Journal of Cellular Physiology

p8/nupr1 Regulates DNA-Repair Activity After Double-Strand Gamma Irradiation-Induced DNA Damage

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The stress protein p8 is a small, highly basic, unfolded, and multifunctional protein. We have previously shown that most of its functions are exerted through interactions with other proteins, whose activities are thereby enhanced or repressed. In this work we describe another example of such mechanism, by which p8 binds and negatively regulates MSL1, a histone acetyl transferase (HAT)-associated protein, which in turn binds the DNA-damage-associated 53BP1 protein to facilitate DNA repair following DNA γ-irradiation. Contrary to the HAT-associated activity, MSL1-dependent DNA-repair activity is almost completely dependent on 53BP1 expression. The picture that has emerged from our findings is that 53BP1 could be a scaffold that gets the HAT MSL1-dependent DNA-repair activity to the sites of DNA damage. Finally, we also found that, although p8 expression is transiently activated after γ-irradiation, it is eventually submitted to sustained down-regulation, presumably to allow development of MSL1-associated DNA-repair activity. We conclude that interaction of MSL1 with 53BP1 brings MSL1-dependent HAT activity to the vicinity of damaged DNA. MSL1-dependent HAT activity, which is negatively regulated by the stress protein p8, induces chromatin remodeling and relaxation allowing access to DNA of the repair machinery.

J. Cell. Physiol. 221: 594–602, 2009.

The *p8/nupr1* (*p8* herein) gene was first described as over-expressed in acinar cells of the pancreas during the acute phase of pancreatitis (Mallo et al., 1997). Then, we and others found that expression of p8 mRNA is rapid, strong, and transient in response to several injuries, including minimal stresses (Jiang et al., 1999; Garcia-Montero et al., 2001a; Zinke et al., 2002; Taieb et al., 2005; Plant et al., 2006) and in response to several factors such as TGFbeta (Garcia-Montero et al., 2001b) TNFalpha (Kallwellis et al., 2006), glucose (Path et al., 2006), and endothelin (Goruppi et al., 2002). Structurally, p8 is a highly basic 82-amino-acid polypeptide, with a theoretical molecular mass of about 8 kDa, containing a canonical bipartite domain of positively charged amino acids typical of nuclear-targeting signals (NLS) (Vasseur et al., 1999) and its nuclear and/or cytoplasmic location has been established (Igarashi et al., 2001; Su et al., 2001; Ito et al., 2003, 2005a,b; Valacco et al., 2006). In fact, the nuclear or cytoplasmic localization of p8 depends, at least, on growth conditions (Valacco et al., 2006). Moreover, p8 protein contains an N-terminal PEST (Pro/Glu/Ser/Thr-rich) region, suggesting a regulation of p8 expression by the ubiquitin/proteasome system (Goruppi and Kyriakis, 2004).

Homology searching in databases yielded no homology of p8 with other proteins of known function. However, some of its biochemical properties are shared by members of the high mobility group proteins (HMG), particularly by the HMG-I/Y family (Encinar et al., 2001). NMR and CD analyses of recombinant p8 showed absence of stable secondary structure. The protein binds DNA weakly and is a substrate for protein kinase A. The phosphorylated p8 has a higher content of secondary structure than the non-phosphorylated protein and phosphorylated p8 binds DNA strongly (Hoffmeister et al., 2002). Moreover, secondary structure prediction methods indicate the presence, within the region showing homology with HMG proteins, of a basic helix–loop–helix secondary

Abbreviations: HAT, histone acetyl transferase; 53BP1, p53-binding protein 1; MSL1, male-specific lethal protein 1; MOF, males-absent-on-the-first.

Meritxell Gironella and Cedric Malicet contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: INSERM.
Contract grant sponsor: Ligue Contre le Cancer.
Contract grant sponsor: Cancéropole PACA.
Contract grant sponsor: Ecos-Sud.
Contract grant sponsor: Instituto de Salud Carlos III.

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Received 8 May 2009; Accepted 26 June 2009

Published online in Wiley InterScience (www.interscience.wiley.com.), 31 July 2009.
DOI: 10.1002/jcp.21889

structure motif, characteristic of some classes of transcription factors. An architectural role in transcription was proposed (Garcia-Montero et al., 2001b; Hoffmeister et al., 2002; Quirk et al., 2003; Carracedo et al., 2006a,b; Goruppi et al., 2007), but other apparently unrelated functions have also been ascribed to p8 such as being an essential element of the defense program of the cell (Vasseur et al., 2003; Taieb et al., 2005), of the endoplasmic reticulum stress response (Carracedo et al., 2006a,b), and of tumor formation and progression (Vasseur et al., 2002; Mohammad et al., 2004). In addition, p8 is involved in cell-cycle regulation, through its interaction with Jab1 (Malicet et al., 2006a), and apoptosis by interacting with prothymosin alpha (Malicet et al., 2006b,c). To account for these various functions, we have suggested that the small size of the protein, its lack of specific tridimensional structure, and its nuclear-cytoplasm localization allow its interaction with several partners to target different signaling pathways.

In this article we describe a new role for p8. We found that p8 binds to and inhibits MSL1, a protein with 53BP1-dependent DNA-repair activity. Through this interaction p8 regulates DNA repair after double-strand DNA damage induced by γ-irradiation.

Materials and Methods

Cell lines and cell culture conditions

All cell lines were obtained from American Type Culture Collection (ATCC, Molsheim, France). HeLa and the Phoenix amphotropic viral packaging cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. All cell lines were routinely cultivated in humidified 5% CO_2 atmosphere.

Antibodies

Anti-H4K16ac (catalogue # 07-329) and anti-H3K9ac (catalogue # 07-352) antibodies were purchased from Millipore (Molsheim, France). The anti-MSL1 rabbit polyclonal antibody (catalogue # ab61008) was purchased from Abcam (Paris, France). The anti-53BP1 rabbit polyclonal antibody (catalogue # sc-22760), anti-MRG15 (A-13) goat antibody (catalogue # sc-26529), and anti-MORF4L1 (N-19) goat antibody (catalogue # sc-26525) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-β-actin mouse monoclonal antibody (catalogue # A5441) and the rabbit polyclonal anti-Flag (catalogue # F7425) were from Sigma (Saint-Quentin Fallavier, France), the mouse monoclonal anti-HA (catalogue # 1666606) was from Roche (Meylan, France) Diagnostics, and the rabbit polyclonal anti-V5 (catalogue # MCA1360GA) was from Serotech (Oxford, UK). The p8 polyclonal antibody was previously described (Vasseur et al., 1999).

