SUMO-dependent regulation of centrin-2

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Summary
Centrins are multifunctional Ca2+-binding proteins that are highly conserved from yeast to humans. Centrin-2 is a core component of the centrosome of higher eukaryotes. In addition, it is present within the nucleus, in which it is part of the xeroderma pigmentosum group C (XPC) complex, which controls nucleotide excision repair (NER). Regulation of the subcellular distribution of centrin-2 has so far remained elusive. Here we show that centrin-2 is a substrate of SUMOylation in vitro and in vivo, and that it is preferentially modified by SUMO2/3. Moreover, we identify the SUMO E3-like ligase PC2 and CBX4 as essential for centrin-2 modification. Interference with the SUMOylation pathway leads to a striking defect in nuclear localization of centrin-2 and accumulation in the cytoplasm, whereas centrosomal recruitment of centrin-2 is unaffected. Depletion of the XPC protein mimics this situation and we provide evidence that SUMO conjugation of centrin-2 enhances its binding to the XPC protein. These data show that the nucleocytoplasmic shuttling of centrin-2 depends on the SUMO system and indicates that localization of centrin-2 within the nucleus depends on its ability to bind to the XPC protein.

Introduction
Post-translational modification of proteins by SUMO regulates central cellular processes including transcription, cell-cycle regulation and DNA-damage response (Gill, 2004; Hay, 2005; Muller et al., 2004; Watts, 2007). It seems that SUMOylation regulates these distinct processes via modulation of specific protein-protein interactions (Geiss-Friedlander and Melchior, 2007). In vertebrates, three SUMO isoforms are expressed. SUMO1 shares 43% identity with SUMO2 and SUMO3, whereas the latter two are closely related (sharing 97% identity) and are therefore collectively referred to as SUMO2/3. Paralog-specific modification has been reported for various substrates (Vertegaal et al., 2006). SUMO proteins are covalently attached to their targets via an isopeptide bond with the ε-amino group of a lysine residue. The conjugation process involves an enzymatic cascade comprising the E1-activating enzyme Aos1 (also known as Uba2 and Sae1), the E2-conjugating enzyme Ubc9, and additional E3 ligases including protein inhibitor of activated STAT (PIAS) family members, Ran-binding protein 2 (RanBP2) and human polycomb protein 2 (hPC2; also known as PC2 and CBX4) (Anckar and Sistonen, 2007; Johnson, 2004). De-SUMOylation is mediated by the action of SUMO-specific isopeptidases that belong to the family of ubiquitin-like proteases [UlpS; also known as sentrin-specific proteases (SENPs)] (Hay, 2007). Notably, in most cases only a small fraction of a given target protein is SUMOylated, making it difficult to identify novel SUMO substrates and elucidate the functional consequence of the modification (Geiss-Friedlander and Melchior, 2007).

Centrin proteins are highly conserved from yeast to humans and regulate various cellular functions (Bornens and Azimzadeh, 2007; Pereira and Schiebel, 2001; Salisbury, 2007). Humans express at least three centrin isoforms. Centrin-1 is expressed exclusively in male germ cells, certain neurons and ciliated cells. Centrin-2 and centrin-3 are ubiquitously expressed, and both localize to the centrioles and the pericentriolar material (Salisbury, 2007). Centrin-2 has been implicated in centriole duplication (Salisbury et al., 2002) but this view has recently been challenged (Kleylein-Sohn et al., 2007). Remarkably, ~90% of the centrin-2 protein is not associated with centrosomal structures (Paolotti et al., 1996) and instead much of it is present within the nucleus. In line with this localization, centrin-2 has been implicated in mRNA export from the nucleus (Resenades et al., 2008) and this function seems to be conserved (Fischer et al., 2004). Furthermore, within the nucleus, centrin-2 associates with the xeroderma pigmentosum group C (XPC) protein and the human homolog of Rad23 B (HR23B) to form the trimeric XPC complex (Araki et al., 2001). This complex functions as a key component of the nucleotide excision repair (NER) pathway. It detects and binds to DNA-damage sites in a reaction that requires all three subunits (Nishi et al., 2005; Sugasawa et al., 1996), thus representing a first line of defense against carcinogenesis (Batty and Wood, 2000; de Laat et al., 1999; Sugasawa and Hanaoka, 2007; Thoma and Vasquez, 2003). Intriguingly, the XPC protein becomes modified by ubiquitin and SUMO in response to DNA damage (Sugasawa et al., 2005; Wang et al., 2007; Wang et al., 2005), and this has been shown to depend on an intact proteasome system (Wang et al., 2005). Modification of the XPC protein was reported to alter the DNA-binding properties of the complex and to be essential for efficient NER (Sugasawa et al., 2005; Wang et al., 2007).

