

# Differential Localisation of PARP-1 N-Terminal Fragment in PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> Murine Cells

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Human PARP family consists of 17 members of which PARP-1 is a prominent member and plays a key role in DNA repair pathways. It has an N-terminal DNA-binding domain (DBD) encompassing the nuclear localisation signal (NLS), central automodification domain and C-terminal catalytic domain. PARP-1 accounts for majority of poly-(ADP-ribose) polymer synthesis that upon binding to numerous proteins including PARP itself modulates their activity. Reduced PARP-1 activity in ageing human samples and its deficiency leading to telomere shortening has been reported. Hence for cell survival, maintenance of genomic integrity and longevity presence of intact PARP-1 in the nucleus is paramount. Although localisation of full-length and truncated PARP-1 in PARP-1 proficient cells is well documented, subcellular distribution of PARP-1 fragments in the absence of endogenous PARP-1 is not known. Here we report the differential localisation of PARP-1 N-terminal fragment encompassing NLS in PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> mouse embryo fibroblasts by live imaging of cells transiently expressing EGFP tagged fragment. In PARP-1<sup>+/+</sup> cells the fragment localises to the nuclei presenting a granular pattern. Furthermore, it is densely packaged in the midsections of the nucleus. In contrast, the fragment localises exclusively to the cytoplasm in PARP-1<sup>-/-</sup> cells. Fluorescence intensity analysis further confirmed this observation indicating that the N-terminal fragment requires endogenous PARP-1 for its nuclear transport. Our study illustrates the trafficking role of PARP-1 independently of its enzymatic activity and highlights the possibility that full-length PARP-1 may play a key role in the nuclear transport of its siblings and other molecules.

## INTRODUCTION

PARP-1 (EC 2.4.2.30) is a nuclear enzyme found in all eukaryotes except yeast with approximately 10<sup>6</sup> molecules per cell. It is a prominent member of a family of 17 PARPs in humans. On limited proteolysis of human PARP-1 using papain and  $\alpha$ -chymotrypsin three functional domains were identified: 46 kDa amino terminal DNA binding domain (DBD), 22 kDa central automodification domain and 54 kDa carboxy-terminal NAD<sup>+</sup> binding domain (Kameshita et al., 1984). DBD encompasses two Cys-Cys-His-Cys zinc finger motifs (FI and FII) that are involved in DNA binding and a nuclear localisation signal (NLS) (de Murcia et al., 1994; Gradwohl et al., 1990; Lamarre et al., 1986; Molinete et al., 1993). PARP-1 has been implicated in a number of functions such as DNA replication and repair, cell death, maintenance of genome integrity, inflammation and cell proliferation (Barnes and Lindahl 2004; Bjelland and Seeberg, 2003; Caldecott, 2008; Dizdaroglu et al., 2002; Svilar et al., 2011).

