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ROLE OF PARP-1 ENZYME IN THE CONTROL OF QUIESCENCE

Candidate:
CASSANDRA MOSTOCOTTO

Scientific tutor:
Prof.ssa Rossella Maione "Sapienza" University of Rome

Director of Doctoral Program:
Prof.ssa Paola Londei "Sapienza" University of Rome

Board of examiners:
Prof. ssa Grazia Graziani
Department of Neuroscience, University of Rome Tor Vergata

Prof. Sergio Pimpinelli
Department Genetics and Molecular Biology, Sapienza University of Rome

Prof. Carlo Rodolfo
Department of Biology, University of Rome Tor Vergata

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A Nonno Mario

ABSTRACT

Poly(ADP-ribose)polymerase-1 (PARP-1) catalyses the polymerization of ADP-ribose units from donor NAD+ molecules on target proteins, resulting in the attachment of linear or branched polymers. The negative charges of poly(ADP-ribose) change the target protein affinity for DNA. PARP-1 exerts numerous functions in cellular physiology, from maintenance of DNA stability and integrity to transcriptional regulation and cell cycle control but its role in the G0-G1 transition is not yet completely defined.
The exit from quiescence is a highly regulated and conserved process started by extra cellular stimuli. These stimuli, for instance serum stimulation, trigger a signal cascade, including MAPK activation, that culminates in the transcriptional induction of Immediate Early Genes (IEGs).
Our group has recently reported that PARP-1 activity promotes cell cycle re-entry through the induction of a set of IEGs, such as c-myc, c-fos, junB and Egr-1.

On the basis of these previous finding we studied the mechanism by which PARP-1 modulates IEGs in fibroblast cells. We highlighted that PARP-1 affects the IEG expression at transcription level. Then analyses of chromatin status of c-myc promoter evidenced that this region is more condensed in absence of poly(ADP-ribosyl)ation upon mitogen stimulation of resting fibroblasts. Further, ChIP experiments showed a complex dynamics of PARP-1 binding and chromatin poly(ADP-ribosyl)ation at the same region during G0-G1 transition. Indeed PARP-1 is associated with silent c-myc promoter during quiescence but, following mitogen

stimulation, activated PARP-1 is displaced from it in concomitance with chromatin poly(ADP-ribosyl)ation. These PARP-1 activities are associated with the switch of transcription factor occupancies on the c-myc promoter. Moreover the dynamics of PARP-1 binding at the promoter suggested a possible implication of the enzyme in the repression of c-myc gene during G0 instauration. According with this hypothesis we found that overexpression of PARP-1 accelerates c-myc shut off during G0 entry.

Since many early events induced during cell-cycle re-entry are shared by different cell lineages in several physiological condition, it was investigate whether PARP activity plays a role in other cell systems undergoing G0–G1 transition. The attention was focused on skeletal myoblasts made quiescent by suspension culture. This muscle cell system mimics the function of muscle satellite reserve cells and can be activated by restoring cell adhesion to substrate. We found that in the myoblast context, the inhibition of PARP-1 activity delays the induction of proliferation and interfers with both the upregulation of IEGs and the expression of the myogenic factor MyoD that normally occurs following reserve cell activation. This kind of analysis may open new ways of investigation in the study of cell cycle exit control that characterize stem cell differentiation or quiescence.

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INTRODUCTION

G0 phase of cell cycle or quiescence

Cell cycle consists of four main phases: G1, S, G2, M. G1 phase corresponds to the interval between mitosis and initiation of DNA replication. During G1, the cell is metabolically active and continuously grows but does not replicate its DNA. G1 is followed by S phase, during which DNA replication takes place. The completion of DNA synthesis is followed by the G2 phase, during which cell growth continues and proteins are synthesized in preparation for mitosis. The chromosomes are separated in the M phase and the cell divides into two daughter cells. G0 phase entry takes place in the G1 phase, before or at the restriction point. Quiescence is defined as reversible cell cycle arrest where cells are poised to re-enter the cell cycle. In an adult organism, most somatic cells (fibroblasts, lymphocytes, hepatocytes and adult stem cells) maintain the quiescent state for long periods of time. However, in response to injury or specific extracellular stimuli, these cells can enter the cell cycle and proliferate. In this way, quiescence can be distinguished from an irreversibly arrested state such as senescence or terminal differentiation. For instance, memory lymphocytes are quiescent as they circulate and survey the body, and they divide only when stimulated by cognate antigen to trigger immune response (Yusuf and Fruman, 2003). Similarly, in the skin, dermal fibroblasts are for the most part quiescent. Injury to the skin stimulates fibroblasts to rapidly proliferate; once tissue repair has been accomplished, the cells re-enter G0 phase (Ito et al., 2005). Moreover, satellite cells, the muscle stem cells, are quiescent. They are the main responsible for postnatal growth and repair of muscle, in fact,

in response to various regeneration signals e.g. muscle injury or exercise they are activated, express muscle regulatory factors and proliferate to initiate the myogenic program. However, a small fraction of activated satellite cells down-regulate myogenic factors, exit the cell cycle and return to the quiescent state in order to maintain the self-renewal potential (Dhawan and Rando, 2005; Le Grand and Rudnicki, 2007).

The ubiquity of quiescence as a central feature of cell life suggests that its regulation may be critical to normal development, degenerative diseases, and cancer. In addition to the lack of cell division and 2N DNA content, quiescent cells exhibit systematic differences in their metabolism (Bauer et al., 2004), chromatin organization (Grigoryev et al., 2004) gene expression (Coller et al., 2006; Liu et al., 2007) and propensity for differentiation. In cultured cells the quiescent state can be obtained through growth factor deprivation, contact inhibition, or loss of adhesion. Each conditions can induce a shared set of genes, indicating the potential existence of a quiescence-specific transcriptional program (Coller et al., 2006). Several tumor suppressor genes, such as Rb, PTEN and cyclin-dependent kinase inhibitors are required for quiescence maintenance (Sage et al., 2003; Groszer et al., 2006). However, ectopic expression of CDK inhibitors leads to an irreversible senescent-like state and does not recapitulate the transcriptional signature of quiescent cells (Coller et al., 2006), which suggests that cell cycle arrest and cellular quiescence are not functionally equivalent. Sang and colleagues have recently reported that reversibility of quiescence is not a passive property. Concerning this they have found that expression of the gene encoding the basic helix-loop-helix protein HES1 is required for the reversibility of quiescence by preventing both premature senescence and inappropriate differentiation (Sang et al., 2008).

The exit from quiescence is a highly regulated and conserved process initiated by extra cellular proliferative stimuli. These stimuli trigger a complex signal cascade, including MAPK pathway, which produces phosphorylation-dependent activation of transcription factors and culminates in IEG induction (Treisman, 1996). The IEGs encode among others, transcription factors belonging to the Myc, Fos and Jun families and are characterised by their rapid and transient expression in response to extracellular proliferative stimuli (Thomson et al., 1999). Is important to note that the transcriptional induction of these genes is independent of *de novo* synthesized proteins indicating that the modification of pre-existing components of signal transduction cascades are responsible for the induction (Greenberg et al., 1986). The coordinate transcriptional activation of the IEGs, that initiate the G0 exit, can be achieved through general mechanisms such as modulation of chromatin structure. Covalent post translational modifications of the histone tails and direct remodelling of nucleosomes involving ATP-dependent complex may explain how chromatin is able to change its conformation rapidly according to cell needs. One or specific combinations of histone modifications including acetylation, phosphorylation, ubiquitination, methylation and ADP-ribosylation, could affect distinct downstream events by altering the structure of the chromatin and/or generating a binding platform for protein effectors.

In particular, acetylation and phosphorylation of histone tails are associated with gene expression. Whereas histone acetylation is widely connected to transcriptional regulation (Grunstein, 1997), phosphorylation of histone H3 at serine 10 is restricted to the activation of rapidly inducible genes like the IEGs c-fos, c-jun, and c-myc (Thomson et al., 1999).

Poly(ADP-ribosyl)ation

Poly(ADPribosyl)ation is a post-translational modification of proteins mediated by a family of enzymes named Poly(ADP-ribose)polymerases (PARPs). These enzymes initiate the reaction by converting the substrate nicotinamide adenine dinucleotide (NAD+) to ADP-ribose, and then catalyze ADP-ribose polymerization on glutamate/aspartate residues of acceptor proteins (D'Amours et al., 1999). The resulting Poly(ADP-ribose) or PAR consists of a linear or branched polyanion of variable size whose monomers are linked to each other via glycosidic ribose-ribose bounds (Figure I).

PAR can act as a site-specific covalent modification or as a protein-binding matrix that recruits specific factors. In fact, attachment of PAR is thought to alter the activity of target proteins through both steric and charge effects, ultimately preventing protein-protein interactions, protein-nucleic acid interactions, enzymatic activity, or subcellular localization (Schreiber et al., 2006; Hassa and Hottiger, 2008).

The catabolism of PAR in the cell is regulated mainly by a specific exo-/endo-glycohydrolase (PARG), which catalyzes the hydrolysis of PAR into free ADP-ribose and thus controls the level of poly(ADPribosyl)ated proteins. Furthermore, (ADP-ribosyl)proteinlyase cleaves the final remaining ADP-ribose monomer from the target protein.

PARP activity has been found in a vast variety of organisms raging from archaebacteria to mammals but it is absent in yeast (Hassa et al., 2006; Rolli et al., 2000). To date, 17 members of PARP superfamily have been identified in mammalian genome. They have different structural domains and functions but all share a PARP signature motif that forms the active site (Amè et al., 2004). Recently, Hottiger and colleages (Hottiger et al., 2010) have proposed a new structure-based classification of PARP family members based on their catalytic domains: PARPs 1-5, which are bona fide PARPs containing a conserved

glutamate (Glu 988 in PARP-1) that defines the PARP catalytic activity; PARPs 6–8, 10–12, and 14–16, which are confirmed or putative momo(ADP-ribosyl)transferase; and PARPs 9 and 13, which lack key NAD+-binding residues and the catalytic glutamate, and are likely inactive (Kleine et al., 2008).

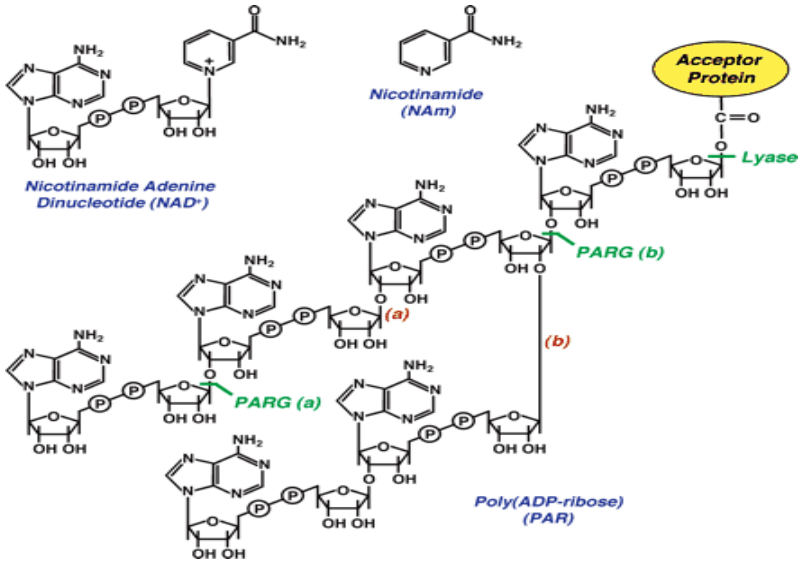

Figure I: Chemical structures of NAD$^+$, nicotinamide (NAm), and PAR. PAR is a branched polymer synthesized on acceptor proteins by PARPs using NAD+ as a donor of ADP-ribose units. The ADP-ribose units in the linear PAR chains are linked by 1″ → 2′ ribose-ribose glycosidic bonds whereas the ADP-ribose units at the branchpoints are linked by 1‴ → 2″ ribose-ribose glycosidic bonds (adapted from Kim et al., 2005).