Here we identify centrin-1 and centrin-2 as novel substrates of SUMO. We demonstrate that SUMOylation regulates centrin-2 localization to the nucleus and we identify the SUMO E3-like ligase hPC2 and SUMO2/3 (but not SUMO1) to be involved in this process. Centrin-2 SUMOylation occurs independently of factors that regulate SUMO modification of its binding partner, the XPC protein. Yet, cells depleted of the XPC protein phenocopy the mislocalization of centrin-2 that is seen upon inhibition of the
SUMOylation system and we show that the SUMOylation status of centrin-2 influences its binding to the XPC protein. We conclude that the nucleocytoplasmic shuttling of centrin-2 as well as its binding to the XPC protein are regulated by the SUMO system.

Results
Centrin proteins are modified by SUMO in vitro and in vivo
Preliminary data obtained in our laboratory suggested that centrin-2 might be a target of SUMOylation (our unpublished observations). To explore this possibility, we first used yeast two-hybrid assays to monitor binding of the essential SUMO E2-conjugating enzyme Ubc9 to the three human centrin isoforms. Interestingly, all centrin proteins showed binding to Ubc9, whereas no binding between the control protein INCENP and Ubc9 was observed (Fig. 1A). Additionally, centrin-1 and centrin-2 preferentially bound to SUMO2 in this assay, whereas centrin-3 did not interact with the SUMO isoforms (Fig. 1B). Next, we analyzed centrin modification by SUMO in an in vitro SUMOylation assay. We detected conjugation of centrin-1 and centrin-2, but not centrin-3, to SUMO3 (Fig. 1C). In order to analyze SUMO conjugation in vivo we overexpressed the Myc-tagged centrin isoforms in combination with His-tagged SUMO2. SUMOylation of centrins was monitored by nickel nitrilotriacetate (Ni-NTA) pulldown of His-SUMO2 conjugates followed by anti-Myc immunoblotting. In agreement with our in vitro SUMOylation assay, only centrin-1 and centrin-2 but not centrin-3 were present in the His-SUMO2 pulldown fractions (Fig. 1D). These data identify centrin proteins, in particular centrin-1 and centrin-2, as novel targets of SUMO modification.

Centrin-2 localization to the nucleus (but not to the centrosome) depends on the SUMOylation system
Because centrin-1 is not expressed ubiquitously, we focused our further studies on the elucidation of the mechanism and function of centrin-2 SUMOylation. To analyze a potential effect of the SUMO system on centrin-2 localization to the centrosome, we first depleted the essential SUMO E2-conjugating enzyme Ubc9 by siRNA. Effective knockdown was verified by western blotting (Fig. 2A). We then used immunofluorescence microscopy to analyze centrin-2 localization in cells that had been pre-extracted with 0.3% Triton X-100 prior to methanol fixation. Under these conditions, centrin-2 localization to the centrosome was not detectably impaired by the depletion of Ubc9 (Fig. 2B). By striking contrast, we noticed that the nuclear pool of centrin-2 was less abundant in Ubc9-depleted cells than in GL2-treated controls (Fig. 2B). Because the total levels of centrin-2 protein were unaffected by Ubc9 depletion (see Fig. 2A), it appeared likely that, upon Ubc9 knockdown, nuclear centrin-2 was directed to the cytoplasm and was then extracted during sample preparation. To substantiate this conclusion, we analyzed nuclear centrin-2 in methanol-fixed cells that had not undergone