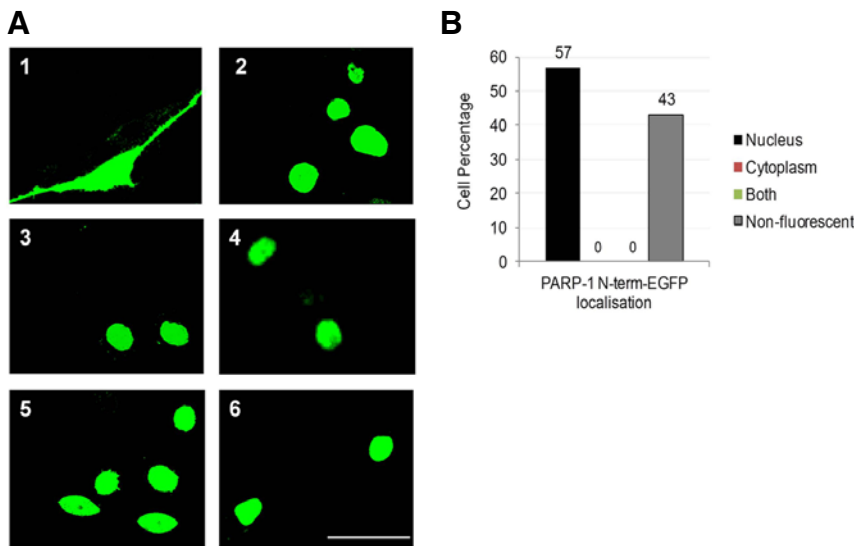
The primary function of a cell is to preserve genomic stability when faced with challenges that cause DNA damage. Failure to repair oxidative DNA lesions can lead to cancer and accumulation of damaged DNA can contribute to ageing and neurodegeneration (Caldecott, 2008; Maynard et al., 2009). PARP-1 binds to sites of DNA break and using NAD<sup>+</sup> it catalyzes the poly-(ADP-ribosylation) of PARP-1 itself as well as histones, nuclear proteins, DNA repair proteins, transcription factors and chromatin modulators (Burkle, 2006; Luo and Kraus, 2012; Ogata et al., 1981; Schreiber et al., 2006). The mechanism of DNA damage induced polymer synthesis by PARP-1 has been elucidated. It engages with DNA as a monomer leading to distortion and destabilisation of its catalytic domain that in turn facilitates its automodification (Langelier et al., 2012). Other studies show that the 40 kDa carboxy-terminal domain forms homo and hetero dimers in the absence of DNA (Mendoza-Alvarez and Alvarez-Gonzalez, 2004).

Excessive activation of PARP-1 can lead to exhaustion of NAD<sup>+</sup> as observed in conditions including reperfusion injury, inflammation and heart attack leading to apoptosis or necrosis. Furthermore, during apoptosis PARP-1 undergoes proteolytic cleavage and generates a 24 kDa N-terminal fragment containing DBD and 89 kDa fragment containing both the automodification and catalytic domains. These fragments have been shown to persist in apoptotic cells several days after the onset of cell death (Lazebnik et al., 1994; Nicholson et al., 1995; Rosenthal et al., 1997; Soldani and Scovassi, 2002; Tewari et al., 1995).

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**Fig. 1.** PARP-1 N-term-EGFP localises to the nuclei in PARP-1<sup>+/+</sup> MEFs. (A) PARP-1 N-term-EGFP construct was introduced into PARP-1<sup>+/+</sup> cells by electroporation. EGFP vector was used as control. After 24 h cells were examined live using CLSM. Panel 1, cell expressing EGFP. Panels 2-6, fields of cells expressing PARP-1 N-term-EGFP. The scale bar represents 35  $\mu$ m. Data is representative of at least 100 fields of cells from three separate transfections. (B) Percentage of cells expressing PARP-1 N-term-EGFP 24 h post-transfection. Data is representative of three separate experiments.

Deficiency or inactivation of PARP-1 can cause telomere shortening and reduced PARP-1 activity was observed in ageing human samples (Chevanne et al., 2007; Muiras et al., 1998; Serrano and Blasco, 2007; Tong et al., 2001). Hence for cell survival, maintenance of genomic integrity and longevity presence of intact PARP-1 in the nucleus is paramount. The objective of this study was to investigate the intracellular trafficking role of full-length PARP-1 by examining the subcellular distribution of exogenous PARP-1 N-terminal fragment in PARP-1 wild type and knock-out cells. To visualise the fragment by non-invasive fluorescence microscopy we generated a DNA construct of PARP-1 N-terminal fragment (750 bp) tagged to pEGFPN1 and introduced into PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> mouse embryo fibroblasts (MEFs).

## MATERIALS AND METHODS

Forward and reverse primers were obtained from Genset. pEGFPN1 and pGEMT-Easy were from Clontech and Promega respectively. Vent DNA polymerase and T4 DNA Ligase were from New England Biolabs. *Bam*HI and *E. coli* DH5 $\alpha$  strain were obtained from Gibco-BRL and *Hind*III and calf intestinal alkaline phosphatase from Roche Diagnostics Ltd. pTG PARP and mouse embryo fibroblasts were kind gifts of Dr. G. de Murcia (Ecole Supérieure de Biotechnologie, France). Maxi prep and DNA purification kits were purchased from Qiagen and Qbiogene-Alexis Ltd respectively. MEFs were grown in DMEM supplemented with 10% (v/v) foetal calf serum. All chemicals used in this study were of analytical grade.