Yeast two-hybrid screenings

Using a PCR-based strategy we subcloned the complete coding sequence of human p8 (Vasseur et al., 1999) into the *Mlu*I restriction site of the pSos vector to generate the fusion protein pSos-p8. MSL1 was PCR amplified and subcloned into the pSos vector using the same approach as for pSos-p8. These constructs were used as bait to screen, according to the protocols provided by the manufacturer (Stratagene, La Jolla, CA), a human testes library constructed into the pMYR vector.

Co-precipitation assays

The full-length sequence of MSL1 was cloned into the mammalian expression vector pcDNA4 using the Gateway approach to produce the MSL1-V5 recombinant protein following recommendations of the supplier (Invitrogen, Cergy-Pointoise, France). p8-Flag was generated by in-frame ligation of the full-length sequence of human p8 into the *Xho*I and *Eco*RV restriction sites of the pcDNA3-Flag vector. DNA constructs were systematically sequenced to confirm their correct sequence. Plasmids were transfected into 293T cells using the lipofectamine 2000 reagent following the manufacturer's recommendations (Invitrogen). After lysis in 50 mM Tris–HCl, pH 8.0, 0.5% Nonidet P-40 with protease inhibitors, p8 was immunoprecipitated by adding rabbit antibodies against Flag epitope and MSL1 with an anti-V5 polyclonal antibody by rocking 2 h at 4°C. Then, 20 μl of protein A-Sepharose or protein G-Sepharose conjugate (Zymed Laboratories Inc, San Francisco, CA) was added and incubated for an additional 2 h at 4°C. The Sepharose beads were washed three times followed by SDS–PAGE and Western blotting by using the anti-V5 or anti-Flag antibodies. Immunoprecipitation of endogenous proteins was performed using an anti-p8 rabbit antibody (Vasseur et al., 1999). Cell lysate prepared with a buffer containing 1% Triton X-100 was used for precipitation with Sepharose beads. The precipitated material was immunoblotted with anti-MSL1, anti-MRG15, or anti-MORF4L1 antibodies.

Full-length 53BP1 HA-tagged cDNA cloned into the pCMH6K plasmid (Iwabuchi et al., 2003) was a gift from Kuniyoshi Iwabuchi (University of Kanazawa, Japan) and Aidan Doherty (University of Sussex, UK). Interaction of 53BP1 with MSL1 in vivo was analyzed by transfecting pCMH6K-53BP1-HA and pcDNA4-MSL1 plasmids into 293T cells and processed as described above with the exception that anti-HA antibody was used instead of anti-Flag. Immunoprecipitation of endogenous proteins was performed using an anti-53BP1 rabbit antibody. Cell lysate prepared with a buffer containing 1% Triton X-100 was used for precipitation with Sepharose beads. The precipitated material was immunoblotted with an anti-MSL1 antibody.

siRNA transfection

p8 siRNA sequence (sense 5′-GGAGGACCCAGGACAGGAUd(TT)-3′) and MSL1 siRNA sequence (sense 5′-TGAGATCCGCGGTGTTCAAd(TT)-3′) were chosen among 4 and 3 sequences, respectively, for their highest efficacies. 53BP1 siRNA (sense 5′-GCCAGGUUCUAGAGGAUGAd(TT)-3′) was previously reported (Wang et al., 2002). Kinetics of silencing by these siRNAs showed that maximum knockdown was reached within the first 48–72 h, recessing thereafter. These siRNAs were obtained from Eurogentec (Serain, Belgium), annealed and ready to use after rehydration. The day before transfection, cells were plated to give 60–80% confluence. After removal of the medium, cells were washed once with serum-free medium and transfection was done in serum-free medium by addition of a mix composed of XtremeGENE (Roche Diagnostics) and p8 siRNA, MSL1 siRNA, 53BP1 siRNA, or control siRNA diluted in serum-free medium. After 4 h of incubation at 37°C, the transfection medium was replaced by fresh medium. Twenty-four hours later cells were plated for 24 h then γ-irradiated with 7 Gy or HAT activity measured after an additional 24-h period.

Lentivirus production

The lentivirus vectors used in this study were based on the pCCL self-inactivating vector kindly provided by Cedric Raoult (University of Marseille, France). In this vector, the viral promoter was crippled through the deletion of the viral enhancers and TATA box in the U3 promoter region of the 3′LTR. In brief, lentiviral vectors were produced by transient transfection into 293T cells. 293T cells were seeded in 10-cm diameter dishes 24 h prior to transfection and were grown in DMEM with 10% fetal bovine serum in a 5% CO_2 incubator. A total of 30 μg plasmid DNA was used for the transfection of one dish: 10 μg of pMDG-VSV-G envelope plasmid, 10 μg of the pCMVΔR8.93 packaging plasmid, and 10 μg of the transfect vector plasmid (pCCL-p8 or pCCL-MSL1). The lentiviral vectors were constructed as follows: full-length human p8-EGFP and MSL1-EGFP fusion proteins were subcloned into *Bam*HI/*Xho*I restriction sites of the pCCL vector. DNA constructs

were sequenced to confirm their correct sequence. The transfection was done using the polyethyleneimine (PEI) reagent in accordance with the manufacturer's protocol. The medium (10 ml) was replaced after 14–16 h; the conditioned medium was collected after another 24 h and filtered through 0.22-mm pore size cellulose acetate filters. Viral supernatant was used to infect HeLa cells supplemented with 8 μg of polybrene (Sigma)/ml. Infection was done twice. As control, cells were infected with the pCCL empty vector and with an EGFP-expressing lentivirus (pCCL-EGFP). EGFP expression was used to measure the transduction efficiency. For cross-infections we mixed equal volumes of each lentivirus supernatant.

Clonogenic survival assay

HeLa cells were transfected with 2 μg of total DNA containing pcDNA3-p8, pcDNA4-MSL1-V5, pcDNA3-p8 + pcDNA4-MSL1-V5 alone or in combination with pCMH6K-53BP1-HA using the Fugene HD transfection reagent following manufacturer's recommendations (Roche Diagnostics). Empty vectors were used as control or to complete to identical amounts the mass of transfected DNA. After 24 h, cells were γ-irradiated with 7 Gy and 45,000 cells were seeded in duplicate on tissue culture dishes (6 cm) maintained in a humidified incubator at 37°C and 5% CO_2 until cell colonies were formed. After 9 days, colonies were fixed with 75% (v/v) methanol, 25% (v/v) acetic acid for 30 min, rinsed twice with PBS and once with distilled water, stained with crystal violet (1 mg/ml in distilled water) for 10 min and rinsed abundantly with distilled water. When stained cultures were dried, visible colonies were counted. The experiment was repeated three times.