Structure of PARP-1 enzyme

Poly(ADP-ribose)polymerase-1 (PARP-1) is a ubiquitous and abundant enzyme as well the most studied member of PARP family. Known targets of PARP-1 catalytic activity include PARP itself histones (especially H1, H2A, and H2B) transcription factors, nuclear enzymes, DNA repair proteins and nuclear structural proteins. (D'Amours et al., 1999; Kim et al., 2005).

PARP-1 has a highly conserved structural and functional organization (Figure II). It is composed of three main domains: an N-terminal DNA-binding domain (DBD), a central automodification domain, and a C-terminal catalytic domain (D'Amours et al., 1999; Rolli et al., 2000).

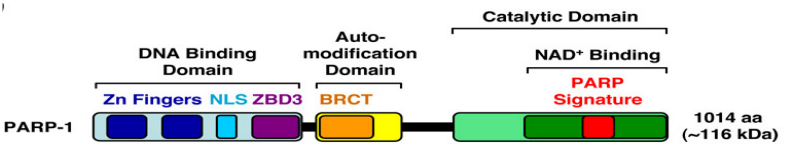

Figure II : Schematic representation of PARP-1's structural and functional organization. The DBD domain, the automodification domain with BRCT motif and the catalytic domain of PARP-1 are represented (adapted from Kraus, 2008).

The DBD contains two Cys-Cys-His-Cys zinc fingers (FI/Zn1 and FII/Zn2) responsible for the binding to DNA, a nuclear localization signal (NLS), a caspase-3 cleavage site, and a third zinc binding domain (FIII/Zn3) that mediates inter-domain contacts important for DNA-dependent enzyme activation (Langelier et al., 2008). PARP zinc fingers are structurally and functionally unique, since they can recognize altered structures

in DNA including single and double strand breaks, crossover and cruciforms rather than particular sequences.

The automodification domain of PARP-1 is located in the central region of the enzyme, between residues 374 and 525 (human protein) (Kurosaki et al., 1987). This domain is rich in glutamic acid residues, consistent with the fact that poly(ADP-ribosyl)ation occurs on such residues. Although automodified PARP-1 loses its activity (D'Amours et al., 1999), it gains the ability to bind proteins through conserved PAR-binding domains in a non-covalent manner (Pleschke et al., 2000; Karras et al., 2005). This domain also contains a protein-protein interaction module called BRCT (breast cancer 1 protein C-terminus motif) that is present in many DNA damage repair and cell-cycle checkpoint proteins.

The catalytic domain is located in the C-terminal part of the enzyme and, in human PARP-1, spans residues 526-1014. The CD is the most conserved domain across the PARP family and contains the PARP signature motif which binds NAD+. X-ray diffraction of this domain showed that the NAD+ binding site consists of a β-α-loop-β-α structural motif (Ruf et al., 1998). This catalytic domain catalyzes multiple distinct reactions in the PAR synthesis: 1) attachment of the first ADP-ribose moiety on acceptor protein; 2) elongation of PAR and 3) branching of PAR (D'Amours et al., 1999). Together, the structural and functional domains of PARP-1 confer the activities required for the broad range of functions of PARP-1 in the nucleus. Although PARP-1 was originally characterized as a key sensor of DNA damage, more recent studies have linked the enzyme to the regulation of chromatin structure and transcription, DNA methylation and imprinting, insulator activity, and chromosome organization.

Regulation of PARP-1 activity

PARP-1's basal enzymatic activity is very low, but is stimulated by a variety of allosteric activators, including damaged DNA, some undamaged DNA structures, nucleosomes, and a variety of protein-binding partners (D'Amours et al., 1999; Kraus, 2008).

PARP-1 was originally characterized as a DNA damage sensor, since its catalytic activity is strongly stimulated by binding with single and double-strand breaks. PARP-1 has been implicated in multiple DNA repair pathways, including single strand breaks (SSB), double strand breaks (DSB), and base excision repair (BER) pathways (Dantzer et al., 1999). The PAR production after DNA damage recognition leads to chromatin loosening and so increase the access to breaks of repair proteins. Several additional stimuli for PARP-1 activation, in absence of DNA damage, have been identified. First, a fast activation of PAR synthesis is evoked upon heat or steroid hormone exposure, in Drosophila. This PARP activity is required for normal chromatin puffing, chromatin loosening at polytene chromosomes, and gene expression (Tulin and Spradling, 2003). Second, It has been reported that the PARP-1, included as a component of a transcriptional co-regulator complex, is activated downstream the PDGF-induction of a specific calcium-dependent protein Kinase pathway, in proliferating neuronal progenitors (Ju et al., 2004). Moreover, a previous work from our laboratory has reported PARP-1 activation upon serum stimulation of resting cells highlighting an important role of this enzyme in the cell cycle reactivation of quiescent cells (Carbone et al., 2008).

A number of molecular mechanisms for PARP-1 activation have been elucidated. For example poly(ADP-ribosyl)ation has been shown to be induced downstream of the Extracellular-Signal Regulated Kinases (ERK) cascade signalling trough the direct interaction of the enzyme with the phosphorylated form

of ERK-2 (Cohen-Armon et al., 2007). Then PARP-1 could be recruited to specific genes and activated by interaction with DNA binding factors as in the case of the transcription factor YY1 which binds to the BRCT motif of PARP-1 and stimulates PARP activity (Griesenbeck et al., 1999). In previous studies conducted in our laboratory, it has been demonstrated that the interaction between PARP-1 and structural viral proteins can activate poly(ADP-ribosyl)ation and that this PAR synthesis is used for efficient viral infection (Carbone et al., 2006).

PARP-1 catalytic activity is also regulated by post translational modifications. These include poly(ADP-ribosyl)ation, phosphorylation, acetylation, ubiquitylation, and SUMOylation; (reviewed by Krishnakumar and Kraus 2010b).

Automodification of PARP-1, may occur as an extensive addition of ADP-ribose in chains >200 units in length or as a more modest addition of a single unit or chains up to 20 units in length (D'Amours et al., 1999; Mendoza-Alvarez and Alvarez-Gonzalez, 1999). Extensive automodification of PARP-1, which occurs for example in response to DNA damage, inhibits its DNA-binding and catalytic activities (D'Amours et al., 1999). Phosphorylation by ERK1/2 or JNK1 enhances its catalytic activity (Kauppinen et al., 2006; Zhang et al., 2007). The former is needed to maximize PARP-1 activation after DNA damage in neurons and astrocytes, whereas the latter promotes sustained PARP-1 activation during hydrogen peroxide-induced non apoptotic cell death PARP-1 is acetylated by the acetyltransferases p300/CBP and PCAF (Hassa et al., 2003, 2005; Rajamohan et al., 2009). Acetylation of PARP-1 was first identified in the context of NF-kBdependent transcription in immune cells, where it plays a critical role in regulating NF-kB target genes (Hassa et al., 2003, 2005). In cardiomyocytes, PARP-1 is acetylated as an endpoint of stress responses, resulting in the DNA damage-independent activation of PARP-1 (Rajamohan et al., 2009).

PARP-1 and transcription

In addition to the well-established role in DNA damage repair, growing amounts of evidence have demonstrated a role for PARP-1 in transcriptional regulation in response to biological, chemical or physical stimuli. Recently chromatin immunoprecipitation coupled to hybridization to genomic microarrays (i.e. ChIP-chip) has shown that PARP-1 binding is enriched at the promoters of expressed RNA polymerase II-transcribed promoters in MCF-7 cells (Krishnakumar et al., 2008). However, this does not imply necessarily a stimulatory role for PARP-1 at all of these promoters, but rather indicates that PARP-1 localizes to sites of ongoing transcription, exerting stimulatory or inhibitory effects (Krishnakumar et al., 2008). Moreover, in a study exploring gene expression profiles in embryonic stem cells and livers from Parp-1-/- mice, 3.5% of the transcriptome was regulated by PARP-1, with approximately 60–70% of the genes being positively regulated (Ogino et al., 2007). This regulation can be achieved through different mechanisms (Figure III).

a) Opening of the chromatin structure by the removal of histones after their poly(ADP-ribosyl)ation, as it occurs at DNA breaks (Schreiber et al., 2006). First of all, PARP-1 acts to exclude H1 from the promoters of some PARP-1-regulated genes, possibly by competing with H1 for binding to nucleosomes or by poly(ADP-ribosyl)ating it (Krishnakumar et al., 2008). For example, this PAR-mediated chromatin loosening is observed at larval salivary-gland polytene-chromosome puffs (Tulin & Spradling, 2003). It has been proposed a model by which PARP-1 can direct the reversible modulation of chromatin structures. Using in vitro approaches they have found that PARP-1, acting like H1, incorporates into

chromatin and promotes the formation of compact, transcriptionally repressed structure. In the presence of NAD+ the enzyme automodifies and dissociates from chromatin resulting in the formation of decondensed transcriptionally active chromatin structures (Kim et al., 2004). Moreover, during estrogen-induced transcription of the TFF1 gene, PARP-1 not only promotes the removal of H1 but also increases the levels of HMGB1, a chromatin architectural protein that enhances transcription (Ju et al., 2006). It has been recently reported that PARP-1 poly(ADP-ribosyl)ates Drosophila ISWI, an ATP-dependent nucleosome remodeller associated with chromatin compaction, and that this modification is involved in the induction of *hsp70* gene upon heat shock (Sala et al., 2008). More recently, it has been shown that PARP-1 leads to a transcription permissive chromatin environment by preventing demethylation of H3K4me3 through the poly(ADP-ribosyl)ation, inhibition, and exclusion of the histone demethylase KDM5B (Krishnakumar and Kraus, 2010a).

b) Many of the initial studies describing direct effects of PARP-1 on the transcriptional regulation of target genes focused on the binding of PARP-1 to specific DNA sequences or structures in the regulatory regions of the genes. In these cases, PARP-1 functions like a classical enhancer-binding factor (Kraus, 2008). For example, it has been examined the role of PARP-1 in the regulation of CXCL1 (Amiri et al., 2006) and BCL6 (Ambrose et al., 2007) genes. PARP-1 binds to specific sequences immediately upstream of the CXCL1 promoter and in the first intron of BCL6 to repress transcription. For CXCL1, PARP-1 prevents the binding of NF-κB to an adjacent element, an effect that is reversed upon PARP-1 activation and automodification, that results in a loss of PARP-1 binding to the promoter.

c) PARP-1 has been found to interact with different transcriptional regulators (Schreiber et al., 2006), such as nuclear receptors, NF-κB, HES1, B-Myb, Oct-1, HTLV Tax-1, Sp1, NFAT, Elk1, and others. In most of these cases, the DNA-binding factor is thought to recruit PARP-1 to relevant target