### Construction of PARP-1 N-term-pEGFP

DNA fragment consisting of 750 base pairs or 250 amino acids from the N-terminal end of human PARP-1 was synthesised by PCR using forward primer 5'-ACAACTGCAGGGATCCATGGC GGAGTCTTCGGAT-3' containing *Bam*HI and *Pst*I restriction enzyme sites and reverse primer 3'-GACTAGACCTTGTAG TTCCTGCCCTAGGACGGGCCCTAAC-5' containing *Bam*HI and *Xma*I restriction sites respectively. PCR product was cloned into PGEM-T Easy vector according to Promega, instruction manual. PARP-1-250 insert was excised out using *Bam*HI and ligated into *Bam*HI digested pEGFPN1 vector using

T<sub>4</sub> DNA ligase as described in recent paper (Rajiah, 2013).

### Transient transfection

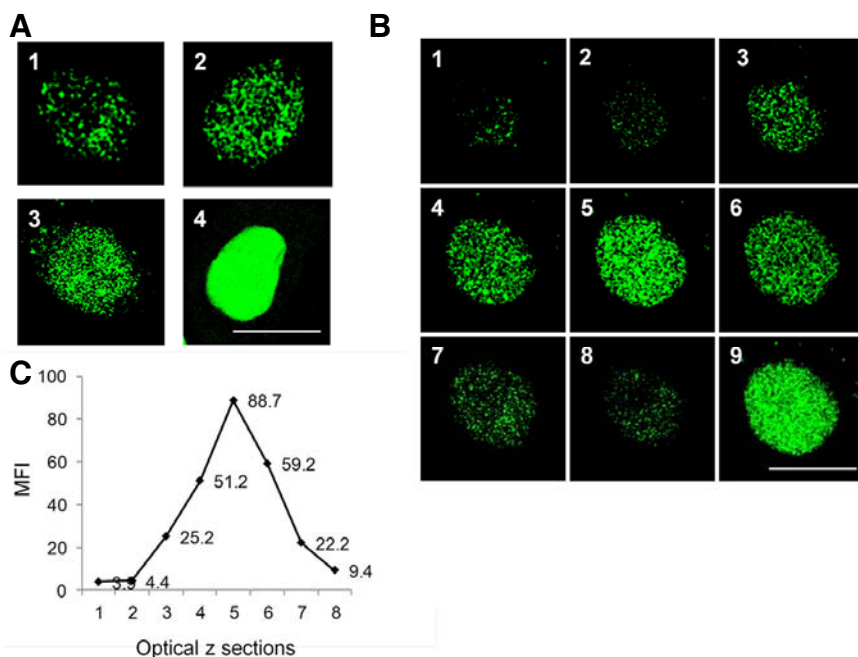
Large scale preparations of the plasmid were made using Qiagen maxiprep kit. 40  $\mu$ g/ml final concentration of DNA was introduced into mouse embryo fibroblasts by electroporation using Gene Pulser from Biorad as described in the instruction manual. Cells were seeded on coverslips in complete DMEM and maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub>.