HAT activity assay

HeLa cells were transduced with lentivirus pCCL-p8 and/or pCCL-MSL1 lentivirus. After appropriate antibiotic selection, nuclear extracts were prepared using the Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA). In separate experiments, lentivirus-transduced cells were also transfected with pCMH6K-53BP1-HA plasmid. Bradford method (Bio-Rad protein assay; Bio-Rad, Hercules, CA) was used to quantify protein concentration on each nuclear extract and 30 μg of each condition was used to analyze histone acetyl transferase (HAT) activity using the HAT activity colorimetric assay kit (BioVision) following the manufacturer's recommendations. Plates were incubated at 37°C for 3 h and color development was read at 440 nm in a plate reader.

Analysis of p8 and MSL1 mRNA expression after irradiation

HeLa cells were γ-irradiated at 3, 7, or 10 Gy and total RNA extraction was performed with the Trizol reagent (Invitrogen) at 2, 6, 12, 18, or 24 h after irradiation. Analysis of p8 and MSL1 mRNA expressions was done using qRT-PCR analysis on a LightCycler detection system (Roche Applied Science, Meylan, France). Expression levels of GAPDH gene were used as an internal control. First strand cDNA was synthesized from 1 μg of total RNA using random hexamers and expanded by reverse transcriptase according to the manufacturer's instructions (ImProm-II Reverse Transcription System; Promega, Charbonnières, France), subsequently diluted 1:10 with water, and stored at −20°C until use. MSL1, p8, and GAPDH PCR products were detected by quantitative real-time PCR using the SYBR Premix Ex Taq (Takara Bio, Inc., Gennevilliers, France) following the manufacturer's instructions. Five microliters of diluted cDNA template was mixed with 10 μl SYBR Premix Ex Taq (including Taq polymerase, reaction buffer, $MgCl_2$, SYBR green I dye, and deoxynucleotide triphosphate mix) and 0.4 μM forward and reverse primers, in a final volume of 20 μl. The following primers were used: MSL1 forward 5′-GCCTCTAAGGGACCCAAATC-3′ and MSL1 reverse 5′-TGGTCGTCCAAATGCTACAA-3′; p8 forward 5′-TAGAGACGGGACTGCG-3′ and p8 5′-GCGTGTCTATTTATTGTTGC-3′ reverse; GAPDH forward 5′-GGGAAGCTCACTGGCATGGCCTTCC-3′ and GAPDH reverse 5′-CATGTGGGCCATGAGGTCCACCAC-3′. After an initial Taq activation for 10 sec at 95°C, LightCycler PCR was done using 55 cycles with the following cycling conditions: 95°C for 5 sec, 58°C for 7 sec, and 72°C for 14 sec. Each sample was analyzed in duplicate and the experiment was repeated three times. Results were analyzed using RealQuant data analysis software (Roche Applied Science, Meylan, France).

p8 and MSL-1 recombinant protein productions

Human p8-His6 was obtained as previously described (Encinar et al., 2001). Human MSL1 full-length cDNA was subcloned into the pGEX-4T-1 vector (Amersham Pharmacia Biotech, Orsay, France), downstream of the glutathione-*S*-transferase (GST) sequence and used to transform *E. coli* BL21 strain in order to produce recombinant MSL1-GST protein. Recombinant proteins were produced as described in Encinar et al. (2001).

Surface plasmon resonance

Surface plasmon resonance (SPR) analysis was performed using the BIAcore 3000 system (BIAcore AB, Uppsala, Sweden). p8-His6 was immobilized by amine coupling onto a CM5 chip (BIAcore AB) as previously described (Bousquet et al., 2006). Following immobilization, the chip was washed for 30 min with HBS buffer. The net increases in signal for p8-His6 were 800 RU, where 1,000 RU is equivalent to ∼1 ng of protein/mm^2. For interaction measurements, MSL1-GST was injected at a flow rate of 30 μl/min during 3 min, dissociation was then evaluated by passing HBS running buffer alone over the chip at 30 μl/min for 3 min. After each run, the chip was regenerated with one short pulse of 0.005% SDS and two pulses of HBS. One activated/deactivated channel was used as a negative reference.

Acid extraction of proteins from HeLa cells

Twenty-four hours after transfection with p8-Flag and MSL1-V5, alone or in combination, HeLa cells were pelleted and lysed with lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1.5 mM PMSF, HCl 0.2 M) and incubated on ice for 30 min. After centrifugation at 11,000*g* for 10 min at 4°C, the supernatant fraction of acid-soluble proteins was recovered and dialyzed twice against 0.1 M acetic acid for 2 h and three times against H_2O for 1 h, 3 h, and overnight. Finally, concentration of acid-soluble protein extracts was quantified by the Bradford method.

SDS–PAGE and Western blotting

Fifty micrograms of protein was submitted to 15% SDS–PAGE then blotted following standard methods. Migration was calibrated using Bio-Rad standard proteins with markers covering a 7–240 kDa range. Non-specific binding to the membrane was blocked by 5% BSA in TBS for 1 h at 4°C. Blots were incubated overnight at 4°C with polyclonal anti-H4K16ac and anti-H3K9ac antibodies diluted in 5% BSA. Then, membranes were washed with TBS–0.1% Triton and incubated with a secondary goat-anti-rabbit-HRPO antibody (1:3,000) obtained from Santa Cruz Biotechnology diluted in 5% dry non-fat milk in TBS for 1 h at room temperature. Finally, membranes were washed with TBS–0.1% Triton, developed with the ECL-detection system (Santa Cruz Biotechnology), quickly dried, and exposed to ECL film.

Densitometric analysis

ImageJ 1.32 software from http://rsbweb.nih.gov/ij/download.html was used to quantify the intensities of the bands obtained in Western blots.

Statistics

Statistical analysis was performed by ANOVA and post hoc analysis with Student–Newman–Keuls test. Results shown represent mean ± SD.

Results

Identification of MSL1 as a partner of p8 by a two-hybrid system

We identified proteins interacting with p8 by screening a cDNA library with the CytoTrap two-hybrid system (Stratagene). The conventional yeast two-hybrid screening system was not suitable because p8 being a co-transcriptional factor would not need a partner to induce transcription of selection factors. The CytoTrap Sos system generates fusion proteins whose interaction allows cell growth through activation of the Ras pathway. The p8 cDNA subcloned into pSos provided the bait to screen a testes cDNA library constructed in pMyr. After co-transfection into *Saccharomyces cerevisiae*, strain cdc25H, 5×10^6 clones were screened and 51 positives identified. All clones were PCR amplified. Their sequences were identified by comparison with the GenBank repertoire (Table 1, Supplementary Data).