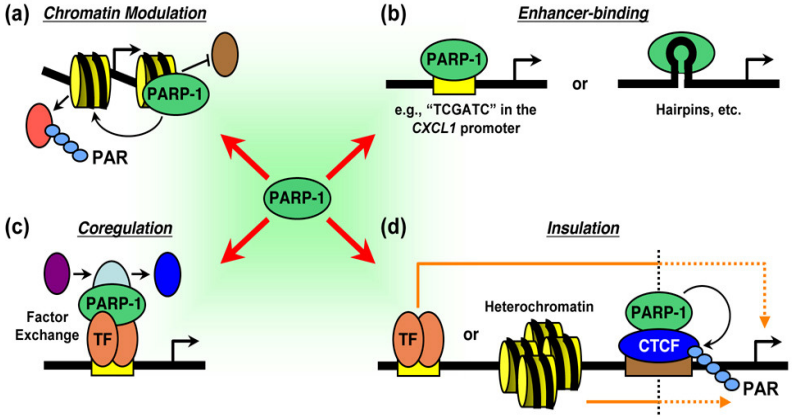

Figure III. Multiple modes of transcriptional regulation by PARP-1. PARP-1 regulates transcription in perhaps as many as four modes, as indicated. **(a)** PARP-1 can modulate chromatin structure by binding to nucleosomes, modifying histone proteins, or regulating the composition of chromatin. **(b)** PARP-1 can act as an enhancer-binding factor that functions in a manner similar to classical sequence-specific DNA-binding activators or repressors. In this mode, PARP-1 may bind to specific sequences or structures in the DNA. **(c)** PARP-1 can function as a transcriptional coregulator in a manner similar to classical coactivators and corepressors. In this mode, PARP-1 may function as a promoter-specific "exchange factor" that promotes the release of inhibitory factors and the recruitment of stimulatory factors during signal-regulated transcriptional responses. TF, DNA-binding transcription factor **(d)** PARP-1 can function as a component of insulators, which act to limit the effects of enhancers on promoters or by preventing the spread of heterochromatin. In this mode, the PARylation of CTCF by PARP-1 is likely to play a role in the maintenance of insulator function. (from Kraus, 2008)

promoters. In some cases, PARP-1 enzymatic activity is not required for its co-regulatory activity (e.g. with NF-κB, B-Myb, and HTLV Tax-1). Otherwise, the DNA-binding factor or other components of the co-regulatory complex are targets for PARP-1-dependent poly(ADP-ribosyl)ation. A key question regarding PARP-1 co-regulatory activity is the effect that it has on the transcription complexes assembled at target promoters. Recent studies have shown that PARP-1 can function as a promoter-specific 'exchange factor' that promotes the release of inhibitory factors and the recruitment of stimulatory factors during signal-regulated transcriptional responses (Ju et al., 2004; Pavri et al., 2005). More recently, PARP-1 was shown to promote the recruitment of topoisomerase IIβ (TopoIIβ) to hormone-regulated promoters, leading to concomitant promoter DNA cleavage, factor exchange, and transcriptional activation (Ju et al., 2006).

d) Last, recent studies have implicated PARP-1-dependent poly(ADP-ribosyl)ation of CTCF, a ubiquitous DNA-binding protein that functions at insulators, in the preservation of insulator function. Insulators are DNA elements that help to organize the genome into discrete regulatory units by limiting the effects of enhancers on promoters or by preventing the spread of heterochromatin (Yu et al., 2004).

Together, these studies highlight the diverse and probably non-exclusive mechanisms of PARP-1 co-regulator function, which are likely to vary in an activator-specific and gene-specific manner.

Previous results and aims of the work

Poly(ADP-ribosyl)ation has been implicated in several distinct processes regulating chromatin structure and transcriptional

activity. PARP-1, the major member of the PARP family, functions both as a structural component of the chromatin and as a modulator of chromatin function, in part through its ability to poly(ADP-ribosyl)ate histone proteins and transcription regulators. This enzyme is recognised to be important in many other cellular functions such as chromosome stability, cell cycle and apoptosis.

In our laboratory it was demonstrated that PARP-1 is involved in the fibroblasts exit from G0 phase. We showed that increased poly(ADP-ribosyl)ation, mainly involving the activation of PARP-1, is transiently detectable within 15 min after serum stimulation of quiescent fibroblasts.

The functional importance of the prompt PARP activation in quiescent cells stimulated with serum was first suggested by the observation that PARP inhibitors interfere with the ability of these cells to re-enter cell cycle. PJ-34 treatment causes a dose-dependent interference with cell cycle reactivation, which correlates with a dose-dependent inhibition of PARP activity.

Analysis of the early phases of the response to serum stimulation revealed that PARP activation correlates with and is required for the timely up-regulation of IEGs and that this regulation occurs at RNA level. We confirmed also, using a specific immunoprecipitation, that PARP-1 is responsible of the most cellular poly(ADP-ribosyl)ating activity during the early response. Moreover, using small interfering RNA, we showed that PARP-1 is the PARP family member playing the most prominent role in IEGs activation (Carbone et al., 2008). Collectively all these previous data provide a functional link between PARP-1 activation and fibroblast cell cycle re-entry. On the basis of these findings the work reported in the present thesis aims to investigate the molecular mechanism by which PARP-1 regulates the expression of IEGs.

To this end we studied the involvement of PARP activity in the accessibility and transcription factors binding to c-myc promoter by performing DNase accessibility and ChIP assays. Then we analysed the IEG induction during G0-G1 transition in PARP-1 knock-down cells and the effect of PARP-1 exogenous expression on c-myc shut off during quiescence establishment.

The proper regulation of the quiescent state it is implicated in controlling differentiation, preserving stem cells function and preventing tumorigenesis. Indeed, it is critical for tissue homeostasis. The reversible transitions between quiescence and proliferation are accompanied by rapid changes of the transcription programs, resulting in the activation or repression of specific genes involved in cell growth and differentiation. The possible implication of PARP-1 activity in other cell systems undergoing G0–G1 transition was studied in reserve cells which mimic the skeletal muscle stem cells. These cells are able to proliferate and undergo myogenic differentiation or return in a quiescent undifferentiated state. We investigated the effects of PARP inhibition and PARP-1 silencing in the reactivation of reserve cells which is characterized by IEGs upregulation as well as by the induction of the myogenic factor myoD.

RESULTS

PARP-1 regulates early response genes in serum-stimulated fibroblasts at the RNA level

It has been previously demonstrated that PARP-1 activity is involved in the fibroblasts exit from a quiescent state. This is a multistep process that begins with the immediate early response to mitogens and extends into an early G1 phase. PARP-1 activity is involved in this step through the regulation of IEGs (Carbone et al., 2008).

To directly verify whether PARP-1 affects the expression of IEGs at the RNA level, we analysed the effects of its knock-down on the kinetics of IEGs induction. Therefore, mouse fibroblasts were serum-starved for 36 h to induce the quiescent state and then transfected with a specific small interfering RNA (siRNA) designed to reduce PARP-1 expression or a control siRNA. After 48 h, cell cycle reactivation was stimulated through serum addition. The silencing of PARP-1 was assessed by western blot. Total RNA was extracted at different times from stimulation and the induction of the IEGs c-myc, c-fos and junB was analysed with RT-qPCR. The plots reported in Figure 1 show that the specific knock-down of PARP-1 is associated with a significant reduction of c-myc, c-fos and junB upregulation respect to the control cells. These results confirm that PARP-1 is required for the correct induction of IEGs during quiescence exit playing a role at the RNA level, likely through transcription regulation.

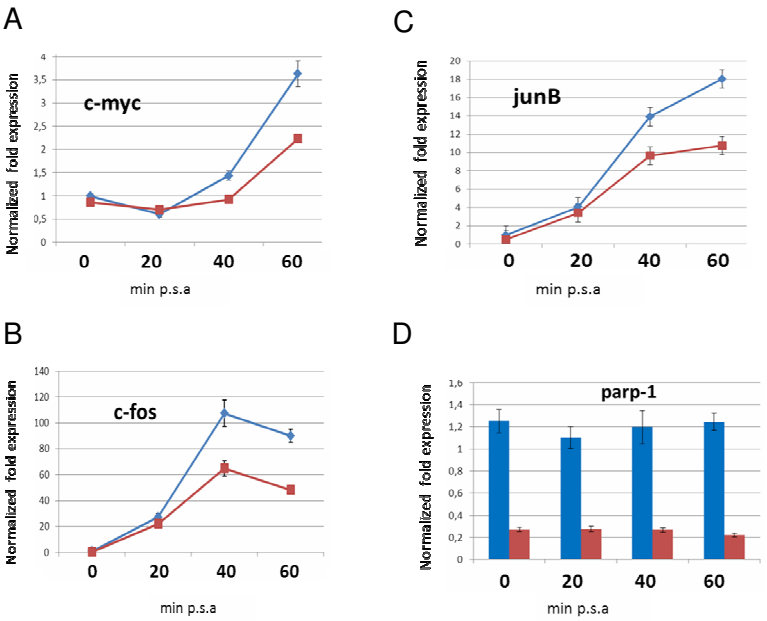

Figure 1. PARP-1 knock-down affects IEGs serum induced up-regulation

Quiescent mouse embryo fibroblasts were transfected with siRNA specific for PARP-1 (red lines) or nonspecific control siRNA (blue lines). RNA levels were quantized by RT-qPCR. Forty-eight hours later, the cells were stimulated by adding 10% fetal bovine serum and lysed at the indicated times for RNA extraction.

A-C) RNA levels of c-myc, c-fos, junB were expressed as fold increase respect to control T0 (quiescent) samples.; min p.s.a on horizontal axes means minutes post serum stimulation and the error bars represent the SEM of three replicates.

D) The PARP-1 mRNA levels were analyzed to confirm the silencing efficiency.

PARP activity changes c-myc promoter accessibility during G0-G1 transition

According to the role of PARP-1 in modulating chromatin structure and function, we examined the effects of PARP inhibition on the status of c-myc promoter through DNAseI accessibility assays. Three regulated DNAse I-hypersensitive sites have been mapped upstream the c -myc gene, in a region that overlaps P1 and P2 c-myc promoters (Figure 2 A). The intensity of cleavage at these sites parallels the synthesis of c-myc mRNA (Levens et al., 1997; Wierstra and Alves 2008).

For the experiment, fibroblasts were made quiescent by serum starvation and then the IEG response was induced by serum stimulation. To inhibit PARP activity, PJ-34 (a competitive inhibitor) was added at the same time as serum. Next entire nuclei, isolated from quiescent and serum-stimulated cells, were treated with DNAseI at increasing concentrations. Then DNA was purified and quantified. A same amount of template was used in PCR assays to assess the extent of DNAseI digestion. A primer set specific for the P1/P2 promoter region, that contains the hypersensitive sites, was used. In this way the amount of PCR product reflected the resistance to DNAseI digestion.

As expected, serum stimulation caused a significant decrease in the DNAseI resistance respect to quiescent cells. This suggested an increase in accessibility or chromatin de-condensation according with the induction of the gene. Remarkably, cells stimulated in presence of the PARP inhibitor showed a degree of DNAseI resistance higher than non-treated control cells, and similar to quiescent cells (Figure 2 B). Thus the inhibition of PARP activity prevents the increase in DNA digestion associated to the transcription of c-myc gene. The results are consistent with a model in which poly(ADPribosyl)ation participates in the IEGs activation

modulating the accessibility of promoters to transcription factors.

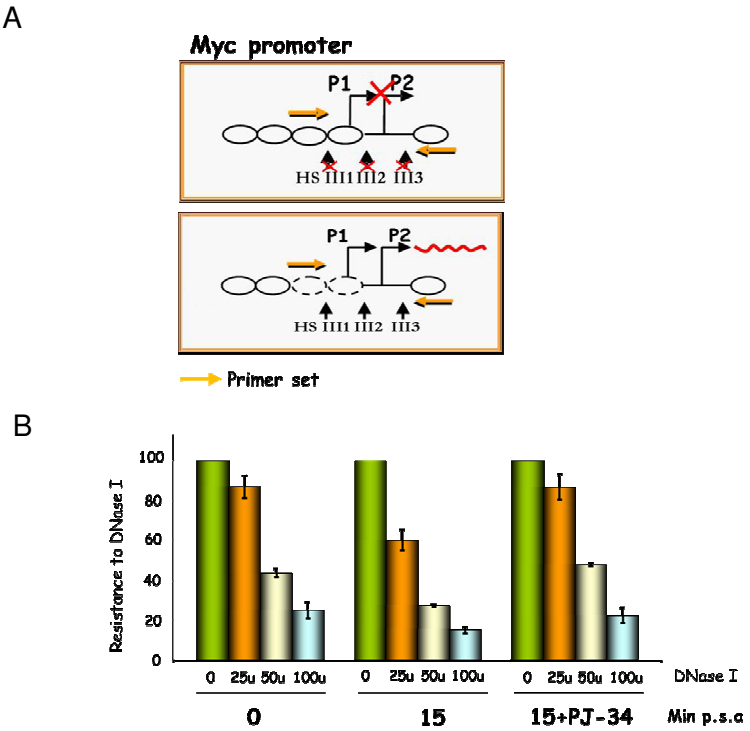

Figure 2 PARP activity modulates DNAseI accessibility at c-myc promoter.