### Confocal laser scanning microscopy (CLSM)

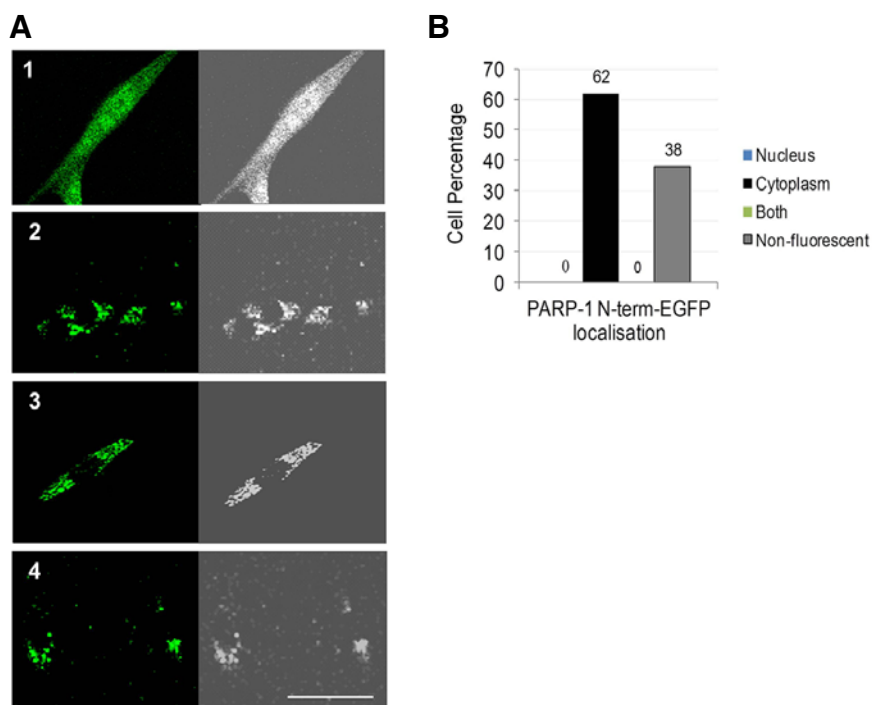
Imaging was carried out using Bio-Rad MRC-600 confocal unit (Bio-Rad Laboratories Ltd.) that was mounted onto an inverted microscope. PARP-1 N-term-EGFP expression was visualised by excitation with the krypton-argon ion laser line at 488 nm and emission at 510-530 nm. The laser power, gain and black level were recorded for each experiment and kept constant when comparisons were made. Specimens were viewed and sectioned using an oil immersion  $\times$  60 (1.4 numerical aperture) objective lens. Optical sections along the z axis were taken at 0.8  $\mu$ m intervals and images collected by Kalman filtering of six scans. Images were processed and analysed using NIH ImageJ and Zeiss LSM image softwares.

## RESULTS

Although localisation of full-length and truncated PARP-1 in PARP-1 proficient cells has been well documented, subcellular distribution of PARP-1 fragments in the absence of endogenous PARP-1 is not known. Therefore, to examine the localisation and pattern of distribution of exogenous PARP-1 N-terminal fragment in PARP-1 deficient cells, PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> mouse embryo fibroblasts were transiently transfected with PARP-1 N-term-EGFP plasmid and cells expressing the tagged protein were examined live using CLSM. At least one hundred fields of cells were analysed and 57% were fluorescent, 43% non-fluorescent and within 100% of the fluorescent cells the tagged protein was present exclusively in the nucleus. EGFP was expressed within the entire cell (Figs. 1A and 1B). Imaging at high magnification revealed a granular pattern of distribution



**Fig. 2.** PARP-1 N-term-EGFP is distributed heterogeneously along the z axis in PARP-1<sup>+/+</sup> MEFs. (A) PARP-1 N-term-EGFP expressing PARP-1<sup>+/+</sup> cells were examined live 24 h post-transfection using CLSM. Panels 1-4, Midsections of cells at high magnification. (B) Panels 1-8, Optical z sections of cell expressing the tagged protein collected at 0.8  $\mu$ m intervals. Panel 9, maximum intensity projection. The scale bars in (A) and (B) represent 10  $\mu$ m. (C) Plot of MFI values of optical z sections. Data is representative of at least 60 cells from three separate experiments.