![Fig. 1.](image-url)
any pre-extraction step. Under these conditions, centrin-2 could clearly be visualized in the cytoplasm of Ubc9-depleted cells, whereas it was mostly absent from the nucleus (Fig. 2C). Quantitative analysis revealed that 84.4% of total centrin-2 could be detected in the nucleus in control depleted cells compared with only 25.8% in Ubc9-depleted cells (Fig. 2D). Of note, the centrin-2 interaction partner in the nucleus, the XPC protein, has been shown to be modified by SUMO (Wang et al., 2007; Wang et al., 2005). However, in contrast to centrin-2, Ubc9 depletion did not alter the localization of the XPC protein to the nucleus (89.4% nuclear localization in control depleted cells versus 89.2% in Ubc9 knockdown) (see Fig. 2D,E). Additionally, depletion of Ubc9 did not detectably affect the nucleocytoplasmic distribution of centrin-3 (Fig. 2F).

To analyze endogenous SUMOylation of centrin-2 and address the possibility of a paralog-specific modification in vivo, we performed immunoprecipitation experiments with either anti-SUMO1 or anti-SUMO2/3 antibodies. We detected a single anti-centrin-2-reactive band indicative of mono-SUMOylation in SUMO2/3 but not in SUMO1 immunoprecipitates (Fig. 3A). Conversely, modified RanGAP1 was found in SUMO1 immunoprecipitates, whereas XPC did not show SUMO modification under these conditions (Fig. 3A). We attempted to exploit this difference to investigate a paralog-specific role for SUMOylation in the in vivo regulation of centrin-2 localization. To this end, we depleted either SUMO1 or SUMO2/3 by specific siRNA duplexes. Western blotting showed effective knockdown of SUMO1 or SUMO2/3 conjugates (Fig. 3B). Depletion of SUMO2/3 resulted in a strongly reduced nuclear localization of centrin-2 and a concomitant accumulation of centrin-2 in the cytoplasm (Fig. 3C, bottom row), in line with the effect of Ubc9 knockdown (Fig. 2C). By contrast, no effect on centrin-2 localization was observed in...
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response to depletion of SUMO1 (Fig. 3C, middle row). This indicates that centrin-2 localization to the nucleus requires an intact SUMO system and, in particular, SUMO2/3.

The SUMO E3-like ligase hPC2 is essential for the SUMOylation of centrin-2

To identify factors that regulate centrin-2 modification by SUMO, we analyzed binding of centrin-2 to candidate SUMO E3 ligases in the yeast two-hybrid assay. Among the tested candidates, only hPC2 showed interaction with centrin-2 (Fig. 4A). Interestingly, hPC2 is associated with Polycomb bodies within the nucleus but only few SUMO substrates have previously been identified to undergo hPC2-mediated modification (Wotton and Merrill, 2007). To address a putative SUMO E3-ligase function of hPC2 towards centrin-2, in vitro translated Myc-tagged hPC2 and the Myc-tagged catalytic fragment of the control E3 ligase RanBP2 (RanBP2<sup>ΔFG</sup>) were added to an in vitro SUMOylation reaction on centrin-2. The assay was performed under limiting E1 and E2 concentrations, because high concentrations can bypass the need for E3 ligases in this set-up. Under these conditions, basal E1-E2-mediated centrin-2 SUMOylation was weak but significantly enhanced in the reaction containing hPC2 (Fig. 4B, compare lanes 2 and 3). The addition of RanBP2<sup>ΔFG</sup> did not change the level of centrin-2 SUMOylation (Fig. 4B, compare lanes 2 and 4). In order to address the requirement of hPC2 for the SUMOylation of centrin-2 in vivo, cells expressing Myc-tagged centrin-2 and His-tagged SUMO2 were depleted of hPC2 or control proteins. Knockdown of transfected Myc-tagged hPC2 was reproducibly observed with the siRNA duplexes used (Fig. 4C). SUMOylation of centrin-2 was monitored by Ni-NTA pulldown of His-SUMO2 conjugates followed by immunoblotting with anti-Myc antibodies (Fig. 4D). Importantly, depletion of hPC2 resulted in a loss of centrin-2 SUMOylation similar to the result obtained after knockdown of Ubc9. By contrast and attesting to the specificity of this assay, centrin-2 SUMOylation was unaltered when cells were depleted of the α- and β-forms of the control E3 ligase PIAS2 (Fig. 4D). Immunofluorescence analysis of hPC2-depleted cells showed a defect in nuclear targeting of centrin-2 (Fig. 4E), which exactly mirrors the phenotype seen in cells depleted of Ubc9 and SUMO2/3 (see Fig. 2C; Fig. 3C). Taken together, these data suggest that hPC2 acts as a SUMO E3 ligase towards centrin-2.