A) Schematic representation of nucleosomal structure of inactive and active c-myc promoter. Shown are the promoters P1 and P2, the DNAse I-hypersensitive sites (HS) and the primer set used in the subsequent assay. The HS were only present on active c-myc gene (adapted from Wierstra and Alves, 2008).

B) DNAse I accessibility assays were performed on quiescent and serum stimulated human fibroblasts in the presence or absence of PARP-1 inhibitor PJ-34. The entire nuclei were isolated from quiescent cells (0) and 15 minutes (15) post serum addition and then treated with increasing quantities of DNaseI (0-100 units). Semi-quantitative PCR shows the different amounts of the fragments after DNAseI digestion. Min p.s.a stand for minutes post serum stimulation.

PARP activity influences transcription factors binding to c-myc promoter

Since we demonstrated the implication of PARP activity in c-myc promoters compaction, we investigated if poly(ADP-ribosyl)ation can influence also transcription factors exchange. To this aim chromatin immunoprecipitation assays (ChIP) were performed in quiescent and in serum stimulated fibroblasts in the presence or absence of the PARP inhibitor PJ-34. Then the occupancy of c-myc promoter region by transcription factors was analyzed by using PCR amplification with specific primers. Among all proteins that are known to modulate c-myc expression we focused our attention on Sp1 and CTCF. Both proteins are targets of poly(ADP-ribosyl)ation (Zaniolo et al., 2007; Yu et al., 2004) and can bind to specific sequences in the same region analysed for DNAseI accessibility, the c-myc P1/P2 promoter region (Chernukhin et al., 2000; Filippova et al., 1996; Klenova et al., 1993). In particular Sp1 has a positive effect on c-myc transcription (Majello et al., 1995) whereas CTCF is known to represse it (Filippova et al., 1996).

As reported in Figure 3, ChIP experiments showed that *c-myc* promoter was not occupied by Sp1 in quiescent cells but the induction of c-myc transcription by serum stimulation resulted in the SP1 binding to the promoter, according to its involvement in mitogen-dependent c-myc induction. Interestingly the association of Sp1 with the promoter was prevented in PJ-34 treated cells (*i.e* in absence of PARP activation). On the other side, the c-myc repressor CTCF bound to the promoter during the quiescent state, but was released 15 min after serum stimulation, consistent with the activation of c-myc gene expression. Interestingly, when PARP activity was inhibited by PJ-34, CTCF remained associated with the promoter despite serum addition. All these evidences

indicate that the dynamics of poly(ADP-ribose) synthesis is associated with change in transcription factors binding to c-myc promoter.

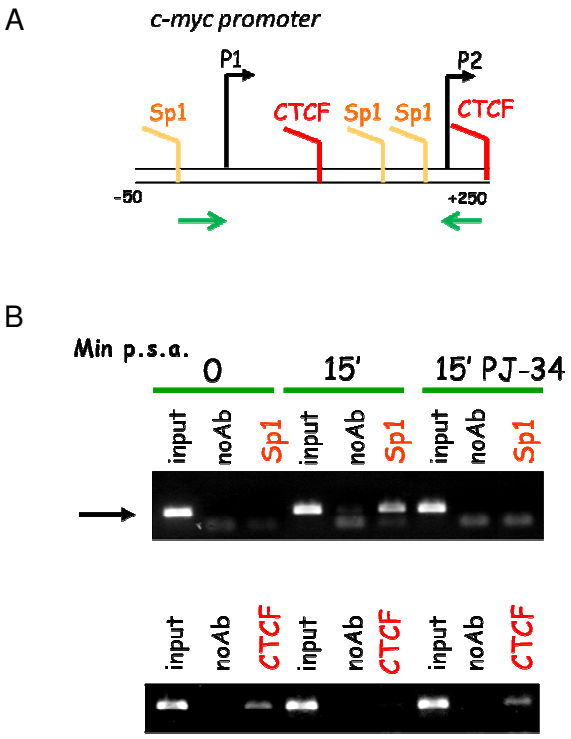

Figure 3. Poly(ADP-ribosyl)ation influences the transcription factor exchange at c-myc promoter.
A) Schematic representation of Sp1 and CTCF binding sequences on *c-myc* promoter. Primers used in the ChIP assays are reported.
B) Sp1 and CTCF ChIP assays**.** Quiescent and activated fibroblasts, in the presence or absence of PJ-34, were fixed at the indicated minutes post serum addition. Sonicated chromatin from these samples was immunoprecipitated with an antibody to Sp1 (upper panel) or to CTCF (lower panel). Then DNA extracted from the immunoprecipitated fractions was amplified by PCR with primers specific for the region between P1 and P2. PCR analyses show the desired-size products. DNA sample lane

descriptions are as follows; Input: samples isolated from cell lysates prior to antibody pull down as an internal positive control; No Ab: samples isolated after pull down with no antibody; Sp1: samples isolated after Sp1 immunoprecipitation; CTCF: samples isolated after CTCF immunoprecipitation).

PARP-1 binds to and modifies c-myc promoter

To test a direct implication for PARP-1 in chromatin remodeling and transcription factor exchange at c-myc promoter, ChIP assays were performed. Chromatin samples from proliferating, quiescent, and serum stimulated cells in the presence or absence of PJ-34, were immunoprecititated using antibodies directed against PARP-1 or its enzymatic product PAR. The presence of c-myc promoter sequences in the immunoprecipitated chromatin fractions was analysed by PCR with primers specific for the region between P1 and P2. Remarkably, as shown in figure 4, PARP-1 was associated with silent c-myc promoter in quiescent cells but not in proliferating cells when the gene was expressed at a basal level. Moreover, following serum stimulation of quiescent cells, PARP-1 was displaced from c-myc promoter in concomitance with chromatin poly(ADP-ribosyl)ation of the same region, probably due to PARP-1 specific activity. According to this hypothesis, in cells stimulated in the presence of PJ-34, poly(ADP-ribosy)lation was impaired and PARP-1 continued to occupy c-myc promoter (Figure 4). These results indicate that during the reversible transitions between quiescence and proliferation the enzyme interacts in a complex and dynamic manner with cell cycle-controlling genes, at least in part through its ability to poly(ADP-ribosyl)ate chromatin.

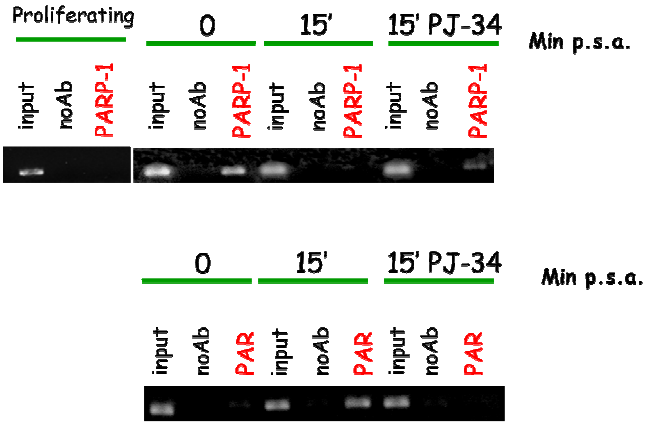

Figure 4. PARP-1 binds to c-myc promoter in G0 cells and is released in concomitance with poly(ADP-ribosyl)ation of the promoter.
PARP-1 and PAR chromatin immunoprecipitation. Quiescent fibroblasts were stimulated to re-enter the cell cycle by serum in the presence or the absence of PJ-34. Then they were fixed at the indicated minutes post serum addition. Sonicated chromatin from these samples was immunoprecipitated with an antibody to PARP-1 (upper panel) or to PAR (lower panel). Then DNA extracted from the immunoprecipitated fractions was amplified by PCR with primers specific for the region between P1 and P2 c-myc promoters. PCR analyses show the desired-size products. DNA sample lane descriptions are as follows. Input: samples isolated from cell lysates prior to antibody pull down as an internal positive control; No Ab: samples isolated after pull down with no antibody represent the aspecific bounded DNA; PARP-1: samples isolated after PARP-1 immunoprecipitation; PAR: samples isolated after polyADP-ribose immunoprecipitation.

Different mechanism of regulation between c-myc and c-fos

The finding that PARP-1 binds to c-myc promoter during the quiescent state prompted us to study the effect of the enzyme silencing at different times in relation to growth arrest. To this aim PARP-1 was knocked-down in proliferating fibroblasts with a specific siRNA and subsequently these cells were serum-starved. After 48 h, cell cycle re-entry was stimulated by serum addition. Total RNA was extracted from quiescent and

serum-stimulated cells. Then the induction levels of the IEGs c-myc and c-fos were analysed with RT-qPCR. Surprisingly PARP-1 knock-down, performed before cell cycle exit, had different effects on the two IEGs. Indeed c-myc induction was not significantly influenced by PARP-1 silencing compared to the control (Figure 5B), while c-fos was affected (Figure 5C), suggesting some differences between PARP-1-dependent regulation of c-fos and c-myc gene expression.

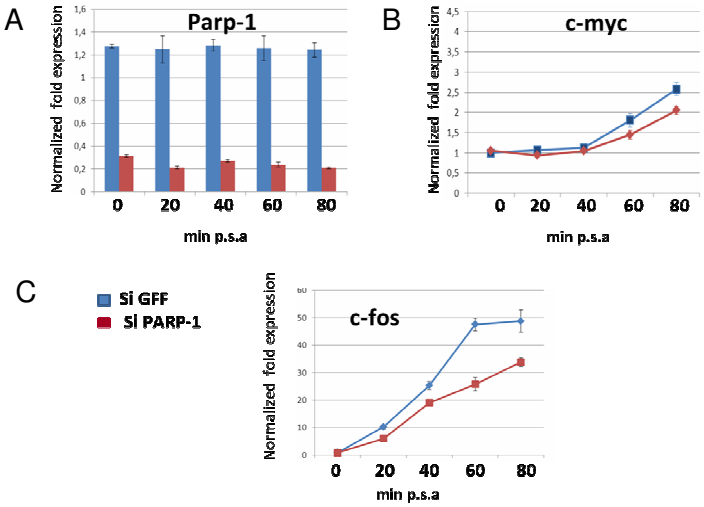

Figure 5. PARP-1 silencing before quiescence entry has a different effect on c-fos and c-myc up-regulation.
(A-C) RT-qPCR quantization of PARP-1 and IEGs mRNA levels. Proliferating MEF fibroblasts were transfected with siRNA specific for PARP-1 (red lines) or control siRNA (blue lines). Then, the cells were growth arrested by serum withdrawal. RNA samples were prepared after serum-stimulation to G0-G1 transition of cells. The Horizontal axis values represent the minutes post serum stimulation (min p.s.a.). The vertical axis values represent the expression fold change respect to control T0 sample, RNA levels of parp-1 c-myc, c-fos, were normalized on 18S. The error bars represent the SEM of three replicates.

