Dual Role for SUMO E2 Conjugase Ubc9 in Modulating the Transforming and Growth-promoting Properties of the HMGA1b Architectural Transcription Factor*

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Members of the HMGA1 (high mobility group A1) family of architectural transcription factors, HMGA1a and HMGA1b, play important roles in many normal cellular processes and in tumorigenesis. We performed a yeast two-hybrid screen for HMGA1-interacting proteins and identified the SUMO E2 conjugase Ubc9 as one such partner. The Ubc9-interacting domain of HMGA1 is bipartite, consisting of a proline-rich region near the N terminus and an acidic domain at the extreme C terminus, whereas the HMGA1-interacting domain of Ubc9 comprises a single region previously shown to associate with and SUMOylate other transcription factors. Consistent with these findings, endogenous HMGA1 proteins and Ubc9 could be co-immunoprecipitated from several human cell lines. Studies with HMGA1b proteins containing mutations of either or both Ubc9-interacting domains and with Ubc9-depleted cell lines indicated that the proline-rich domain of HMGA1b positively influences transformation and growth, whereas the acidic domain negatively influences these properties. None of the changes in HMGA1 protein functions mediated by Ubc9 appears to require SUMOylation. These findings are consistent with the idea that Ubc9 can act as both a positive and negative regulator of proliferation and transformation via its non-SUMO-dependent interaction with HMGA1 proteins.

The HMGA1 (high mobility group A1) proteins are a subgroup of non-histone, chromatin-associated, “architectural” proteins that participate in chromatin folding and modeling, DNA binding, and transcription (1, 2). As a result, these proteins exert profound effects over basic cellular functions such as proliferation, differentiation, apoptosis, and genotoxic stress responses (1, 3). As befitting their role in these processes, HMGA1 proteins are widely expressed, with particularly high levels occurring in rapidly proliferating, undifferentiated, and neoplastic cells (4–6).

HMGA1 proteins occur in two major isoforms, HMGA1a and HMGA1b, which arise from alternate splicing of a common parent gene transcript (1–3). The former protein contains a unique 11-amino acid insert between residues 34 and 35 that likely accounts for the differential post-translational modification of the two isoforms (7, 8). Other important characteristics of both proteins include their relatively small sizes (~11–12 kDa), highly acidic C-terminal domains, and three reiterated “AT hooks” of 10–12 residues each. The latter mediate cooperative DNA binding to AT-rich regions in the minor groove of DNA, particularly if these exist within a structurally distorted context (2, 9, 10). In many cases, these sites lie within the promoters of genes whose transcript levels are modulated by HMGA1 proteins (11). HMGA1 proteins can thus regulate target gene expression by virtue of their modification of chromatin and DNA structure, which in turn alters the accessibility or function of other transcription factors (11). More recently, an alternate way for HMGA1 proteins to regulate transcription has been shown by demonstrating that they interact with the oligomerization domains of the tumor suppressor p53 and its relatives, p63 and p73, resulting in the inhibition of their DNA binding and transcriptional regulatory activities (12, 13).

The deregulated overexpression of HMGA1 proteins has also been shown to contribute directly to the development of certain experimental neoplasms (14–17). Conversely, siRNA2 or antisense-mediated depletion of HMGA1 proteins is associated with a less aggressive tumor cell phenotype (18, 19). Rearrangements or otherwise abnormal expression of the HMGA1 gene have also been implicated in a variety of naturally occurring human tumors (20–24). Further consistent with its putative role in experimental and naturally occurring cancers, the HMGA1 gene is a direct downstream transcriptional target for both the c-Myc and c-Jun oncoproteins (14, 18, 25). The importance of this relationship is underscored by the fact that inhibition of HMGA1 expression blocks both c-Myc and c-Jun transformation (14, 18, 25). Furthermore, we have previously shown that ectopic HMGA1b expression partially phenocopies c-Myc in c-Myc nullizygous fibroblasts, rendering them more highly proliferative and transformed (26).

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2 The abbreviations used are: siRNA, small interfering RNA; AD, activation domain; HA, hemagglutinin; FBS, fetal bovine serum; HOS, human osteosarcoma; mAb, monoclonal antibody; IP, immunoprecipitation.
Modulation of HMGA1b Function by Ubc9

Because of the central importance of HMGA1 proteins to transformation, particularly with respect to that mediated by c-Myc, we conducted a yeast two-hybrid-based screen for novel HMGA1b-interacting proteins. As a result of these studies, we have identified Ubc9 as an avid HMGA1 partner whose interaction with these proteins dramatically alters their function.

