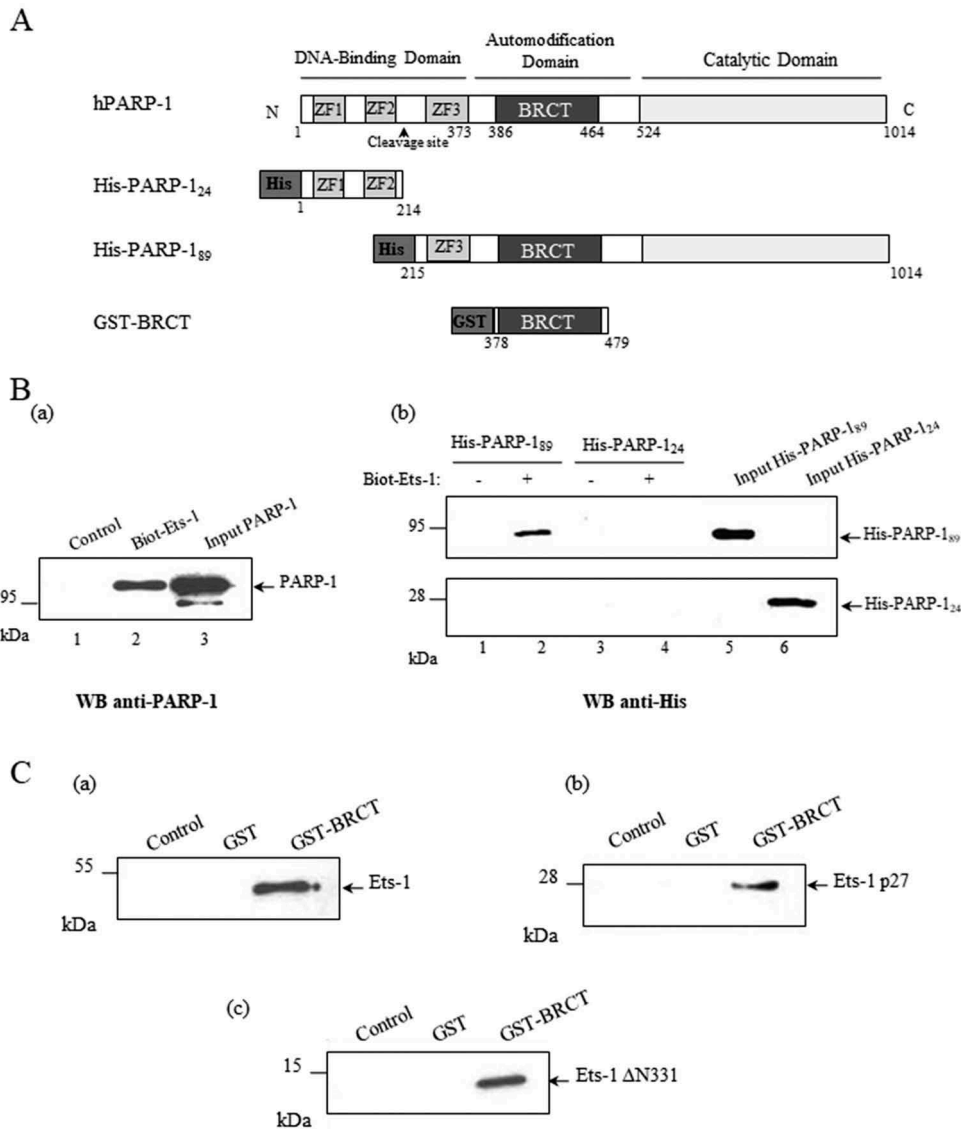
Our previous work showed that Ets-1 p27 isoform containing the ETS domain surrounded by two inhibitory domains interacts directly with PARP-1 [10]. To test whether this interaction is mediated by PARP-1 BRCT domain, GST pull-down assay was used. The result shows that like Ets-1, Ets-1 p27 interacts with the BRCT domain (Figure 3(c-b)). To further delimit the interaction zone, we carried out GST pull-down with GST-BRCT domain and Ets-1  $\Delta$ N331. As shown in Figure 3(c-c), Ets-1  $\Delta$ N331 is pulled down with GST-BRCT. These results indicate that PARP-1 BRCT domain and the Ets-1 C-terminal region containing ETS domain are required for their interaction.

## Discussion

In the previous studies, we found that Ets-1 interacted with DNA-PK components [9] and with PARP-1 [10]. In the present study, we further analyzed how Ets-1 interacts with these DNA repair proteins and



**Figure 2.** The interaction of Ets-1 with Ku70 is mediated by its C-terminal region including the ETS domain. Interaction experiment was performed with the GST forms of Ku70 proteins including the C-terminal (GST- $\Delta$ N469 and GST- $\Delta$ N524) or the N-terminal (GST- $\Delta$ C326) parts of Ku70 and with Ets-1  $\Delta$ N331 deletion mutant (top panel). After 1 h of incubation of Ets-1  $\Delta$ N331 (1  $\mu$ g) with each of GST-Ku70 derivatives (on beads), the collected beads were washed, and re-suspended in Laemmli buffer. The bound fractions were separated by SDS-PAGE and subjected to Western blot analysis using a C-20 anti-Ets-1 antibody directed against its C-terminal region.