Because of these differences, the binding of PARP-1 to the c-fos promoter was investigated by performing ChIP assays. Chromatin samples were prepared from quiescent cells and at different times from serum stimulation. The presence of c-fos promoter sequences in the fraction of PARP-1 bound chromatin was detected using primers specific for a promoter region encompassed between the serum responsive element and the transcription start site. PARP-1 bound to this region from 15 to 45 min after serum stimulation but not in quiescence. Therefore, differently from what happens for c-myc promoter that is bound by PARP-1 in quiescent cells, PARP-1 does not occupy c-fos promoter when the gene is repressed. According with the above-mentioned RT-qPCR data, these ChIP results support the existence of different mechanism by which PARP-1 regulates c-fos and c-myc gene expression during the early phase of cell cycle reactivation.

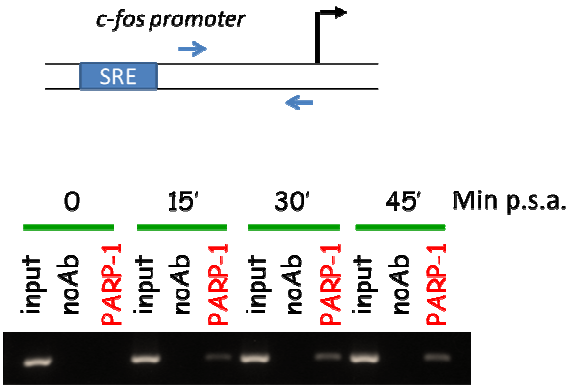

Figure 6. PARP-1 occupies c-fos promoter during G0-G1 transition.
A) Schematic representation of c-fos promoter. The PCR primers used in the subsequent ChIP assays are showed. SRE: serum responsive element.

B) PARP-1 ChIP assays on c-fos promoter region. Quiescent and serum stimulated fibroblasts were fixed at the indicated minutes after serum addition. Sonicated chromatin samples from these cells were immunoprecipitated with an antibody to PARP-1. Then DNA extracted from the immunoprecipitated fractions were amplified by PCR with primers specific for the c-fos promoter. PCR analyses show the desired-size products. DNA sample lane descriptions are as follows;. Input: samples isolated from cell lysates prior to antibody pull down representing the internal positive control; No Ab: samples isolated after pull down with no antibody representing the aspecific-bound DNA; PARP-1: samples isolated after PARP-1 immunoprecipitation.

PARP-1 contributes to c-myc repression during quiescence entry

We observed that in quiescent cells c-myc promoter is occupied by PARP-1, while after mitogen stimulation the enzyme is released, in concomitance with the poly(ADP-ribosyl)ation of the same region. Moreover Kim e colleagues have reported an in vitro model in which PARP-1, promotes transcriptional repression in a manner similar to histone H1 while following activation and auto-poly(ADP-ribosyl)ation, PARP-1 detaches from chromatin, leading to decompaction and transcriptional activation (Kim et al., 2004). According to Kim's model and in light of the results of our ChIP experiments on c-myc promoter, we hypothesized that PARP-1 may have a role in suppressing c-myc expression during quiescence establishment. To verify this hypothesis we analysed the effects of overexpressing the enzyme in parp-1 -/- fibroblasts.

At first we assessed if the knock-out cells can enter quiescence when serum-starved. The levels of G1 phase markers including cyclin D1 and c-myc were determined by western blot while the cell cycle distribution was analysed by FACS on propidium iodide-stained cells. As reported in Figure 7 A-B, fibroblasts

down-regulate cyclin D and c-myc during quiescence entry and accumulate in G0/G1 after 48h from serum withdrawal.
Secondly, fibroblasts were transfected with an expression vector coding for PARP-1 enzyme. The overexpression of exogenous PARP-1 was verified by western blot. Then cells were serum starved and the kinetics of c-myc down-regulation was followed over the time by quantifying its coding RNA by RT-qPCR. PARP-1 overexpression did not affect the basal levels of c-myc in proliferating cell but accelerated c-myc decrease during quiescence entry indicating a possible involvement for PARP-1 in the repression of c-myc transcription (figure 7C).

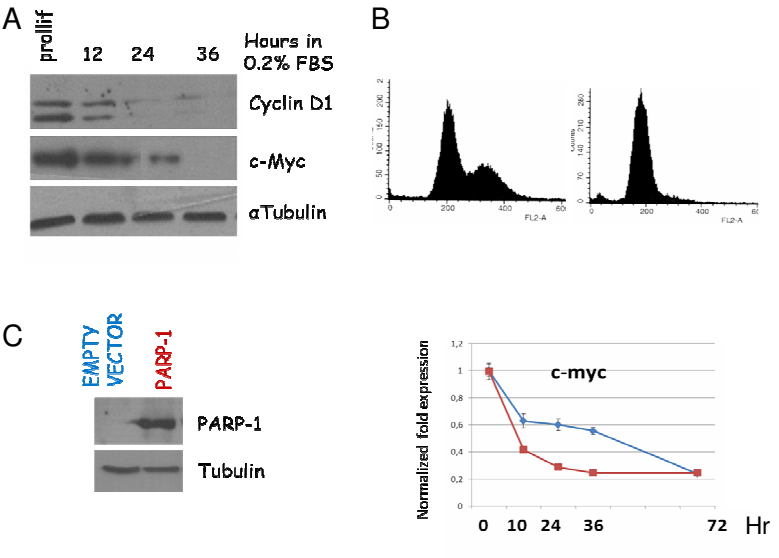

Figure 7. Parp-1 contributes to c-myc repression during quiescence entry.
Proliferating parp-1 -/- fibroblasts were serum starved to induce cell cycle arrest.

A) Western blot analysis of proliferation markers at different hours from serum withdrawal. Tubulin represent the loading control.
B) Flow cytometric analysis of DNA content. A proliferating population of fibroblasts shows the characteristics two-peak distribution (left). After 48 hours in 0.2% serum cells accumulates in the G0/G1 peak (right).
C) Western blot analysis of exogenous PARP-1 expression in knockout fibroblasts (left panel) and RT-qPCR quantization of c-myc mRNA levels (right panel). Fibroblasts were transfected with a vector for PARP-1 expression under the control of CMV promoter (red lines) or the empty vector (blue lines). Forty-eight hours later, cells were shifted in 0.2% serum. RNA levels of c-myc were normalized on 18S. The horizontal axis values represent the hours post serum withdrawal. The vertical axis values represent the fold expression change respect to control T0 sample and the error bars represent the SEM of three replicates.

PARP-1 activity is involved in muscle reserve-cell activation

To highlight the possible role of PARP-1 in regulating quiescence in a stem cell type, we focused our attention on muscle satellite cells. They are quiescent mono-nucleated cells resident between myofibres and basal lamina (Armand et al., 1983; Schultz, 1976) whose activation in response to hypertrophic stimuli or trauma is the first step of growth, repair, and maintenance of skeletal muscle (Grounds and Yablonka-Reuveni, 1993).

To circumvent experimental difficulties deriving from an in *vivo* approach to study satellite cell quiescence, we took advantage of C2 myoblasts, a satellite-derived myoblast cell line. These cells represent a well described in vitro model system that recapitulates many of the functional properties of satellite cells. When cultured in low serum, they are able to undergo myogenic differentiation (Yaffe and Saxel 1977). By contrast, abolishing C2 myoblasts adhesion to substrate reversibly arrests their cell cycle. This condition leads to a quiescent, undifferentiated “reserve cell” state, which is characterized by the expression of satellite cell markers and by the repression of muscle regulatory factors. This state can be

reverted by restoring cell adhesion in the presence of growth factors (Sachidanandan et al., 2002; Sambasivan et al., 2008).
To obtain a homogeneous population of quiescent reserve cells, proliferating C2 myoblasts were cultured in suspension in methylcellulose-containing growth medium for 48 hours. We performed experiments in which gene expression of quiescent and activated reserve cells was assessed by RT-qPCR and western blot. As expected, in absence of substrate adhesion, reserve cells expressed no S-phase markers such as cyclin A, indicating the cell cycle withdrawal, and no detectable levels of the c-myc and c-fos IEG mRNA (Figure 8). Furthermore, the expression of the key muscle regulator MyoD was suppressed at both protein and RNA levels (Figure 8C-D) indicating that cell cycle arrest is uncoupled from differentiation in this condition.
To establish whether restoring the adhesive contacts resulted in their activation, reserve cells were re-plated in growth medium after methylcellulose removal. The fast up-regulation of c-myc and c-fos, detected by RT-qPCR, indicated their cell cycle re-entry. Moreover western blot analysis of cyclin A showed that these cells can resume proliferation. Indeed this S-phase marker was re-expressed within 20h from plating (Figure 8D). Importantly cells not only rapidly up-regulated the IEGs but also restored MyoD expression, as reported by RT-qPCR and western blot in Figure 8 C-D.
The absence of MyoD in quiescent cells and its subsequent induction during cell cycle re-entry is consistent with the fact that these cells represent a suitable model for studying the activation of muscle satellite cells.
To determine the possible implication for PARP activity in cell cycle re-entry of satellite cells, firstly we evaluated whether the PAR levels were modulated upon adhesion-dependent reserve cell activation. By performing a western blot analysis we found that PAR, indicative of PARP-activity, was almost

undetectable in G0 quiescent cells, while rapidly accumulated within 2 hours upon cell cycle re-entry (Figure 9A).

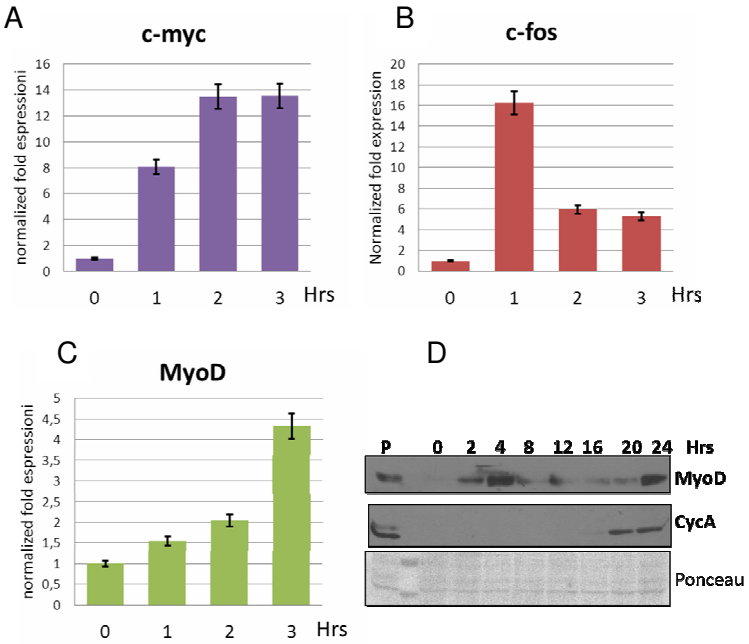

Figure 8. reserve cell activation.

C2 myoblasts were arrested in G0 through suspension culture in methyl-cellulose containing medium to induce the “reserve cell” state. Then cells were re-activated by replating in tissue culture dishes. Samples were collected at the indicated hours after replating.

A-C) RT-qPCR quantization of c-myc, c-fos and MyoD. RNA levels are represented as mean fold changes respect to control T0 sample and the error bars represent the SEM of three replicates. The horizontal axis values represent the hours post replatinig . 18S and L34 were used as normalizing genes.

D) Western blot analysis of MyoD and cyclin A protein levels in proliferating myoblasts (P), quiescent (0) and reactivated reserve cells at the indicated hours after replating. Ponceau staining of nitrocellulose membrane was used as loading control.