The interaction between p8 and MSL1 was confirmed by transforming *S. cerevisiae* with both pMyr-MSL1 and pSos-p8 constructs and allowing the transformants to grow on synthetic drop-out (SD) glucose and galactose agar plates lacking leucine and uracil [SD/glu(−LU) and SD/gal(−LU)] at the stringent temperature of 37°C. Clones growing on SD/gal(−LU) plates but not on SD/glu(−LU) plates at 37°C are interaction-positive clones. Growth was observed when pSos-p8 and pMyr-MSL1 were both present, but not when pSos-p8 or pMyr-MSL1 was used separately. Negative and positive controls were grown as suggested by the manufacturer with expected results. These data show that p8 interacts with MSL1 and that the interaction is specific.

pSos-p8-1-46 and pSos-p8-41-82 constructs encode the N-terminal or C-terminal parts of the human p8. These constructs were co-transfected with the pMyr-MSL1 plasmid to identify the interacting region. However, whereas complete p8 interacts with MSL1, neither the N-terminal or C-terminal parts of the protein was able to interact with MSL1 indicating that the complete p8 protein is necessary for this interaction (data not shown).

Interaction of p8 and MSL1 in cells

Interaction between p8 and MSL1 was controlled by co-immunoprecipitation assays. 293T cells were transfected with MSL1-V5 and p8-Flag, alone or in combination. MSL1-V5 and p8-Flag were immunoprecipitated from cell extracts with anti-V5 or anti-Flag antibodies, respectively, and analyzed by Western blot. Anti-V5 antibody was used to detect tagged MSL1 and anti-Flag was used to detect tagged p8. MSL1-V5 was detected in the complex containing p8 while p8-Flag was detected in the complex containing MSL1-V5. Tags were not detected in the negative control (Fig. 1). These results confirm that MSL1 interacts with p8 in 293T cells.

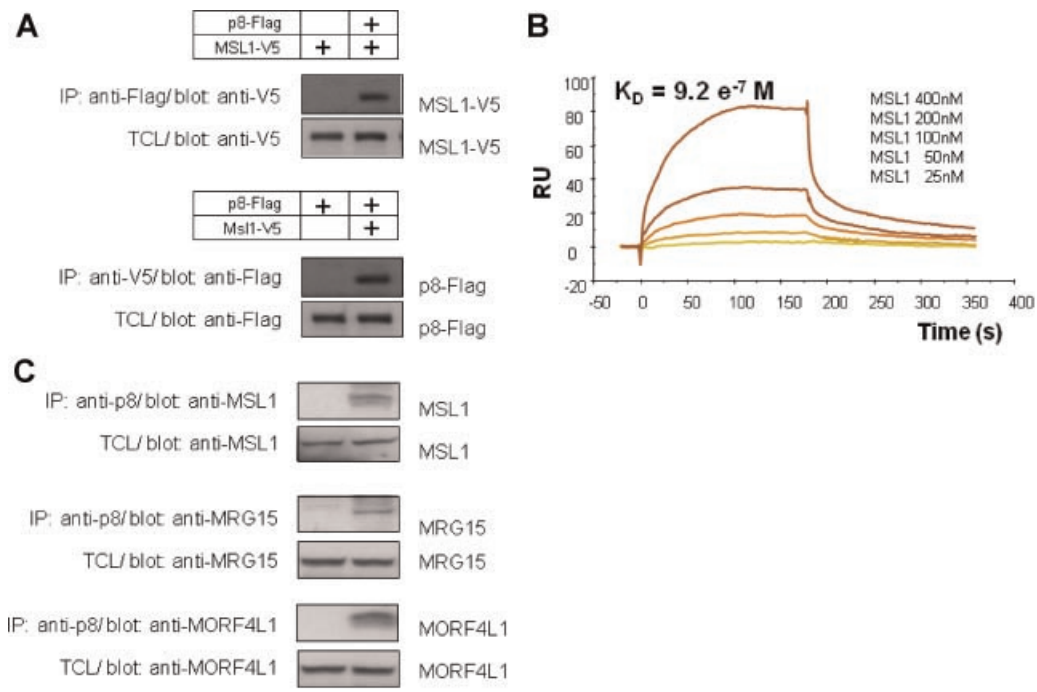

Fig. 1. Interaction of p8 with MSL1: (A) 293T cells were co-transfected with 2 μg of pcDNA4-MSL1-V5 and p8-Flag as indicated. Immunoprecipitation was performed using an anti-Flag or an anti-V5 rabbit polyclonal antibody. Cell lysates prepared with a buffer containing 0.5% Nonidet P-40 were used for precipitations with Sepharose beads. The precipitated material was immunoblotted with anti-V5 or anti-Flag antibodies. TCL, total cell lysate. B: BIAcore analysis of the p8/MSL1 complex: Indicated concentrations of MSL1-GST were injected over immobilized p8-His6. Differential sensograms are obtained by subtracting the response from a free channel to the response from the p8-His6 channel. Affinity constant of the interaction was evaluated using the Biaeval 4.0.1 software (BIAcore AB). RU stands for resonance units. C: Endogenous p8 and MSL1 interaction: Immunoprecipitation of endogenous proteins was performed using an anti-p8 rabbit antibody. Cell lysate prepared with a buffer containing 1% Triton X-100 was used for precipitation with Sepharose beads. The precipitated material was immunoblotted with anti-MSL1, anti-MRG15, or anti-MORF4L1 antibodies. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

BIAcore analysis

To test if a direct interaction exists between MSL1 and p8, we used the surface plasmonic resonance technique as described in the Materials and Methods Section. Recombinant protein p8-His6 was coated on the BIAcore sensor chip. Figure 1 shows that the purified MSL1-GST recombinant protein, used as an analyte, interacts with the immobilized p8-His6. Binding of recombinant MSL1 to p8-His6 was dose-dependent allowing assessment of their affinity ($k_d = 0.92\ \mu M$). By contrast, no significant interaction was observed between p8-His6 recombinant protein and GST (not shown), indicating the specificity of the interaction between p8-His6 and MSL1-GST.

Interaction with endogenous proteins

We controlled the interaction of endogenous p8 and MSL1 in HeLa cells by a co-immunoprecipitation approach using specific antibodies (Fig. 1). As expected, when p8 was immunoprecipitated the MSL1 protein was found in the complex, but not when an irrelevant antibody was used. Interestingly, we also found that MRG15 and MORF4L1 proteins, two components of the HAT hMSL complex (Smith et al., 2005) co-precipitate with p8 (Fig. 1), indicating that p8 is associated with the HAT hMSL complex through its interaction with MSL1.

p8 and MSL1 mRNA expressions after γ-irradiation

p8 and MSL1 mRNA expressions were measured by qRT-PCR in HeLa cells, after 3, 7, or 10 Gy irradiation. As shown in Figure 2, p8 mRNA expression was activated within 2 h after treatment with 7 or 10 Gy, in a dose-dependent manner. Then, a progressive and sustained decrease and inhibition of its expression was observed. p8 mRNA activation was not sensitive to 3 Gy treatment. MSL1 mRNA expression was also transiently activated by γ-irradiation in a dose-dependent manner. Activation was observed after exposure to 3 Gy, suggesting that MSL1 expression is more sensitive than p8 expression to γ-irradiation.