Ubc9 is the sole E2 conjugase for the small ubiquitin-related protein SUMO-1 and is itself frequently overexpressed in certain tumors (27, 28). Whereas ubiquitinylation invariably results in proteasome-mediated degradation of the targeted protein, SUMOylation more typically alters target protein activity or subcellular trafficking (29–35). Several instances have also been described of proteins that interact with Ubc9 without being SUMOylated (36–39). It is this latter group into which the HMGA1 proteins appear to fall. Despite its apparent lack of physical modification by Ubc9, we show that the function of HMGA1b is nevertheless dramatically altered as evidenced by changes in the growth-promoting and transforming activity of HMGA1b when its interaction with Ubc9 is perturbed or when endogenous cellular Ubc9 stores are depleted. Together, these results provide evidence for a novel interaction involving HMGA1b and Ubc9 that has profound consequences for the HMGA1b-transformed cell.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assays—General procedures for yeast two-hybrid screening and validation and quantitative β-galactosidase assays have been previously described (40, 41). To screen specifically for HMGA1b-interacting proteins, the entire murine HMGA1b coding region was amplified by PCR with the forward and reverse primers engineered to contain EcoRI and Sall restriction sites, respectively. The digested product was cloned directionally into the pGBK7 “bait” vector (BD-Clontech, San Diego, CA) so as to be in-frame with a Gal4 DNA-binding domain and a c-Myc epitope tag encoded by the vector. The sequences and reading frames of all mutants were confirmed by DNA sequencing.

Together, these results provide evidence for a novel interaction involving HMGA1b and Ubc9 that has profound consequences for the HMGA1b-transformed cell.

For the directional cloning of wild-type or mutant HMGA1b expression vectors into the previously described pLXSN-EYFP bicis- tronic retroviral vector containing a 3′-c-Myc epitope tag (26), Phoenix amphotropic packaging cells were transfected with retroviral constructs using Superfect reagent (Qiagen) according to the directions of the supplier. Supernatants were harvested 48–72 h later for infection of recipient Rat1a cells. After an additional 48–72 h, EYFP-positive cells were purified by cell sorting as previously described (26), pooled, and utilized in all further studies.

The creation of HMGA1b-Ubc9 fusion protein expression vectors was performed in two steps. In the first step, the coding region, including the termination codon of murine Ubc9 was amplified by PCR using XhoI- and Sall-linked forward and reverse primers, respectively. The fragment was cloned into the XhoI site in the polylinker of the pcDNA3.1+ vector (Stratagene), and expression of Ubc9 was verified by immunoblotting in a transient transfection assay. In the second step, the Ubc9 expression vector was digested with HindIII and XhoI to allow for the directional cloning of wild-type or mutant HMGA1b fragments, each of which had been amplified with forward and reverse PCR primers that contained engineered HindIII and XhoI sites, respectively. The reverse primers also lacked termination codons so as to allow for continuity of the HMGA1b and Ubc9 reading frames. The resultant vectors were sequenced to confirm the in-frame fusions between HMGA1b and Ubc9 coding sequences and to verify the identities of the HMGA1b moieties.

Inhibition of endogenous HMGA1 in HOS cells was performed using an siRNA duplex sequence (SiGenome duplex number D-004597-01-005, Dharmacon, Inc. Lafayette, CO) at a final concentration of 100 nM. Transfections were performed using DharmaFECT1 transfection reagent according to the directions of the supplier. Lysates for co-immunoprecipitation experiments and SDS-PAGE were performed 48 h after transfection.

Co-immunoprecipitation Experiments—These were performed essentially as described previously (41, 44). Briefly, 107 cells were washed twice in phosphate-buffered saline and resuspended in 1 ml of ice cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 μM ZnCl2, 10 mM EDTA, 1% SDS, 0.1% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 10 μg/ml aprotinin).
mm sodium vanadate, 0.1% Nonidet P-40, 50 µg/ml phenylmethylsulfonyl fluoride, and 100 µg/ml each of aprotinin, leupeptin, and pepstatin). After a 30-min incubation on ice, the suspension was subjected to two 30-s bursts with the microtip of a Branson sonifier (setting 7). Following clarification at 10,000 × g for 10 min, the supernatant was pre-cleared with 5 µl of nonimmune rabbit IgG followed by 30 µl of a slurry of protein G-agarose (Sc-2002; Santa Cruz Biotechnology, Santa Cruz, CA). The cleared supernatant was then incubated overnight at 4°C with 10 µl (2 g) of rabbit polyclonal IgG against HMGA1a/b (Sc-8982; Santa Cruz) or an equivalent amount of control IgG. Following precipitation with 30 µl of protein G-agarose for 3 h, the beads were washed five times with lysis buffer. The final pellet was subjected to SDS-PAGE and immunoblotting as described below.