Secondly, we analysed the effects of the enzyme inhibition on the S-phase entry kinetics. Cells were re-activated in the presence or in the absence of the PARP inhibitor PJ-34 and DNA synthesis was monitored by BrdU labelling. As aforementioned reserve cells synchronized in quiescence by suspension re-entered S phase in an adhesion dependent manner. However, the addition of PJ-34 impaired the progression to S phase (Figure 9B). Moreover by performing RT-qPCR analysis, we examined also the effect of PARP inhibition on c-myc and MyoD expression. As shown in Figure 9C, PJ-34 treatment impaired the up-regulation of both genes.
Taken together these observations indicate that the activation of reserve cells requires PARP activity.
To Assess the contribution of PARP-1 enzyme in the activation of reserve cells we analysed the effects of its knockdown on IEGs and myogenic regulatory factors. Mouse myoblasts were transduced with a retroviral construct expressing a specific shRNA designed to reduce PARP-1 expression (Figure 10A) or a control vector. Then cells were cultured in suspension to induce the quiescent reserve cell state and, after 48h, were reactivated by re-plating in growth medium.
The western blots reported in Figure 10B show that the reduction of PARP-1 levels was associated with a significant inhibition of c-Myc and MyoD protein accumulation during cell cycle entry. Furthermore the response of c-myc and c-fos genes was analysed by RT-qPCR. As in fibroblasts, in the case of myoblast reserve cells PARP-1 knock-down impaired the induction of the two genes (Figure 10D). Taken together these data suggest the importance of PARP-1 in the re-activation of muscle reserve cells.

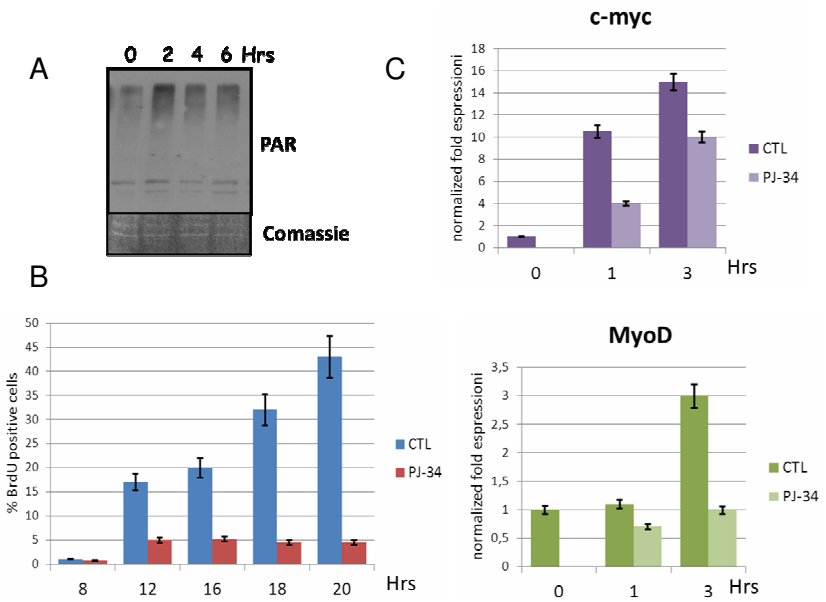

Figure 9 PARP activity is required for reserve cell activation.
C2 myoblasts were arrested in G0 through suspension culture in methyl-cellulose containing medium to induce the "reserve cell" state. Then the cells were re-activated by replating in tissue culture dishes in the presence or in the absence of PARP-1 inhibitor PJ-34. Samples were collected at the indicated hours after activation.
A) western blot analysis of PAR accumulation in reserve cells during their activation. PAR level peaks two hours post cells plating.
B) S phase entry kinetics. Percentage of BrdU-positive nuclei on total DAPI-stained nuclei. Cells was activated in the absence (Blue rectangles) or in the presence of the PARP inhibitor PJ-34 (red rectangles) and fixed for immunostaining at the indicated hours (horizontal values). At least 400 nuclei were counted for each sample and the results are the mean of three experiments.
C) RT-qPCR quantization of c-myc and MyoD RNAs induction during reserve cell activation. Untreated samples are represented in violet or green, PJ-34 treated samples are reported in the light respective colours. On the horizontal axis the hours post cell activation are reported. The vertical axis values represent the mean fold changes respect to T0 sample and the error bars represent the SEM of three replicates. Target mRNAs are normalized on 18S and L34 levels.

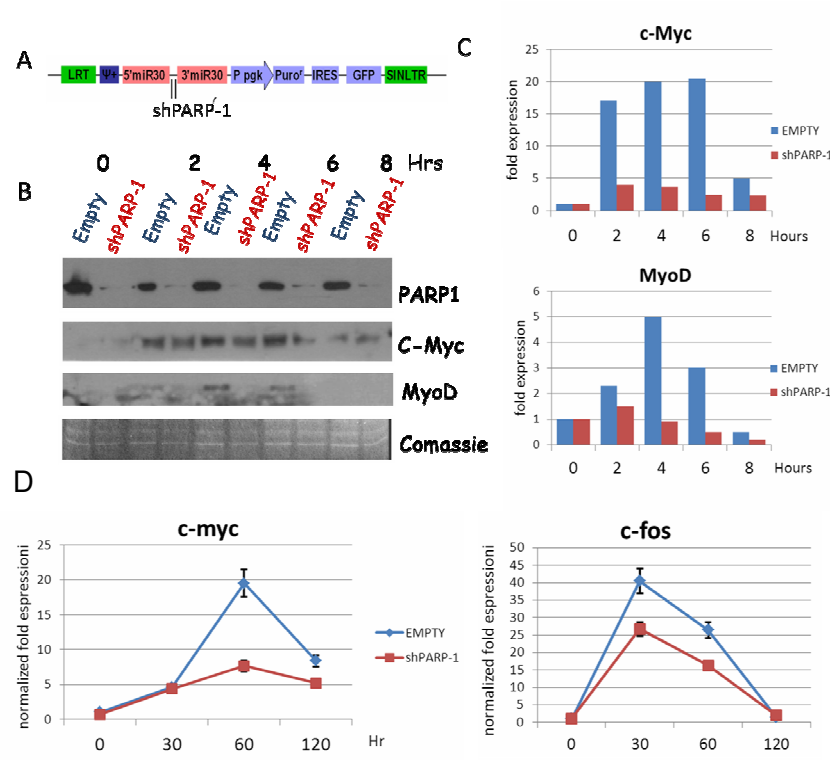

Figure 10. PARP-1 is implicated in reserve cell activation.
A) Schematic representation of the retroviral construct used to silence PARP-1 expression.
B) Western Blot analysis of PARP-1, c-Myc and MyoD in myoblasts infected with a retrovirus encoding for a shRNA specific for PARP-1 (shPARP-1) or with the empty vector (Empty). Samples were collected at the indicated time after adhesion-dependent reactivation of reserve cells. Comassie staining represents the loading control.
C) Densitometry quantification of the c-Myc and MyoD protein level of the western blot reported in B. Vertical axis values represent the fold expression changes respect to each T0 sample.
D) Myoblasts expressing the ShRNA direct against PARP-1 (red lines) or no shRNA (blue lines) were growth arrested and then reactivated. RNA levels of c-myc and c-fos were normalized on 18S and L34 and reported as fold

changes respect to control T0 sample. The error bars represent the SEM of three replicates The horizontal axis values represent the minutes after cell plating.

DISCUSSION

An increasing number of evidence from literature demonstrated that PARP-1, originally characterized as a key factor in DNA repair pathways, has a role in the regulation of gene expression and cell cycle progression. Specifically, PARP-1 can act as an integral part of cellular signaling pathways that culminate in gene-regulatory outcomes (D'Amours et al., 1999).
Even though the best established functions of PARPs during cell cycle concern the control of chromosome segregation at mitosis, other reports indicate that these enzymes, particularly PARP-1, are involved in regulating cell cycle progression to other phases. PARP-1 acts at the S phase, both by participating in a multiprotein DNA replication complex (Simbulan-Rosenthal et al., 1996) and by acting as a positive regulator of E2F1-mediated transcription and, hence, of S-phase gene expression (Simbulan-Rosenthal et al., 1999, 2003). Moreover, previous evidence correlated increased PARP expression and activity with liver regeneration (Cesarone et al., 1990), PBMC activation (Menegazzi et al., 1992) and thymocyte proliferation (Wein et al., 1993), but the role of PARPs in cell cycle re-entry was not further investigated.

Our group previously highlighted a functional link between PARP-1 activation and fibroblast cell cycle re-entry (Carbone et al., 2008). In this work it was shown that increased poly(ADP-ribosyl)ation is transiently detectable after serum stimulation of quiescent fibroblasts and that PARP-1 is responsible for the most cellular poly(ADP-ribosyl)ating activity during the early response. More importantly inhibition of PARP activity by a competitive inhibitor named PJ-34

causes a dose-dependent interference with cell cycle reactivation. Analysis of the early phases of the response to serum stimulation revealed that PARP-1 activation correlates with and is required for the timely up-regulation of IEGs.

Once demonstrated that PARP-1 activity is stimulated by mitogen treatment of resting fibroblasts and contributes to G0-G1 transition through the induction of IEGs, we investigated how PARP-1 may regulate these genes.
At first, the specific silencing of PARP-1 in quiescent fibroblasts confirmed that this enzyme is the PARP family member playing the most prominent role in IEGs activation and that this regulation occurs at the RNA level.
Recent studies have revealed the role of PARP-1 in distinct ways of transcriptional regulation: this protein can act as an enhancer-binding factor, can function as a transcriptional coregulator and can play a role in the maintenance of insulator function (Kraus 2008). Further, PARP-1 can modulate the chromatin structure by binding to nucleosomes, modifying histone proteins or regulating the composition of chromatin.
To go inside into potential molecular mechanisms by which PARP activity may acts locally on IEGs promoters regulation we focused our attention on c-myc promoter. All crucial aspects of cell proliferation, cell growth, and tumorigenesis are positively regulated by c-Myc. Consequently, the fine regulation of c-Myc expression is essential for normal cell function. Such a tight control is achieved through a regulation at multiple levels: transcription initiation and elongation, translation, mRNA and protein stability and post translational modification and interacting proteins. Besides, chromatin remodeling provides an important additional level for control (Wierstra and Alves, 2008).
C-myc gene is transcribed from the dual P1 and P2 promoters (located 160 bp apart), with a dominance of P2. Moreover, the

nucleosomal structure of this promoter region undergoes a reversible change after gene activation (reviewed by Wierstra and Alves, 2008). To elucidate the mechanism by which PARP-1 may regulate transcription locally, we performed a DNAse accessibility assay, in the presence or in absence of PARP activity. Generally, the differential sensitivity to DNAseI has been identified as a characteristic feature that distinguishes the chromatin of transcribed and silent genes and is presumed to reflect alterations of factor binding, nucleosome positioning, or DNA conformation (Levens et al., 1997; Liu and Levens, 2006). Specifically, we observed that after serum stimulation of quiescence fibroblasts the chromatin of c-myc domain including P1 and P2 promoters undergoes a structural change, from a closed and transcriptionally inactive state to an expanded and transcription factor-accessible state, in a PARP-activity dependent manner. Thus, the DNAse accessibility assay of c-myc promoter gave us the first demonstration of a local involvement of PARP activity in causing chromatin to expand and indicated poly(ADP-ribosyl)ation as a specific epigenetic mark for c-myc regulation during G0-G1 transition.