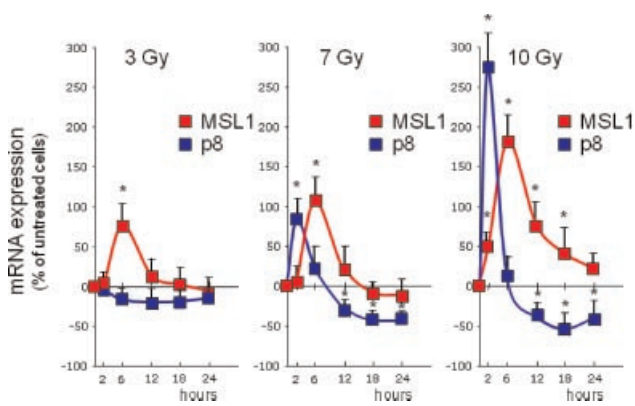

Fig. 2. p8 and MSL1 mRNA expressions after irradiation: HeLa cells were γ-irradiated at 3, 7, or 10 Gy and total RNA extraction was performed at 0, 2, 6, 12, 18, or 24 h after irradiation. p8 and MSL1 mRNA expressions were monitored by qRT-PCR analysis on a LightCycler detection system as described in the Materials and Methods Section. mRNA values are represented as percent of values obtained with untreated cells and expressed as the mean ± SD of combined results from three independent experiments performed in duplicate (*$P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

p8 and MSL1 expressions alter HAT activity

HeLa cells were transduced with lentivirus-expressing p8-EGFP (pCCL-p8), MSL1-EGFP (pCCL-MSL1) or p8-EGFP and MSL1-EGFP, to generate cells constitutively over-expressing these proteins. p8-EGFP and MSL1-EGFP protein expressions were confirmed by Western blot (data not shown). Total HAT activity in nuclear extracts from these cells was also analyzed. As shown in Figure 3, cells over-expressing p8-EGFP presented only with a slight increase in HAT activity, compared to cells infected with control lentivirus. In contrast, cells over-expressing MSL1-EGFP presented with about 35% increase in HAT activity. Surprisingly, cells over-expressing simultaneously p8-EGFP and MSL1-EGFP showed a strong reduction in HAT activity when compared to cells over-expressing MSL1-EGFP alone, suggesting a negative role for p8 expression on MSL1 function. In the same way, we found that p8 siRNA treatment of HeLa cells slightly decreased HAT activity, whereas MSL1 siRNA decreased that activity by about 30% as shown in Figure 3. Altogether, these results indicate that MSL1 participates in the regulation of HAT activity that the direct effect of p8 is minor but that p8 interaction with MSL1 inhibits the effect of MSL1 on HAT activity.

Specificity of p8 and MSL1 activities on histone acetylation

To study the specificity of this HAT activity, we assessed by Western blot the acetylation of H4K16 and H3K9 in HeLa cells after forced expression of p8-Flag and MSL1-V5, alone or in combination. Whereas p8 over-expression did not induce significant changes, MSL1 over-expression increased acetylation of H4K16 but not of H3K9. However, over-expression of p8 together with MSL1 prevented the increase on H4K16 acetylation as shown in Figure 3. These results suggest that MSL1 and p8 regulate HAT activity in a target-specific way.

Clonogenic test after γ-irradiation

To assess the effect of p8 and MSL1 on survival after γ-irradiation, we examined the ability of p8 and MSL1 alone or together to alter colony formation after cell exposure to a 7 Gy irradiation. Cells transfected with the empty vector could generate colonies after irradiation. Their number was not modified by p8 expression but it increased by 50% in cells expressing MSL1. However, when MSL1 was transfected together with p8 its activity on colony formation was completely suppressed, as shown in Figure 4. To confirm these findings, we inhibited p8 or MSL1 expression by specific siRNAs and found that p8 knockdown did not alter colony formation, whereas MSL1 suppression decreased it by about 45%. Taken together, these results indicate that p8 expression prevents the resistance to γ-irradiation induced by MSL1 expression. To verify that this represents a bone fide effect, we analyzed the effect of knocking down p8 and MSL1 mRNAs, alone or together and found no significant effect on cell growth (Supplementary Fig. 1).

Two-hybrid screening reveals that 53BP1 is a partner of MSL1

To find a mechanistic explanation for the protective role of MSL1 against γ-irradiation, we used the CytoTrap approach to look for partners of MSL1. Several partners were identified (see Supplementary Table 2). Among them, 53BP1 was the most interesting candidate because this protein is strongly involved in DNA repair after exogenous induction of DNA damage. The interaction between MSL1 and 53BP1 was confirmed by transforming *S. cerevisiae* with both pMyr-53BP1 and pSos-MSL1 constructs as described above.