Immunoblotting—General procedures for SDS-PAGE and immunoblotting have been previously described (42). Antibodies included monoclonal antibody (mAb) 9E10 directed against the c-Myc epitope tag (Sc-40, Santa Cruz), the anti-HA tag mAb (Sc-7392, Santa Cruz), and a mAb directed against human Ubc9 (number 610748; BD Biosciences, San Jose, CA).

RESULTS

Identification of Ubc9 as an HMGA1b-interacting Protein—A full-length human HMGA1b coding sequence, fused in-frame to the yeast Gal4 DNA-binding domain and a c-Myc epitope tag, was expressed in the AH109 yeast strain (Fig. 1A). This fusion protein alone was not self-transactivating, as evidenced by its inability to confer histidine and adenine prototrophy or to induce the expression of β-galactosidase at levels above background (Fig. 1B). This yeast strain was then used to screen a day-11 murine embryo cDNA library in which in-frame cDNAs are expressed as fusions with the Gal4-binding domain and a c-Myc epitope tag in AH109 yeast. This strain also expressed a fusion between full-length Ubc9, the Gal4 AD, and an HA tag. The ability of each strain to be propagated on medium lacking histidine and adenine was then tested, and quantitative β-galactosidase assays were performed in triplicate with <5% variation in all cases (not shown). As negative controls, the same HMGA1b deletions were expressed in yeast carrying the empty pGADT7 vector. Under these conditions, none of the HMGA1b deletion mutants demonstrated detectable self-transactivation potential. C, immunoblotting of yeast lysates. 50 µg of total cell lysate from yeast expressing the indicated HMGA1b proteins were resolved by SDS-PAGE and immunoblotted using the 9E10 monoclonal antibody directed against the c-Myc epitope in the pGBK7 vector. Note that most proteins were expressed at similar levels and were of the sizes predicted.

FIGURE 1. The Ubc9-interacting domain of HMGA1b is bi-partite. A, the upper portion of the figure depicts the entire amino acid sequence of HMGA1b. Important landmarks include the three 10–12-amino acid AT hooks (underlined), the proline-rich segment between AT hooks 1 and 2 (PPKEPSEVPTP), and the C-terminal acidic segment (italicized). Note that HMGA1a differs from HMGA1b by virtue of an 11-amino acid insert between residues 34 and 35 of the sequence shown. B, each of the deletions shown in A was expressed as a fusion with the Gal4-binding domain and a c-Myc epitope tag in AH109 yeast. The ability of each strain to be propagated on medium lacking histidine and adenine was then tested, and quantitative β-galactosidase assays were performed in triplicate with <5% variation in all cases (not shown). As negative controls, the same HMGA1b deletions were expressed in yeast carrying the empty pGADT7 vector. Under these conditions, none of the HMGA1b deletion mutants demonstrated detectable self-transactivation potential. C, immunoblotting of yeast lysates. 50 µg of total cell lysate from yeast expressing the indicated HMGA1b proteins were resolved by SDS-PAGE and immunoblotted using the 9E10 monoclonal antibody directed against the c-Myc epitope in the pGBK7 vector. Note that most proteins were expressed at similar levels and were of the sizes predicted.
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A. Ubc9 FL 1-110 62-110 110-158 134-158

B. Growth on His– Ade– medium Relative β-galactosidase activity

| HMGA1b | pGBKT7 | HMGA1b | pGBKT7 |
|--------|--------|--------|--------|
| Ubc9 FL | + | - | 1 | ≤0.05 |
| 1-110 | - | - | 0.04 | ≤0.05 |
| 62-110 | - | - | 0.037 | ≤0.05 |
| 110-158 | + | - | 0.83 | ≤0.05 |
| 134-158 | - | - | 0.16 | ≤0.05 |
| pGADT7 | - | - | 0.02 | ≤0.05 |

C. [Image]

FIGURE 2. Deletional analysis identifies the HMGA1b-interacting domain of Ubc9. A, the indicated deletions of murine Ubc9 were expressed as HA-tagged AD-fusion proteins in the pGADT7 vector. Each of these was introduced into the AH1109 yeast strain that contained either a full-length HMGA1b-binding domain-c-Myc epitope fusion (Fig. 1) or a control pGBK7 vector. FL, full-length. B, growth on histidine and adenine-deficient medium was then determined, and quantitative β-galactosidase assays were performed as described in the legend to Fig. 1. C, immunoblotting was performed with comparable amounts of total cell lysate (50 μg). Ubc9-AD fusions (arrows) were detected using an anti-HA tag mAb. All Ubc9 fusions (arrows) were generally expressed at comparable levels and were of the predicted sizes. In the last lane, the arrow indicates the HA-tagged Gal4 AD only in the parental vector.