A link between epigenetics and PARPs should not have come as a surprise, since poly(ADP-ribosyl)ation of histone proteins has long been associated with an extended and open chromatin conformation believed to facilitate the access of DNA repair factors to the damage chromatin (D'Amours et al., 1999). The first evidence for the implication of PARP activity in chromatin loosening comes from the study of hsp70 gene activation in Drosophila larval salivary glands. In this work the authors have shown that poly(ADP-ribosyl)ation is required to produce polytene chromosome puff formations associated with gene induction (Tulin and Spradling, 2003). Indeed, it's well established that histones are amongst the main substrates and that poly(ADP-ribosyl)ation of these building blocks of the nucleosome reduces their affinity to DNA thereby increasing

the accessibility of the chromatin fibre. On the basis of this evidence, we can suppose several mechanisms by which PARP-1 could affect the condensation status of chromatin at c-myc promoter and the accessibility to transcription: The most obvious would be the direct modification of chromatin structural proteins or transcription regulatory factors. Unfortunately this case cannot be directly verified at the moment since specific antibodies for poly(ADP-ribosyl)ated proteins to be used in ChIP assays are not yet available.
To further study the effects of PARP activity on c-myc regulation we analysed its promoter occupancy by two transcription factors. CTCF and Sp1 are known to be negative and positive regulators of c-myc gene, respectively (Filippova et al., 1996; Majello et al., 1995). Consistently we found that during quiescence CTCF but not Sp1 is present on c-myc promoter. Moreover after serum stimulation, CTCF is displaced while Sp1 is recruited on its regulatory region. Interestingly, the inhibition of PARP activation by PJ-34 treatment of stimulated cells restores the promoter state occupancy of the quiescence, for both transcription factors. These results are consistent with the DNAse accessibility assay experiment, suggesting a role for poly(ADP-ribosyl)ation in mediating the switch of CTCF and Sp1 at the promoter.

Moreover, we found that PARP-1 coimmunoprecipitates with c-myc promoter chromatin in quiescent cells, that virtually do not express c-myc. In contrast cell cycle re-activation by serum stimulation causes PARP-1 promoter detachment, probably due to its automodification, and the poly(ADP-ribosyl)ation of the same region. This local poly(ADP-ribosyl)ation is related with the exchange of specific transcription factors on c-myc promoter. In addition, PARP-1 is not detected on c-myc promoter in proliferating cells when the gene is expressed at a basal level.

The finding that PARP-1 binds to c-myc promoter during the quiescent state prompted us to study the effects of the enzyme knock-down at different times in relation to growth arrest. Unexpectedly PARP-1 silencing, performed before cell cycle exit, does not significantly influence c-myc expression. In addition *parp-1-/-* cells correctly enter and exit the quiescence state suggesting that the PARP-1 dependent mechanism of c-myc gene regulation have to be established before quiescence entry. Hence if the enzyme is already absent in proliferating cells, c-myc up-regulation during G0-G1 transition could occur in a PARP-1 independent manner, involving other chromatine-related mechanisms or perhaps another member of the PARP family.

On the basis of this dynamics of PARP-1 binding and activity to c-myc promoter we hypothesized a possible mechanism involving an active participation of the enzyme first in the repression, when cells have to exit cell cycle, and then in de-repression when cells have to be reactivated. This dual role would be supported by a well established in vitro model in which in the absence of NAD+ PARP-1, by binding to nucleosomes, promotes chromatin compaction and transcriptional repression in a manner similar to histone H1. Following activation and auto-poly(ADP-ribosyl)ation in the presence of NAD+, PARP-1 detaches from chromatin, leading to decompaction and transcriptional activation (Kim et al., 2004). Accordingly the overexpression experiments in *parp-1-/-* fibroblasts showed that exogenous PARP-1 accelerates c-myc shutdown during the establishment of quiescence without affecting the basal levels of c-myc expression in proliferating cell. These data suggest a possible implication for PARP-1 in the repression of c-myc transcription during quiescence. PARP-1, still not activated, either by itself or by regulating other chromatin modifiers may induce chromatin condensation or may promote CTCF inhibitory effects on c-myc. At the

same time, PARP-1, CTCF or the presence of both may prevent Sp1 binding to c-myc promoter. Therefore, 15 min post stimulation PARP-1, now automodified, detaches from the promoter facilitating CTCF removal (either by direct interaction with the factor or by its poly(ADP-ribosyl)ation). Furthermore PARP-1 activity causes chromatin relaxation. In this condition Sp1 and possibly other transcriptional factors may interact with their binding sequences acting as a positive transcription regulators. Collectively our data reveal a functional link between promoter binding and gene-regulatory actions of PARP-1, highlighting the relation between PARP-1 activity and the function of transcription factors (Figure 11).

In any case, we cannot exclude the participation of additional mechanisms that could mediate the PARP-1-dependent changes at IEG promoters. For example, it has been reported that PARP-1 interacts with co-repressor and co-activator complexes which contain histone deacetylases and acetylases (Ju et al., 2004; Ju et al., 2006) and more recently, with the histone demethylase KDM5B (Krishnakumar and Kraus, 2010a). Additional studies are needed to clarify this specific mechanism. ChIP specific for histone modifications or transcriptional factors and cofactors could be helpful for this purpose.

The IEGs expression data from the PARP-1 knock-down experiments performed at different times during growth arrest in addition to ChIP assay results, suggest some differences between PARP-1 dependent regulation of c-myc and c-fos promoters. Certainly PARP-1 knock-down, performed in fibroblasts after growth arrest, affects c-myc as well c-fos serum-induced up-regulation. Otherwise, when the enzyme was silenced before quiescence establishment, only the induction of c-fos was significantly affected. Moreover differently from what happens for c-myc whose promoter binds PARP-1 in

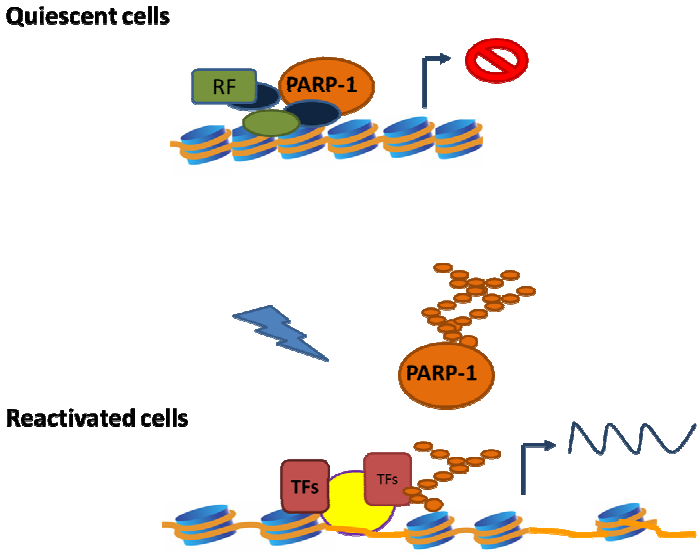

Figure11. Proposed model of PARP-1 action at c-myc promoter
During quiescence PARP-1 binds to c-myc promoter favouring shut off of the gene. The enzyme could induce chromatin compaction or recruit repressive factors (RF). After mitogenic stimulation active PARP-1 poly(ADP-ribosyl)ates the promoter and detaches from it allowing chromatin de-condensation and transcriptional factor (TF) exchanges.

quiescence and is released in concomitance with the induction, PARP-1 coimmunoprecipitates with active c-fos promoter after serum stimulation but not during quiescence when the gene is repressed. These data suggest that PARP-1 is implicated in the prompt induction of c-fos at the early phase of G0 exit but, unlike for c-myc, not for the repression of the gene. This is not completely unexpected because the promoters of the two IEGs are quite different (reviewed by Wierstra and Alves, 2008 ; Janknecht at al.,1995) as well as their patterns of expression show some differences. In fact after a rapid induction

promoting G0/G1 transition, c□myc mRNA declines to a lower level which persists during the whole cell cycle (in continuously proliferating cells) while c-fos mRNA drop to under detectable levels also in cycling cells except that other signals e.g cytokines, ionizing radiation and stress cause its induction.

To identify a specific roles of PARP-1 in the reversible transitions between quiescence and proliferation in a other cellular system the attention was focused on muscle satellite cells, which are quiescent mono-nucleated cells resident between myofibres and basal lamina (Armand et al., 1983; Schultz, 1976). The experimental in vivo approach to study the satellite cell quiescence raises several difficulties not only because these cells represent a very small population, but also because every method for isolating them inevitably leads to their activation. However, there are some reliable in vitro model systems that recapitulate many of the functional properties of satellite cells and allow the generation of homogenous cell populations that can be reversibly arrested in G0. C2 myoblasts, a satellite-derived myoblast cell line (Yaffe and Saxel 1977), when cultured in low serum, are able to undergo a well characterized process of differentiation that recapitulates many features of *in vivo* myogenesis. A subpopulation present in differentiated C2 cell cultures showing many properties of satellite cells is known as C2 myoblast-derived "reserve cells" (Kitzmann et al., 1998; Yoshida et al., 1998). In particular, they are in a quiescent state, have lost the expression of muscle regulatory factors and retain the myogenic potential. When stimulated by serum, "reserve cells" proliferate, re-express myogenic factors and can be induced to differentiation, producing myofibers and once again quiescent reserve cells. In addition, C2 myoblasts can be arrested in G0 through suspension culture in methyl-cellulose.

This condition also leads to an "reserve cell-like" undifferentiated state, which is characterized by growth arrest, expression of satellite cell markers and down-regulation of myogenic determinants (Sachidanandan et al., 2002; Sambasivan et al., 2008).

We investigated PARP-1 involvement in the reactivation of satellite cells using the C2 myoblasts model system synchronized in G0 ("reserve cell-like" state) by suspension in a viscous gel of methylcellulose-containing media and then reactivated by restoring cell anchorage. Our results suggest that poly(ADP-ribosyl)ation may regulate quiescence even in satellite cells. Western blot analysis of cell extracts from methyl-cellulose- synchronized myoblasts shows that poly(ADP-ribose), indicative of PARP-activity, rapidly accumulates within 2 hours upon cell cycle re-entry. Interestingly, PARP activation precedes the induction of c-myc, just like in mitogen-stimulated fibroblasts. Most importantly, the inhibition of the enzyme activity impairs c-myc and MyoD up-regulation and prevents S-phase entry. These observations suggest that the importance of PARP activity in the emergence from quiescence could be also extended to the function of muscle stem cells.

In conclusion in this work we highlight a role of PARP-1 in the regulation of cell quiescence which is of fundamental importance for controlling differentiation, preserving stem cell function and preventing tumorigenesis. The identification of new mechanisms involved in the transitions between quiescence and proliferation will provide valuable information aimed at devising further strategies for cancer treatment or for a controlled stem cells proliferation.

MATERIALS AND METHODS

Cell culture

Mouse embryonic fibroblasts (MEF), mouse embryonic fibroblasts from PARP-/- mice (A1) and human fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Cambrex), 2 mM L-glutamine, 1% pen/strep solution (v/v). For serum stimulation experiments, fibroblasts were grown for 48 hours in DMEM containing 0.2% FBS and then stimulated by incubation in growth culture medium supplemented with 10% FBS. When indicated PJ-34 (Sigma-Aldrich) previously diluted in aqueous solution was added at the final concentration of 30µM.

C2C12 mouse myoblasts were passaged and maintained as subconfluent monolayers in growth medium, high glucose DMEM (GIBCO) supplemented with 20% FBS. To growth arrest myoblasts, cultures were trypsinized and suspended at a final density of 2 x l0^5 cells per ml in DMEM containing 1.3% Methocel (SIGMA), supplemented with 10% FBS. After 72h, suspended cells were recovered for replating by dilution of methylcellulose-containing medium with four volumes of sterile phosphate-buffered saline (PBS) followed by centrifugation at 2000 g, 30 minutes, room temperature. the cells were replated on tissue culture dishes in growth medium (10% FBS) and analysed at the indicated times following replating (Milasincic et al., 1996). When indicated PJ-34 (Sigma-Aldrich) previously diluted in aqueous solution was added to the growth medium at the final concentration of 30µM.