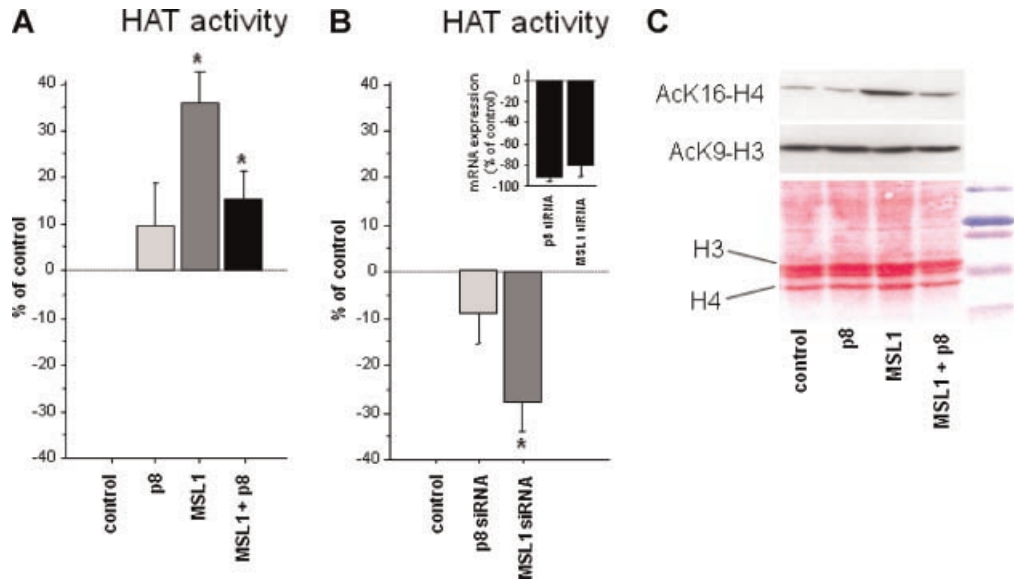

Fig. 3. HAT activity is regulated by p8 and MSL1: (A) HAT activity in cells after over-expressions of p8 and MSL1. HeLa cells were transduced with lentivirus expressing p8-EGFP, MSL1-EGFP or p8-EGFP and MSL1-EGFP. Nuclear extract were prepared to analyze HAT activity using the HAT activity colorimetric assay kit (BioVision) as described in the Materials and Methods Section. B: HAT activity in cells with p8 and MSL1 knockdown. HeLa cells were transfected with p8 and MSL1 siRNAs. Forty-eight hours later nuclear extracts were prepared and HAT activity measured as described above. Inset: HeLa cells were transfected with siRNAs for p8 or MSL1 and 48 h later p8 and MSL1 expressions were measured by qRT-PCR and expressed as percent of control, siRNA-transfected cells. HAT activity was expressed as percent of pCCL-EGFP control cells and expressed as the mean ± SD of combined results from two independent experiments performed in triplicate (*$P < 0.05$). C: HeLa cells were transfected with p8-Flag and MSL1-V5, alone or in combination. Twenty-four hours later, the supernatant fraction of acid-soluble proteins was recovered, submitted to 15% SDS–PAGE, blotted and revealed with specific polyclonal anti-H4K16ac and anti-H3K9ac antibodies following standard methods. Membranes were washed and developed with the ECL detection system. Bottom part: The membrane is stained with 0.5% Ponceau red in 1% TCA and shows molecular weight markers. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MSL1 interacts with 53BP1 in cells

The interaction between MSL1 and 53BP1 observed in yeast was confirmed by co-immunoprecipitation assays. 293T cells were transfected with 53BP1-HA and MSL1-V5, alone or in combination. MSL1-V5 and 53BP1-HA were immunoprecipitated from cell extracts with anti-V5 or anti-HA antibodies, respectively. Anti-V5 antibody was used to detect tagged MSL1 and anti-HA to detect tagged 53BP1. As expected, MSL1-V5 was detected in the complex containing 53BP1 while BP53-HA was detected in the complex containing MSL1-V5. Tags were not detected in negative controls (Fig. 5). We controlled the interaction of endogenous MSL1 and 53BP1 in HeLa cells by a co-immunoprecipitation approach using specific antibodies (Fig. 5). As expected, when 53BP1 was immunoprecipitated the MSL1 protein was found in the complex.

MSL1 DNA-repair activity is 53BP1-dependent

We speculated that the MSL1-dependent DNA-repair activity should be dependent on 53BP1 expression. To test this hypothesis we treated HeLa cells with a siRNA against 53BP1 mRNA while over-expressing p8 and MSL1, alone or in combination. We found that knocking down 53BP1 decreased DNA-repair activity, as evidenced by a decrease in the number of colonies observed after γ-irradiation. Interestingly, that phenomenon was not enhanced after forced expression of p8 and MSL1 alone or in combination (Fig. 6). These results suggest that MSL1 activity on DNA repair is dependent on 53BP1 expression.

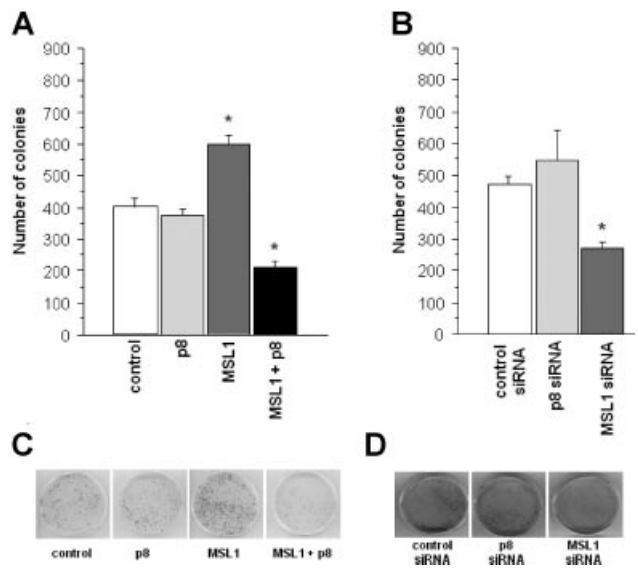

Fig. 4. Colony formation after γ-irradiation is regulated by p8 and MSL1: HeLa cells were transfected with pcDNA3-p8 and pcDNA4-MSL1-V5, alone or in combination (A,C). p8 and MSL1 siRNAs were transfected to HeLa cells (B,D). After 24 h (A,C) or 48 h (B,D), cells were γ-irradiated (7 Gy) and seeded on tissue culture dishes until cell colonies were formed. After 9 days, colonies were fixed, stained, and visible colonies counted. The experiment was repeated three times in duplicate. C,D: Representative images of the results. Values are expressed as the mean ± SD of combined results from three independent experiments performed in duplicate (*$P < 0.05$).

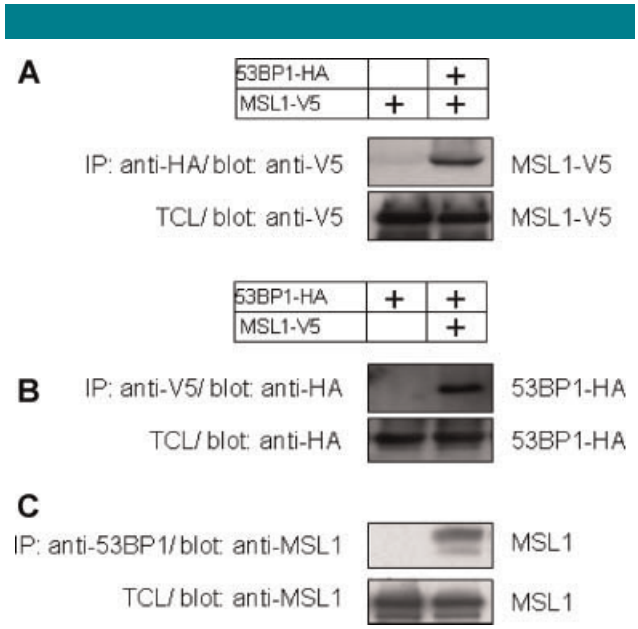

Fig. 5. Interaction of MSL1 with 53BP1: (A,B) 293T cells were co-transfected with 2 μg of pcDNA4-MSL1-V5 and 53BP1-HA as indicated. Immunoprecipitation was performed using anti-V5 or anti-HA-specific antibodies. Cell lysates prepared with a buffer containing 0.5% Nonidet P-40 were used for precipitations with Sepharose bead conjugates. The precipitated material was immunoblotted with anti-V5 or anti-HA antibodies. C: Endogenous MSL1 and 53BP1 interaction: Immunoprecipitation of endogenous proteins was performed using an anti-53BP1 rabbit antibody. Cell lysate prepared with a buffer containing 1% Triton X-100 was used for precipitation with Sepharose beads. The precipitated material was immunoblotted with an anti-MSL1 antibody. TCL, total cell lysate.