**Figure 3.** Mapping of interaction domains between Ets-1 and PARP-1. (A) Schematic representation of human PARP-1 domain structure and constructs used in these experiments. Functional domains are indicated, caspase-3 cleavage site is marked with a head arrow and numbers indicate amino acid positions. (B) Streptavidin beads loaded with biotinylated Ets-1 (Biot-Ets-1) (lane 2, left panel and lanes 2 and 4, right panel) or unloaded beads (lane 1, left panel and lanes 1 and 3, right panel) were incubated with full-length PARP-1 (1  $\mu$ g), His-PARP-1<sub>89</sub> (800 ng) or His-PARP-1<sub>24</sub> (220 ng). Bound proteins were resolved by SDS-PAGE and detected by Western blot (WB) using anti-PARP-1 (left) and anti-His (right) antibodies. Inputs correspond to the amount of proteins used for the pull-down. (C) GST or GST-BRCT were expressed in *E. coli* and purified with Glutathione-Sepharose beads. Purified proteins (on beads) were incubated with Ets-1 (1  $\mu$ g), Ets-1 p27 (500 ng) or Ets-1  $\Delta$ N331 (250 ng). Bound proteins were resolved by SDS-PAGE and detected by Western blot using a C-20 anti-Ets-1 antibody. Lane 1, control with unloaded beads.

we found that Ets-1 interacts through its C-terminal region with DNA-PK via the SAP domain of the regulatory subunit Ku70 and with PARP-1 through BRCT domain.

The two Ku subunits show similarity both in sequence and in topology, each containing three well organized regions: an N-terminal  $\alpha/\beta$  domain, a central  $\beta$ -barrel domain, and a subunit-specific helical C-terminal arm. The human Ku70 subunit has C-terminal domain composed of a highly random and flexible linker (residues 536–560) and a well structured helix-extended loop-helix fold (residues 574–609), also referred to as the SAP domain [24]. Consistently, Ets-1 interacts with Ku70 but not Ku86

via a SAP domain uniquely found in Ku70. This is consistent with other study demonstrating the interaction of the homeoproteins, Dlx1 and Dlx2, with Ku70 but not Ku86 through the SAP domain [14].

Ku is a protein that binds DNA in a non-sequence-specific manner and regulates DNA-PKs catalytic activity. Although DNA was originally shown as a potent activator of DNA-PKs, other studies [14,25] indicate that the multiple protein factors recruited by the Ku subunit may also serve as activators to stimulate DNA-PK activity. For instance, several homeodomain proteins, including Oct-1, have been shown [14] to interact with Ku70 and enhance DNA-PK phosphorylation. Consistent

with their finding, Ets-1 interacts with Ku70 but not Ku86. This interaction may consequently result in stimulation of DNA-PK in the absence of DNA. The DNA-independent stimulation of DNA-PK by direct interaction with Ku70 was also observed in other proteins such as in thyroid hormone receptor-binding protein (TRBP) [13]. It appears that although DNA-PK can be stimulated by DNA ends [12], or by other kinases [26,27], protein interactions via Ku70 may be another more important but previously less appreciated mechanism for DNA-PK activation.

In addition to DNA-PK subunits, we have identified a physical interaction between Ets-1 and PARP-1, and we have shown that the Ets-1/PARP-1 interaction is important for the PARylation of Ets-1 in a DNA-independent manner [10]. In this paper, we further mapped the interacting domains between Ets-1 and PARP-1. We found that PARP-1 associates with Ets-1 through its BRCT domain already described as platforms for protein-protein interaction with several partners [22,23].

DNA damage was shown as a potent activator of PARP-1 [28]. The multiple domains of PARP-1 collapse onto damaged DNA, forming a network of interdomain contacts that introduce destabilizing alterations in the catalytic domain leading to an enhanced rate of poly(ADP-ribose) production [29]. We and others have indicated that protein-protein interaction may also stimulate PARP-1 activity in the absence of DNA damage [10,30,31] and that most of the proteins that are PARylated by PARP-1 interact with the BRCT domain [32,33]. The BRCT domain is not strictly required for DNA damage-dependent activity, but its positioning within the protein will have important implications for how PARP-1 recruits partner proteins which stimulates PARP-1 activity in the absence of DNA, and in turn, PARP-1 PARylates them. It will be interesting to investigate whether interaction with the BRCT domain can lead to the same changes observed in PARP-1 DNA-dependent activation.

In order to better understand Ets-1/Ku70 and Ets-1/PARP-1 interactions and as the solution structures of the PARP-1 BRCT domain [34], the Ku70 SAP domain [35] and the Ets-1 ETS domain [36] were previously determined, it will be interesting to perform studies designed to elucidate these interactions. Therefore, we can attempt to co-crystallize these interacting domains in order to determine the essential contact areas and to determine the structure of the complexes. Furthermore, due to the existence of the structural data for each of the domains, it is also possible to complete this structural approach by molecular modeling of the complexes. These structural studies make it possible to identify the residues essential to these interactions. Moreover, the role of domain-domain interaction in the functional

relevance of Ets-1/Ku70 and Ets-1/PARP-1 interactions, previously characterized [9,10], need further study to broaden our understanding of the functional role of Ets-1 interacting proteins. Nevertheless, we have demonstrated that the short isoform Ets-1 p27, containing only the C-terminal region of Ets-1 including the ETS domain, activates both DNA-PK and PARP-1 by direct interaction and is then phosphorylated and PARylated, respectively, in a DNA-independent manner.

In summary, we have revealed that Ets-1 interacts through its C-terminal region containing the ETS domain with the SAP domain of Ku70 and with the PARP-1 BRCT domain. These observations raise a number of interesting questions on the physiological importance of these interactions and the different cross-talks between these proteins. Addressing these questions will add new insight into the understanding of the biology of Ets-1.

### Author contribution

Choul-li S.: acquisition of data, analysis and interpretation of data, drafting of manuscript

Legrand A.: data collection, analysis and interpretation of data

Vicogne D.: data collection

Villeret V.: critical revision

Aumercier M.: design of the work, critical revision, final approval of the version to be published

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### Disclosure statement

No potential conflict of interest was reported by the authors.

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