SUMOylation of centrin-2 occurs independently of factors that regulate modification of the XPC protein

The binding partner of centrin-2 within the nucleus, the XPC protein, has been reported to be modified by ubiquitylation and SUMOylation. These modifications were shown to occur in response to ultraviolet (UV) irradiation (Sugasawa et al., 2005; Wang et al., 2005) and to require an intact proteasome system, because treatment with the proteasome inhibitor MG132 abolishes them (Wang et al., 2005). We therefore investigated whether UV irradiation and/or treatment with MG132 influence centrin-2 SUMOylation. Cells expressing Myc-tagged centrin-2 and His-tagged SUMO2 were either UV irradiated with 50 J/m<sup>2</sup> and incubated for an additional 30 minutes or treated with MG132 at 20 μM for 1 hour before lysate preparation. In agreement with published data (Sugasawa et al., 2005; Wang et al., 2005), we observed the characteristic upshift of the XPC protein after UV irradiation. Ni-NTA pulldown of His-SUMO2 conjugates revealed that neither UV irradiation nor MG132 treatment detectably influence the SUMOylation status of centrin-2 (Fig. 5A). In addition, immunofluorescence analysis showed no significant effect on overall nuclear localization of either centrin-2 or XPC protein under conditions of proteasome inhibition (Fig. 5B) or UV irradiation (Fig. 5C).

SUMO modification of centrin-2 regulates its binding to the XPC protein

The observation that both the XPC protein and centrin-2 are targets of SUMOylation prompted us to analyze their mutual
interdependence with regards to this modification. Of note, siRNA-mediated depletion of either one of these proteins did not effect the abundance of the other (Fig. 6A), confirming and extending previous data (Nishi et al., 2005). We first analyzed the influence of centrin-2 knockdown on XPC-protein modification and localization. Consistent with the data shown above, UV irradiation resulted in modification of the XPC protein. In agreement with previous reports indicating that Ubc9 and modification of XPC at the SUMO consensus site K655 was responsible for the characteristic XPC upshift (Wang et al., 2007; Wang et al., 2005), the higher-molecular-weight bands reactive with anti-XPC antibodies were largely lost upon knockdown of Ubc9 (Fig. 6B). By contrast, centrin-2 depletion did not detectably interfere with XPC-protein modification generated by UV irradiation (Fig. 6B).

Moreover, the overall nuclear localization of XPC protein was independent of centrin-2 (Fig. 6C). Next, we analyzed centrin-2 modification and localization under conditions of XPC-protein depletion, again using Ubc9 depletion for control. XPC-protein knockdown did not significantly influence either the SUMOylation of centrin-2 in vivo. Myc-tagged centrin-2 and either HA- or His-tagged SUMO2 were coexpressed in HeLa cells treated with siRNA oligonucleotides directed against the indicated proteins. The siRNA directed against PIAS2 targets the α- and β-isoforms. His-SUMO2 conjugates were recovered on Ni-NTA beads and western blotting was performed with the indicated antibodies. The asterisk denotes a PIAS2 cross-reactive band. (E) HeLa cells were treated with siRNA duplexes specific for GL2 or hPC2 and incubated for 72 hours. Immunofluorescence was performed with anti-centrin-2 antibodies. DNA was stained with DAPI. Scale bar: 10 μm.
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fails to localize to the nucleus because of an import defect or, alternatively, because of an inability to bind to nuclear XPC protein. To determine whether SUMOylation of centrin-2 might directly regulate its binding to the XPC protein (and in consequence its nuclear localization), we set up an in vitro binding assay. As shown in Fig. 7A, we combined in vitro transcription and translation with in vitro SUMOylation to prepare Myc-tagged centrin-2 in both a modified and an unmodified form. For control, borealin, another SUMOylation substrate (Klein et al., 2009), was similarly prepared. Immunoprecipitations were carried out with anti-Myc or control antibodies, and reactions were subsequently incubated with in-vitro-translated untagged XPC protein. Samples were analyzed by autoradiography (to detect 35S-labeled centrin-2 and borealin) and probed by western blotting with anti-XPC antibodies for co-precipitation of the XPC protein (Fig. 7B). Anti-SUMO2/3 western blotting was used to confirm SUMO2/3 conjugates. No binding of XPC protein could be seen in immunoprecipitates prepared with control antibodies (Fig. 7B, lane 2) or in anti-Myc immunoprecipitates containing SUMOylated borealin (Fig. 7B, lane 5). Only a modest fraction of XPC protein was found in the immunoprecipitation of unmodified centrin-2, but a striking increase in binding of the XPC protein was observed in immunoprecipitations of SUMOylated centrin-2 (Fig. 7B, compare lanes 3 and 4). To strengthen this finding, we performed centrin-2 immunoprecipitations from control depleted cells and cells that had been depleted of Ubc9. In agreement with the in vitro assay described above, the amount of XPC protein co-precipitating with centrin-2 was substantially higher in control depleted cells compared with cells in which SUMOylation was blocked by Ubc9 knockdown (Fig. 7C, compare lanes 5 and 6). These results suggest that the SUMOylation of centrin-2 enhances its binding to the XPC protein, which in turn is required for efficient accumulation of centrin-2 within the nucleus.