Then, we forced the expression of 53BP1 and, as expected, found an increased resistance to cell death induced by γ-irradiation. The number of colonies formed was twice that of control cells. A further increase was observed when MSL1 was concomitantly over-expressed but not when p8 was expressed alone. The effect of MSL1 was abolished if p8 was also expressed. Taken together, these results suggest that MSL1 activity on cell resistance to DNA damage is dependent on 53BP1 expression.

Regulation of HAT activity by MSL1 is independent of 53BP1

To check whether HAT MSL1-dependent activity was dependent on 53BP1 expression, we over-expressed MSL1, alone or with p8, in HeLa cells in which 53BP1 was either over-expressed by plasmid transfection or knocked down by siRNA treatment. No significant change in HAT activity was observed after over-expressing or knocking down 53BP1 (Fig. 7), indicating that this protein has no influence on MSL1-dependent HAT activity.

Discussion

The stress protein p8 is a small, highly basic, unfolded, and multifunctional protein. We have previously shown that most of its functions are exerted through interactions with other proteins, whose activities are thereby enhanced or repressed (Hoffmeister et al., 2002; Malicet et al., 2006a,c). In this work we describe another example of such mechanism, by which p8 binds and negatively regulates MSL1, a HAT-associated protein belonging to the MSL complex (Smith et al., 2005), which in turn binds to the DNA-damage-associated 53BP1 protein to facilitate DNA repair. We screened a cDNA library to detect new p8 partners and found that MSL1 binds to p8. This interaction was confirmed by co-immunoprecipitation and SPR analysis. *Drosophila* MSL complex is found exclusively on X chromosome in males, where it targets activated genes (Sass et al., 2003) and enhances the level of gene expression by acetylating entire transcriptional domains (Smith et al., 2001). In humans, this complex does not associate with a specific chromosome and appears to have a more dispersed and ubiquitous genomic distribution (Smith et al., 2005). It has been described that the acetylase present in the MSL complex is named MOF and is responsible for H4K16 acetylation in a wide range of higher eukaryotes (Smith et al., 2005). Several studies have shown that loss of MOF in mammalian cells has several consequences such as G2/M cell-cycle arrest, nuclear morphological defects, spontaneous chromosomal aberrations, reduced transcription of certain genes, and an impaired DNA-repair response upon ionizing irradiation (reviewed in Rea et al., 2007). Moreover, MOF is involved in ATM activation in response to DNA damage, and acetylation of p53 by MOF influences the cell's decision to undergo apoptosis instead of cell-cycle arrest (Sykes et al., 2006). Here, we show that p8 acts as a negative regulator of the MSL complex; interacting with MSL1 is able to abrogate the MSL1-dependent H4K16 acetylation.

A second yeast two-hybrid strategy against MSL1 as bait revealed us that MSL1 binds to 53BP1. We confirmed this interaction by co-immunoprecipitation experiments. Human 53BP1 is a polypeptide of 1,972 amino acids that contains two tandem BRCA1 (BRCT) motifs and a tudor domain. 53BP1 binds to the DNA-binding domain of p53 and rapidly forms discrete nuclear foci in response to γ-irradiation (Mochan et al., 2004). Double-strand breaks activate signaling responses at cell-cycle checkpoints, which monitor DNA damage and transduce signals to co-ordinate repair and cell-cycle progression. At the cellular level, damaged DNA that is not properly repaired can lead to genomic instability, apoptosis, or senescence, which can greatly affect the organism's development and aging process. Therefore, it is essential for cells to efficiently respond to DNA damage through co-ordinated and integrated DNA-damage checkpoints and repair pathways.

The role of chromatin acetylation by HAT complexes in transcriptional regulation is well established (Carrozza et al., 2003; Peterson and Cote, 2004). Recent studies have also implicated HATs in DNA-damage detection and DNA repair (Bird et al., 2002; Utley et al., 2005; Murr et al., 2006), but the precise underlying mechanism remains to be established. We hypothesized that MSL1-containing HAT complexes may participate in DNA repair through histone acetylation and reconfiguration of chromatin at break sites. To test this hypothesis we measured, by cell colony assay after γ-irradiation, the role of MSL1 on DNA repair after its forced expression, alone or in combination with p8. As expected, MSL1 over-expression induced a significant increase in cell resistance to DNA damage that was completely inhibited by p8 over-expression. On the contrary, knocking down p8 had no effect whereas knocking down MSL1 decreased cell survival after DNA damage. These results strongly suggest that MSL1 may be involved in DNA repair and that this effect is blocked by interaction with p8. Furthermore, we found that MSL1-dependent DNA-repair activity is dependent on 53BP1 expression as it is inhibited by siRNA-mediated 53BP1 knockdown and, on the contrary, over-expression of 53BP1 strongly increased that activity. In contrast, we found no significant changes in MSL1-dependent HAT activity after over-expression or knockdown of 53BP1, suggesting that 53BP1 is not involved in MSL1-dependent HAT activity.