Flow cytometric analysis of DNA content

Fibroblasts were trypsinized and counted. 5x 10^5 cells were fixed in 70% ethanol for 16 hours incubated with 0.1 mg/ml Propidium Iodide (Sigma-Aldrich) and 2 mg/ml RNAse in PBS for 30' in the dark. Cell cycle profiles of stained cells were obtained by flow cytometric analysis with FACS Calibur (BD Bioscience Pharmingen).

Immunofluorescence analysis of BrdU incorporation

Cells were first incubated for the indicated periods of time with 20uM bromodeoxyuridine (BrdU) and then fixed with 30% methanol/70% acetone for 30 min at -20°C. Cells were treated with 1.5M HCl for 30 min at room temperature. After washing three times with PBS, cells were incubated with anti BrdU antibody (sc-51514 Santa Cruz biotechnology) 1:50 in 3% BSA/PBS for 1 hour. After that, cells were washed three times with PBS and incubated for 45 minutes in the dark with a rodaminated goat anti-mouse antibody (dilution 1:100 in 3% BSA/PBS.) Then the cells were rinsed with PBS. To counterstain nuclei, cells were incubated with DAPI for 5 min at room temperature. immunostained cells were analysed on Nikon microphot FXA equipped with a 20x objective.

RNA interference and overexpression

For PARP-1 siRNA experiments in quiescent cells, MEF cells (2.5×10^5cells) were grown for 36h in DMEM containing 0.2% FBS and then transfected with a mix containing 20µl of Lipofectamine 2000 reagent (Invitrogen Life Science Technologies, CA, USA) plus 150nM of siRNA oligos. At 48h after cells were stimulated by incubation in DMEM with 10% FBS and collected for RNA or protein analysis.

For siRNA experiments in proliferating cells, MEF were seeded in DMEM containing 10% FBS 16h before transfection. 24h after transfection growth medium was replaced with

DMEM containing 0.2% FBS. After 48h cells were stimulated by incubation in DMEM with 10% FBS and collected for RNA or protein analysis

siRNA target sequences:
parp-1 5'-TAAAGAAGCTGACGGTGAA-3'
gfp 5'-GGCTACGTCCAGGAGCGCACC-3'

A1 fibroblasts were transfected with pPARP31 plasmid DNA containing the full length cDNA sequence of PARP-1 or the empty vector as a control and Lipofectamine 2000 reagent according to manufacturer's At 48 h after transfection, the growth medium (10% FBS) was replaced by fresh medium containing 0.2% FBS to induce growth arrest. PARR-1 expression was assessed by western blot 48h after transfection.

Retrovirus production and infection

We designed 3 hairpins targeting PARP-1 gene. Of these, we successfully cloned 2 hairpins. These hairpins were cloned into a retroviral vector (LMP from Open Biosystem) containing a puromycin resistance gene and GFP gene as a marker for retroviral integration. Briefly, synthetic double-stranded oligos that represent a stem-loop hairpin structure were PCR amplificated and cloned into retroviral vector. the expression of a given hairpin produces a shRNA that targets the gene of interest. To obtain high titer recombinant retroviruses expressing, the BOSC 23 packaging cells were transfected with LMP-shPARP-1 as previously described (Fimia et al., 1998). Briefly, 6×10^6 cells were seeded onto 100-mm tissue culture dishes in DMEM supplemented with 10% FBS and grown for 24 h. Just before transfection, 25 μM chloroquine was added to the culture medium and 20 μg of plasmid/100-mm dish were transfected with the calcium phosphate precipitation method. After 10 h the medium was changed and cells were incubated

for an additional 16 h in DMEM-10% FBS. Medium was again replaced with a smaller volume. The retroviral supernatant was harvested 24 h later and, after removal of cell debris, frozen at −80°C for later use. BOSC 23 retroviral supernatants were routinely tested for their ability to infect the cells, by immunofluorescence detection of GFP expression. For retroviral infections, cells were plated 24 h before infection then they were incubated with the specific BOSC 23 retroviral supernatant supplemented with 4 µg/ml polybrene for 8 h and then re-fed with fresh medium. The effect of ShRNA expression was assessed 48h after transduction with western blot analysis of PARP-1 protein levels.

Real Time-PCR

Total cellular RNA was extracted with "High Pure RNA Isolation Kit" according to the manufacturer's instructions (Roche Diagnostics). 1 µg of total RNA was reverse transcribed (Iscript cDNA Synthesis Kit, bio-rad). Real timePCR reaction was performed in 20 µl of reaction buffer containing 1µl of diluted cDNA, 10 µl of GoTaq qPCR Master Mix (Promega) and each primer at the optimized final concentration (150-250nM). The reaction was performed in the termocycler "MiniOpticon Real-Time PCR detection system" (Bio-Rad). The primer pair efficiency, the normalized expressions (ΔΔC(t)) and the Standard Deviation for the Normalized Expression were determined with CFX ManagerTM software (Bio-Rad).

Gene	Accession number	Primer set
c-myc	NM_001177354	5'-TGCCCGCGATCAGCTCTCCT-3' 5'-GGGGCATCGTCGTGGCTGTC-3'
c-fos	NM_010234	5'-AGGGCAGCAGCAGCAACGAG-3' 5'-CTCGGGCAGTGGCACGTCTG-3'

junb	NM_008416.3	5'-ACGACTCTTACGCAGCGGCG-3' 5'-GCCAGGTTGAGCGCCAAGGT-3'
parp-1	NM_007415	5'-GCGAGGTCCAGCAGGCAGTG-3' 5'-ACCTTGGCCTGCACGCTGTC-3'
myod	NM_010866	5'-CTCTGCTGCGCGACCAGGAC-3' 5'-GGGCCGCTGTAATCCATCATGCC-3'
18s	NR_003278	5'-ACGACCCATTCGAACGTCTG-3' 5'-GCACGGCGACTACCATCG-3'
L34	NM_053162	5'-GGA GCC CCA TCC AGA CTC-3' 5'-CGC TGG ATA TGG CTT TCC TA-3'

Western blot

For western blot analysis cells were washed twice with cold PBS and lysed in Leamli 1x. Proteins were resolved by electrophoresis in 8%-10% SDS-PAGE and transferred to nitrocellulose membranes by electro-blotting. Membranes were blocked in 1% non-fat dry milk in TBS containing 0.05% Tween 20 for 1h at room temperature and incubated with primary antibody overnight at 4°C, then membranes were washed three times and incubated in a 1:10000 or 1:20000 dilution peroxidase-conjugated anti-mouse or anti-rabbit respectively (Bio-Rad). Proteins were detected using the ECL chemiluminescence system (Pierce).

The following primary antibodies used for western blot were purchased from Santa Cruz Biotechnology: c-Myc N-262 (sc-764); c-Fos 4 (sc-52); PARP-1 H-250 (sc-7150), cyclin A(C-19)sc-596, cyclin D1 72-13G (sc-450) and α-Tubulin TU-02 (sc-8035).
The following primary antibodies used for western blot were purchased from Enzo biochem: anti-PAR (H-10), monoclonal anti-PARP-1 (C2-10 and F1-23), polyclonal anti-PARP-1, MyoD1 clone 5.8A from Dako

Chromatin immunoprecipitation (ChIP)

Cells were treated with formaldehyde (1% final concentration), added directly to the culture dishes, to cross-link protein complexes to the DNA. The reaction was stopped by adding glycine to a final concentration of 0.125M for 5 min at RT. Cells were washed with cold phosphate buffered saline, scraped and lysed in L1 buffer (2mM EDTA, 50mM Tris-HCl [pH8.1], 0.1% NP40, 10% Glicerol and protease and phosphatase inhibitors) for 20 min at 4°C in rotation. The lysates were homogenized by and then centrifuged at 5000 rpm for 5 min at 4°C. Nuclear pellets were resuspended in L2 buffer (5mM EDTA, 50mM Tris-HCl (pH8.0), SDS 1% and protease and phosphatase inhibitors) and kept on ice for 10 min. Nuclear lysates were sonicated to obtain chromatin fragments of an average length of 200 to 800 bp and centrifuged at 10000 rpm for 10 min at 4°C. The sonicated supernatant fractions were diluted 10 fold with dilution buffer (5mM EDTA, 50mM Tris-HCl (pH8.0), NP40 0.5%, NaCl 200 mM and protease and phosphatase inhibitors). After determining the DNA concentrations, each chromatin sample was divided into aliquots of 100µg of chromatin was incubated with Protein A or G Sepharose for 3 hours at 4°C on a rotating platform. The beads was pre-incubated with BSA and Salmon sperm O.N.. The pre-cleared chromatin samples were centrifuged at 14,000 rpm for 5 min and incubated with antibody or without antibody overnight with gently rotation at 4°C. Before washing, an aliquot of the supernatant of the no antibody control was taken as input sample. After the immunoprecipitate was washed twice with a low salt wash buffer (0.1% SDS, 1% triton X-100, 2mM EDTA, 20mMTris-HCl, pH 8.1, 150mM Nacl), twice with high salt wash buffer (0.1% SDS, 1% triton X-100, 2mM EDTA, 20mMTris-HCl, pH 8.1, 500 mM Nacl) ant with LiCl wash buffer (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mMEDTA, 10mM Tris-HCl, pH 8.1) supplemented with

protease and phosphatase inhibitors. Pellets were dissolved in 300 µl elution buffer (1% SDS, 0.1 M NaHCO3). The samples treated with RNAse A for 10 min at RT were incubated at 67 °C overnight to reverse the protein-DNA cross-linking. Then in each sample the NaHCO3 was neutralized with 6 µl Tris-HCl 1M (pH6-7.5). After treatment with proteinase K, the DNA was extracted with phenol-chloroform, precipitated with ethanol and resuspendend in 200 µl of distilled water. 1 µl of immunoprecipitated were used for PCR.

The following antibodies were used for immunoprecipitation: Sp-1 (sc-59) from Santa Cruz Biotechnology, CTCF (07-729) from Millipore, PARP-1 (pAb 210-302 R100) from Alexis biochemical, PAR (H 10) from Enzo biochem.

c-myc promoter
Left primer 5'-CTTTAAATGCGAGGGTCTGG-3'
Right primer 5'-TGCCTCTCGCTGGAATTACT-3'

DNaseI accessibility assay.

Quiescent or serum stimulated cells were rinsed with PBS and then with ice-cold CSK buffer (10 mM HEPES [pH 7.4], 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2). Cells were scraped from the plate, pelleted by centrifugation at 3000 rpm, and lysed in CSK-Triton buffer (CSK buffer containing 0.5% Triton X-100, 1 µg of leupeptin/ml, 1 µg of aprotinin/ml, 1 mM NaF, 1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride) at 10^7 cells/ml for 10 min on ice. Nuclei were pelleted by centrifugation at 3000 rpm for 5 min at 4°C. Supernatants were removed. The pelleted nuclei then were washed with 1 ml of CSK-Triton buffer, pelleted by centrifugation, and suspended in CSK-Triton buffer containing DNase I (0-100 U) and 50 mM MgCl2 and incubated on ice for 4 min. the digestion was stopped with 9 volumes of DNA lysis Buffer(Tris 10 mM, EDTA 10mM e 0.5% SDS). The samples

were treated with proteinase K for 2 hours 56°C. then the DNA was extracted with phenol-chloroform, precipitated with ethanol and resuspendend in 500 µl of distilled water. 2 µl of DNA samples were used for PCR.

c-myc promoter NG_007161
Left primer 5'-GAGGAGCAGCAGAGAAAGG-3'
Right primer 5'-TCCAGCGTCTAAGCAGCTGCAA-3'

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