Discussion
We report the identification of centrin-1 and centrin-2 as novel substrates of post-translational modification by SUMO. Within the human centrin protein family, centrin-1 and centrin-2 are most closely related (83.7%), making it likely that a common structural feature mediates SUMO conjugation to these two proteins. By contrast, we did not observe SUMOylation of centrin-3, which is only 51.7% identical to centrin-1 and 52.3% identical to centrin-2. Of note, centrin-3 is the closest homolog of Cdc31p (51.4% identity), the single centrin protein expressed in the yeast Saccharomyces cerevisiae. Thus, it should not necessarily be expected that centrin SUMOylation represents a conserved mechanism that also regulates Cdc31p in yeast.

In this study, we have focused on the mechanism and function of SUMO conjugation to centrin-2. Although we have not observed any obvious impact of SUMOylation on the centrosome association of centrin-2, we show that the SUMO system is required for efficient centrin-2 localization to the nucleus, in which this protein is implicated in the NER pathway (Nishi et al., 2005). In particular, we demonstrate that the SUMO E3-like ligase hPC2 is essential for centrin-2 modification and, accordingly, its nuclear localization. hPC2 localizes to Polycomb bodies within the nucleus but its role as a SUMO E3 ligase was recognized only recently (Kagey et al., 2003). A subsequent study has shown that the E3-ligase activity of
hPC2 is not linked to its localization to Polycomb bodies (Kagey et al., 2005). Hence, hPC2-mediated SUMOylation of centrin-2 could well take place outside of Polycomb bodies. It has been speculated that hPC2 might be recruited to DNA via interaction with a DNA-binding protein in order to SUMOylate regulators of transcription (Kagey et al., 2005). In this context, it is intriguing that centrin-2 associates with DNA as part of the XPC complex (Araki et al., 2001), and we show in this study that hPC2 interacts with centrin-2. Thus, it will be interesting to explore whether centrin-2 in turn influences the localization and/or function of hPC2.

Our data suggest a paralog-specific modification of centrin-2. In fact, SUMO2/3 (but not SUMO1) showed interaction with centrin-2, and depletion of SUMO2/3 (but not SUMO1) influenced centrin-2 localization to the nucleus. Analysis of the conserved lysine residues present in the related SUMO substrates centrin-1 and centrin-2 reveals an extensive overlap (19 out of the total 25 lysine residues present in the centrin-2 sequence, with only seven of these not present in the non-SUMO substrate centrin-3), making it difficult to predict the specific lysine residue that might serve as the SUMO acceptor site (supplementary material Fig. S1A). Interestingly, mutation of the five SUMO consensus sites present within centrin-2 did not inhibit SUMOylation substantially (supplementary material Fig. S1B,C). In view of a recent proteomic screen showing that more than half of all SUMO2/3 substrates...
Regulation of centrin-2 identified lacked the established Kx[E/D] consensus motif (Blomster et al., 2009), this is perhaps not surprising. Thus, given the multitude of lysines in centrin-2, it might be difficult to interfere with SUMOylation through specific mutagenesis of this protein. Nevertheless, the observation that depletion of Ubc9, hPC2 or SUMO2/3 (all demonstrated to interact with centrin-2) all abolish centrin-2 localization to the nucleus strongly suggests that SUMOylation of centrin-2 (rather than SUMOylation of another substrate) is directly responsible for its nuclear localization. Our data indicate that a non-SUMOylatable centrin-2 mutant will exhibit defects in XPC-protein binding and targeting to the nucleus.