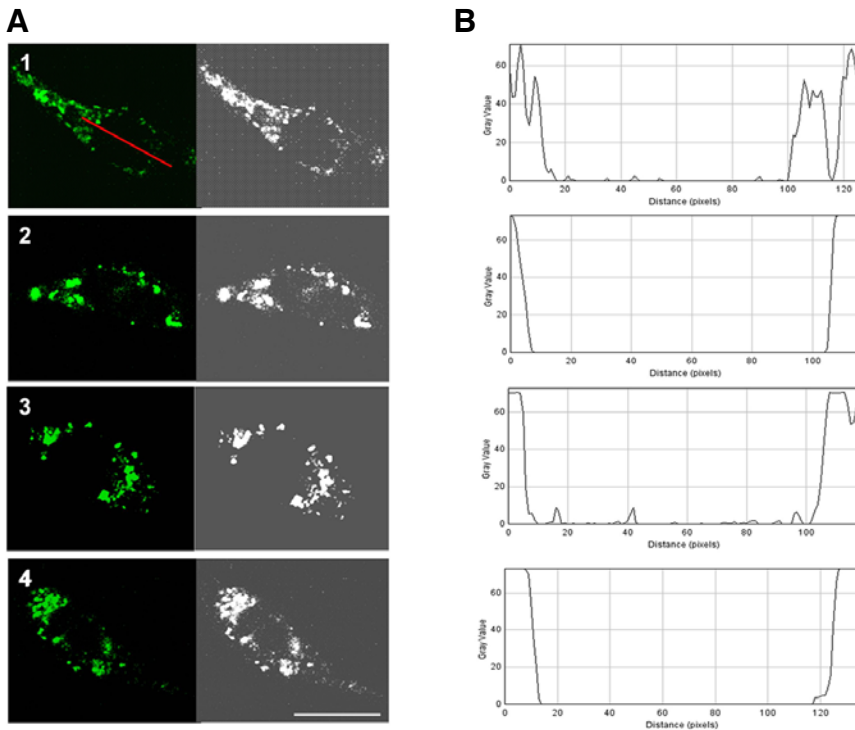


**Fig. 3.** PARP-1 N-term-EGFP localises to the cytoplasm in PARP-1<sup>-/-</sup> MEFs. (A) PARP-1 N-term-EGFP construct was introduced into PARP-1<sup>-/-</sup> cells by electroporation. EGFP vector was used as control. After 24 h cells were examined live using CLSM. Panel 1, cell expressing EGFP. Panels 2-4, fields of cells expressing PARP-1 N-term-EGFP. Each panel is paired with its contrast enhanced 8-bit gray scale image. Data is representative of at least 100 fields of cells from three separate transfections. The scale bar represents 35  $\mu$ m. (B) Percentage of cells expressing PARP-1 N-term-EGFP 24 h post-transfection. Data is representative of three separate experiments.

of the tagged protein (Fig. 2A). Interestingly, optical z sections showed heterogenous distribution through the depth of the cell (Fig. 2B). The intensity of fluorescence of the sections was quantified using ImageJ. MFI of outer sections ranged from 0-10, inner sections from 20-60 and the mid-section gave a peak value of 88.7 indicating dense packaging of the tagged protein in the middle of the nucleus (Fig. 2C).

In contrast to the above data, PARP-1 N-terminal fragment

was present exclusively in the cytoplasm of PARP-1<sup>-/-</sup> MEFs (Fig. 3A). EGFP was localised in the entire cell. At least 100 fields of cells were examined and 62% were fluorescent, 38% non-fluorescent and 100% of the fluorescent cells displayed fluorescence exclusively in the cytoplasm (Fig. 3B). RGB images obtained at high magnification as well as the processed contrast enhanced 8-bit gray scale images confirmed the localisation of the fragment in the cytoplasm. Furthermore, the im-



**Fig. 4.** Localisation analysis of PARP-1 N-term-EGFP in PARP-1<sup>-/-</sup> MEFs. (A) PARP-1 N-term-EGFP expressing cells were examined live 24 h post-transfection using CLSM. Panels 1-4, midsections of cells at high magnification paired with contrast enhanced 8-bit gray scale images. The scale bar represents 13  $\mu$ m. (B) Fluorescence intensity plot along the line selection across the cytoplasm and nucleus as depicted in (A), Panel 1. Data is representative of at least 60 cells from three separate experiments.

ages revealed a granular pattern of distribution similar to PARP-1<sup>+/+</sup> cells (Fig. 4A). Fluorescence intensity plot along the line selection across the cytoplasm and nucleus as depicted in Fig. 4A, Panel 1 was obtained using ImageJ. According to the plot profile, average gray values were 98.6% higher in the cytoplasm compared to the nucleus (Fig. 4B). Thus, both imaging and analytical data provided conclusive evidence for the cytoplasmic distribution of the N-terminal fragment in PARP-1 deficient cells. Optical z sectioning of the cells was not possible due to rapid photobleaching.