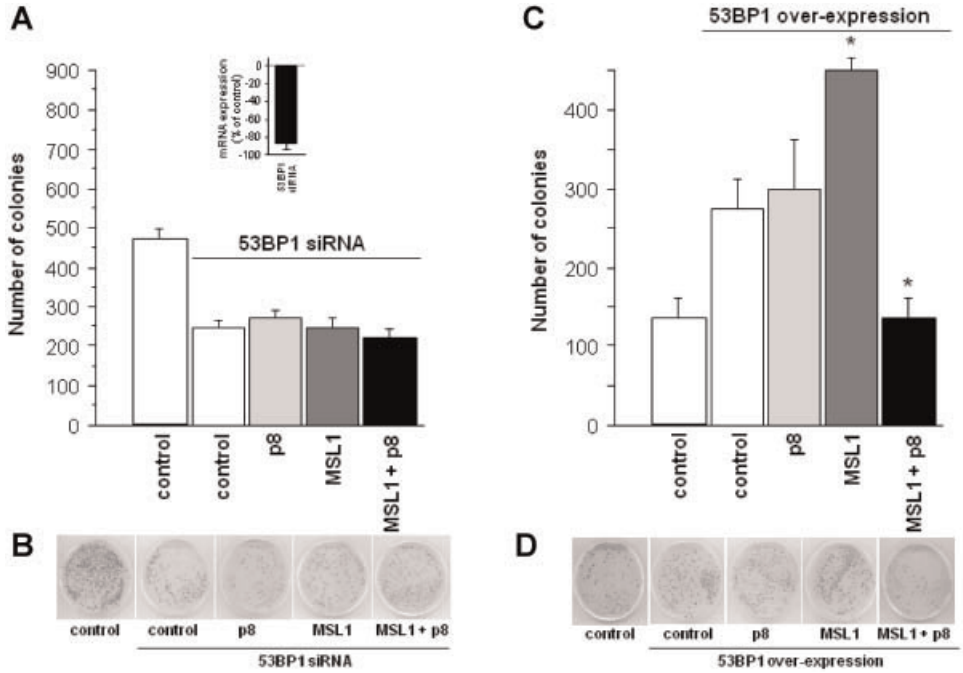

Fig. 6. Colony formation after γ-irradiation requires 53BP1 expression: (A) HeLa cells in which 53BP1 was knocked down with specific siRNA were transfected with pcDNA3-p8 and pcDNA4-MSL1-V5, alone or in combination. Inset: HeLa cells were transfected with siRNA for 53BP1; 48 h later 53BP1 mRNA expression was measured by qRT-PCR and expressed as percent of control siRNA-transfected cells. C: HeLa cells were transfected with pcDNA3-p8 or pcDNA4-MSL1-V5 alone or in combination in cells over-expressing 53BP1. Cells were γ-irradiated (7 Gy) and seeded on tissue culture dishes until cell colonies were formed. After 9 days, colonies were fixed, stained, and visible colonies were counted. The experiment was repeated three times in duplicate. B,D: Representative images of the results. Values are expressed as the mean ± SD of combined results from three independent experiments performed in duplicate (*$P < 0.05$).

It has been shown that cellular exposure to irradiation enhances MOF-dependent acetylation of H4K16 independently of ATM function but that MOF inactivation abrogates ATM activation after irradiation, indicating that MOF acts upstream of ATM (Gupta et al., 2005). It has been described that H4K16 acetylation destabilizes nucleosomes leading to chromatin decondensation (Shogren-Knaak et al., 2006). However, ATM activation needs H4K16 to be specifically acetylated by MOF (Gupta et al., 2005), suggesting that not only changes in the chromatin structure provoked by irradiation-induced H4K16 acetylation are sufficient to activate ATM-DNA-repair pathway; some scaffold proteins would be necessary to link double-strand breaks to ATM activation. Here we show a further link between the MSL complex and the ATM-DNA-repair pathway as MSL1 interacts physically and functionally with 53BP1, suggesting a strong relationship between both phenomena in DNA-damage response. From our findings we suggest that, when irradiation-induced double-strand breaks happen to DNA, 53BP1 brings MSL1 to the vicinity of damaged DNA through its proven interaction. MSL1-containing MSL complexes increase H4K16 acetylation due to increase in HAT activity and this histone modification leads to changes in chromatin structure inducing chromatin remodeling and relaxation, allowing the DNA-repairing machinery to be loaded to damaged DNA sites. Depending on the cell context, p8 would act as a negative regulator of this process by interacting with MSL1 and preventing the formation of the MSL complex. This fact would result in H4K16 acetylation decrease and in the consequent DNA-repair decrease as we could see with the survival assay after irradiation. In this sense, high doses of irradiation provoke a sustained p8 mRNA down-regulation preceded by a rapid short increase in p8 expression. This fast

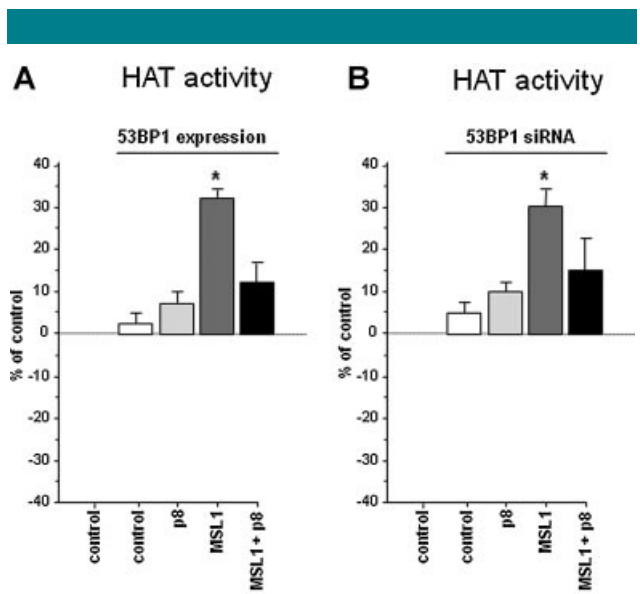

Fig. 7. HAT activity is not influenced by 53BP1 expression: HeLa cells were transduced with lentivirus expressing p8-EGFP, MSL1-EGFP or p8-EGFP and MSL1-EGFP. In these cells, 53BP1 was over-expressed by transfection of a 53BP1-HA DNA construct (A) or knocked down with a specific siRNA (B). Nuclear extracts were prepared to analyze HAT activity as described in the legend of Figure 3. HAT activity was expressed as percent of value in control cells and expressed as the mean ± SD of combined results from two independent experiments performed in triplicate (*$P < 0.05$).

increase suggests that p8 might rapidly prevent MSL1-mediated DNA repair of too much damaged cells, allowing them to undergo apoptosis. The subsequent inhibition of p8 expression would allow surviving cells to be DNA repaired by the MSL1 pathway. Although these results strongly suggest that MSL1-associated HAT activity accounts for the DNA-repair activity of MSL1, conclusive demonstration remains out of reach because, to our knowledge, specific inhibitors of this activity are not available. However, Sun et al. (2006) established that global inhibition of HAT activity with the non-specific inhibitor anacardi acid increased cell sensitivity to ionizing radiation, indicating that HAT activity is very important for DNA repair.

In summary, our results show that MSL1 plays an important role in mediating irradiation-induced DNA repair through formation of HAT complexes and interaction with 53BP1, and p8 would act as a negative regulator of this process by interacting with MSL1 and preventing its role on HAT activity.

Acknowledgments

This work was supported by grants from INSERM, Ligue Contre le Cancer, and Canceropole PACA to J.L.I. and Ecos-Sud to J.L.I. and S.M. M.G. was supported by INSERM, C.C. was from Ligue Contre le Cancer. Ciberehd is funded by the Instituto de Salud Carlos III. We thanks to P. Spoto, P. Berthezene, and J. Tardivel-Lacombe for their technical help.

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