XPC protein, the nuclear centrin-2 interaction partner, is conjugated to ubiquitin and SUMO in response to UV irradiation (Sugasawa et al., 2005; Wang et al., 2007; Wang et al., 2005), and this has been shown to be regulated by the proteasome system (Wang et al., 2005). In yeast, Rad33p was identified recently as a binding partner of Rad4 (the yeast homolog of the XPC protein) and was shown to regulate modification of Rad4 protein under conditions of DNA damage, leading to the speculation that yeast Rad33p might function similarly to mammalian centrin (den Dulk et al., 2008). Here, we demonstrate that SUMOylation of centrin-2 occurs independently of UV irradiation and the proteasome system. Moreover, in contrast to data from yeast (den Dulk et al., 2008), centrin-2 depletion did not influence XPC-protein modification in HeLa cells. We thus conclude that the post-translational modifications of the XPC protein and centrin-2 depend on different factors.

It is striking that SUMO modification of two different subunits of the XPC complex contributes to its regulation. Although SUMOylation of the XPC protein was shown to be required for the recruitment of XPG to sites of DNA damage and regulate the NER efficiency of the complex (Wang et al., 2007), we show here that SUMO modification of centrin-2 is required for efficient interaction with the XPC protein, which in turn seems to result in the accumulation of centrin-2 within the nucleus. However, we also observed binding (albeit to a much lesser extent) of non-SUMOylated centrin-2 to XPC protein, in agreement with previous in vitro binding studies (Araki et al., 2001) and our notion that nuclear localization of centrin-2 was not completely abolished upon interference with the SUMOylation pathway. Structural studies using small peptides showed residues 847-863 of the XPC protein to associate with centrin-2 in vitro (Popescu et al., 2003; Thompson et al., 2006). However, these experiments did not address the post-translational modifications on either XPC protein or centrin-2. Thus, in future structural studies it will be interesting to evaluate binding of the full-length XPC protein and centrin-2 under conditions in...
which the two proteins have undergone post-translational modifications.

Cdc31p/centrin-2 are core components of the yeast spindle pole body or the centrosome of higher eukaryotes, respectively. Although little is presently known about a possible role of SUMO at the centrosome, recent studies have identified several proteins that undergo SUMOylation and are known to localize to both centrosomal structures and the nuclear compartment (Cheng et al., 2006; Haindl et al., 2008; Yun et al., 2008). Our present data suggest that the SUMOylation machinery does not influence centrin-2 localization to the centrosome but is required for the efficient accumulation of the protein in the nucleus, where it functions in NER (Nishi et al., 2005) and mRNA export (Fischer et al., 2004; Resenades et al., 2008). A priori, the nuclear accumulation of centrin-2 could be regulated at the level of transport or retention (or both). The small size of centrin-2 (~16 kDa) would allow for passive diffusion across the nuclear membrane and, consistent with this, the inhibition of Crm-1-mediated nuclear export by leptomycin-B treatment did not change the localization of centrin-2 to the centrosome (data not shown). Our data identifies SUMOylation-dependent binding to the XPC protein as a crucial step for centrin-2 nuclear recruitment. Thus, the equilibrium distribution of centrin-2 between the cytoplasm and the nucleus is most probably determined by its binding to interaction partners in the two compartments. This interpretation is supported by the finding that overexpression of the XPC protein leads to enhanced nuclear accumulation of centrin-2 (Charbonnier et al., 2007).