## DISCUSSION

In addition to DNA damage response and cell survival, members of PARP family play a role in many functions including cell division and regulation of chromatin structure and transcription (reviewed in Krishnakumar and Kraus, 2010; Lord and Ashworth, 2012). Both full-length PARP-1 and truncated DNA binding domain are distributed in the nuclei of PARP-1 proficient cells. PARP-1-DsRed, PARP-1-EYFP and PARP-1-24 kDa-DsRed plasmids are distributed in the nucleoli and nucleoplasm of live COS-7 and HeLa S3 cells (Yung et al., 2004). Early studies have also reported extranuclear PARP activity in the ribosomal and mitochondrial fractions (Kun et al., 1975; Roberts et al., 1975). Our findings of PARP-1 N-terminal fragment in the nucleus of wild type MEFs exhibiting a granular pattern of distribution with patchy areas of strong intensity is consistent with previous reports of PARP-1 in HeLa cells (Fig. 2) (Mosgoeller et al., 1996). Furthermore, in wild type MEFs the fragment was more densely packaged in the mid-sections of the nucleus (Figs. 2B and 2C). We have reported a similar pattern of heterogeneous distribution through the depth of the nucleus in wild type IRK cells (rat embryo fibroblasts) expressing the N-terminal fragment (Rajiah, 2013).

In contrast to wild type cells the fragment localised exclusively to the cytoplasm in PARP-1 knock-out cells and presented a granular pattern of distribution (Figs. 3 and 4). Recently full-length PARP-1 plasmid was expressed in PARP-1<sup>-/-</sup> MEFs and localisation was observed in the nucleus by immunocytochemical method (Patel et al., 2012). This PARP-1 has in addition to NLS, a central automodification and C-terminal catalytic domains which may have facilitated proper folding of the molecule required for nuclear entry. In PARP-1<sup>+/+</sup> MEFs used in our study, these domains would have been provided by endogenous PARP-1 to enable nuclear entry of the N-terminal fragment whereas in PARP-1<sup>-/-</sup> MEFs this facilitation was not available. Recent findings highlight a novel role for PARP-1 in the regulation of intracellular trafficking of antiapoptotic p53 and the key immune response protein, nuclear factor kappa B (NF- $\kappa$ B). PARP-1 inhibition by gene knock-out, knock-down or by chemical inhibitors in smooth muscle cells prevents NF- $\kappa$ B translocation into the nucleus (Zerfaoui et al., 2010). Following the discovery of post-translational modification of NF- $\kappa$ B by PARP-1 and its nuclear translocation during inflammation, the mechanism of NF- $\kappa$ B nuclear retention promoted by PARP-1 leading to modification of its gene regulatory effects has been proposed. In brief, despite the presence of NLS in NF- $\kappa$ B, it requires PARP-1 for nuclear translocation.

Interestingly, cytoplasmic molecules lacking NLS have also been localised in the nucleus. PTEN (phosphatase and tensin homolog) and Ferritin are traditionally cytoplasmic proteins and lack a consensus NLS. However, accumulating evidences show that PTEN and Ferritin are transported into the nucleus and Ferritin is able to bind and protect nuclear DNA from oxidative damage (Alkhateeb and Connor, 2010; Cai et al., 1998; Lian and Di Cristofano, 2005; Thompson et al., 2002). While researchers suggest the existence of a specific nuclear chaperone, what regulates Ferritin entry into the nucleus remains



unclear. In conclusion, our GFP tagged PARP-1 N-terminal fragment has allowed for non-invasive visualisation of its sub-cellular distribution in live cells. Our findings provide confirmatory evidence for the requirement of endogenous PARP-1 for nuclear transport of the fragment. This pilot study highlights an intracellular trafficking role for PARP-1 independently of its enzymatic activity and future work will focus on investigating the requirement of intact PARP-1 for nuclear translocation of its siblings and other molecules.

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