Numerous centrosomal proteins are known to reside also within the nucleus but their nuclear functions, if any, are unknown and the regulation of their subcellular localization have not been addressed. Extrapolating from the data reported here, we consider it attractive to speculate that SUMOylation of centrosomal proteins might represent a general mechanism to influence their nucleocytoplasmic partitioning. Thus, the nucleus might conceivably serve as a reservoir for proteins involved in centrosome function and the regulation of their nucleocytoplasmic distribution could represent an additional level of control. In support of this hypothesis, shuttling of centrosomal proteins has recently been implicated in the process of centrosome duplication (Prosser et al., 2009).

Materials and Methods

Directed yeast two-hybrid assays
cDNAs encoding the respective prey or bait proteins were cloned in frame with the GAL-activation domain of pACT2 or pGAD vectors or the GAL-binding domain of pFBT9 or pGBD vectors and tested for self-activation. Directed yeast two-hybrid assays were performed as described (Klein et al., 2006) with the following primary antibodies: anti-γ-tubulin (Sigma), anti-centrin-2 (Kleylein-Sohn et al., 2007), anti-XPC (Santa Cruz Biotechnology) and anti-pH2AX (Millipore). Anti-centrin-3 antibody was a kind gift of Michel Bornens, Institut Curie, Paris, France. Secondary antibodies were Cy2/Cy3-conjugated donkey antibodies (Dianova). For quantification, the cell body was visualized by α-tubulin (Sigma) staining and DAPI was used to identify the nuclear compartment. Centrin-2 and XPC nuclear fluorescence was defined as fluorescence overlap with DAPI staining. Cytoplasmic fluorescence was calculated as the difference between total cell fluorescence and nuclear fluorescence. All analyses were performed using ImageJ software. The following antibodies were used for western blotting: anti-SUMO1 (clone 21C7, Zymed), anti-SUMO2/3 (clone 1E7, MBL), anti-PIASII (clone 116, Sigma-Aldrich), anti-Ubc9 (clone 50, BD Biosciences), anti-MyC (9E10), anti-centrin-2 (Kleylein-Sohn et al., 2007), anti-XPA (Santa Cruz Biotechnology), anti-XPC (GeneTex) and anti-α-tubulin (DAMA1, Sigma-Aldrich). Anti-RanGAP1 was a kind gift of Franke Melchior, University of Göttingen, Germany. Secondary antibodies conjugated to horseradish peroxidase were obtained from Jackson ImmunoResearch Laboratories. To avoid IgG chain signals in the anti-SUMO2/3 western blot shown in Fig. 7B, secondary anti-mouse TrueBlot (e Bioscience) was used.

Cell culture, transfection and siRNA treatment

HeLaS3 or COS-7 cells were grown under standard conditions. Plasmid transfections were performed using FuGENE 6 reagent (Roche Diagnostics). mRNAs were targeted as follows: Ube9 (Klein et al., 2009); SUMO1 (sc-29498A, Santa Cruz Biotechnology); SUMO2 (targeting SUMO2 and SUMO3), 5'-GUCAUGUGAGGCA-UCACTGT-3'; hpc2#1, 5'-CGUGGCAAUCGGGAAATGTT-3'; hpc2#2, 5'-GUUUGUCAUCGGGAAUUAUdTdT-3' (a mixture of hpc2#1 and hpc2#2 was used to achieve efficient hpc2 knockdown); centrin-2, 5'-GCACAAUGUAUCAAGUUAUATGTT-3'; XPC (Despras et al., 2007); XPA (Santa Cruz Biotechnology); and PIAS2 (targeting the α- and β-isofoms) (Yang and Sharrocks, 2005).

Treatement with MG132 and UV irradiation

UV irradiation was performed using a CL-1000 UV-crosslinker (UVP, Upland, CA). HeLa S3 cells were irradiated with 50 J/m², incubated for an additional 30 minutes and either subjected to the in vivo SUMOylation protocol (see above) or pelleted and directly dissolved in 2× sample buffer (Fig. 6B). HeLa S3 cells were treated with the proteasome inhibitor MG132 (Calbiochem) at a concentration of 20 μM for 1 hour and subjected to the in vivo SUMOylation protocol (Fig. 5A) or processed for immunofluorescence analysis (Fig. 5B).

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References


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