A protein from each strain showed that all fusions were expressed at similar levels and were of the predicted sizes (Fig. 1C). A comparison of these deletion mutants for their ability to grow on medium lacking adenine and histidine and to induce β-galactosidase (Fig. 1B) revealed that HMGA1b contained two independent Ubc9-interacting domains localizing, respectively, to a proline-rich segment between AT hooks 1 and 2 and to the extreme C-terminal acidic domain immediately adjacent to AT hook 3.

Although full-length HMGA1a and HMGA1b behaved identically in the yeast two-hybrid assay, we noted that the 11-amino acid insert that distinguishes the former isoform from the latter occurs within the proline-rich Ubc9-interacting domain. This suggested that the isolated proline-rich domains of the two HMGA1 isoforms might differentially interact with Ubc9. To test this directly, therefore, we examined comparable deletions of murine Ubc9 that described in the preceding section (Fig. 2A). The results of these studies showed that the HMGA1-interacting domain of Ubc9 consisted of a single region between amino acids 110–134 (Fig. 2B). Interestingly, this segment contains a cluster of at least six closely spaced residues that have been previously shown to play a critical role in SUMOylation of proteins relevant to tumorigenesis such as p53, E1b, and PML (Ref. 45 and see “Discussion”). Immunoblotting showed that all deletions were expressed at comparable levels and were of the predicted sizes (Fig. 2C).

Interaction between Endogenous HMGA1 Proteins and Ubc9—To verify the HMGA1-Ubc9 interaction under more physiologic conditions, co-immunoprecipitations (co-IPs) were performed on total cell lysates prepared from human HL60 promyelocytic leukemia, Burkitt’s lymphoma, and HOS cell lines. These were chosen because preliminary experiments indicated that they express extremely high levels of endogenous c-Myc and Ubc9 and have demonstrable deregulation of HMGA1 proteins (not shown). As shown in Fig. 3A, endogenous HMGA1a/b-associated Ubc9 was readily detectable in all three cell lines when an anti-HMGA1 antibody was used in the initial IP but not when a control IgG was used.

To confirm the specificity of the above co-IPs, we transiently knocked down endogenous HMGA1 protein expression in HOS cells using siRNA. SDS-PAGE of cellular lysates performed 2 days after transfection showed a >90% reduction in HMGA1 protein levels compared with lysates derived from cells transfected with a control duplex sequence (Fig. 3B). Furthermore, co-IPs performed on lysates from these cells under the conditions described in Fig. 3A showed that whereas endogenous Ubc9 was again readily detectable in cells treated with the control siRNA, Ubc9 was undetectable in HMGA1 siRNA-treated cells.

To further corroborate the above findings and to verify the relevance of the two Ubc9-interacting domains of HMGA1b, several Myc epitope-tagged variants of the latter protein were stably expressed in Rat1a fibroblasts, which express low to undetectable levels of endogenous HMGA proteins but which can be transformed by their deregulated expression (14). These mutants included either double or triple proline → alanine domain of HMGA1a, exerts little influence over the ability of this region to interact with Ubc9.

Identification of the HMGA1-interacting Domain of Ubc9—To identify the region of Ubc9 responsible for its interaction with HMGA1 proteins, we pursued a deletion-based strategy similar to that described in the preceding section (Fig. 2A). The results of these studies showed that the HMGA1-interacting domain of Ubc9 consisted of a single region between amino acids 110–134 (Fig. 2B). Interestingly, this segment contains a cluster of at least six closely spaced residues that have been previously shown to play a critical role in SUMOylation of proteins relevant to tumorigenesis such as p53, E1b, and PML (Ref. 45 and see “Discussion”). Immunoblotting showed that all deletions were expressed at comparable levels and were of the predicted sizes (Fig. 2C).
(P → A) substitutions in the proline-rich domain (P33A/P34A or P33A/P34A/P37A), a 19-amino acid C-terminal deletion of the acidic domain (1–77), or a combination of triple P → A substitutions and the acidic domain deletion (3P → A/1–77) (Fig. 3D). Immunoblotting of lysates from these cells established that all four proteins were expressed at levels comparable with that of the wild-type protein (Fig. 3E). The ability of each protein to interact with endogenous Ubc9 was then determined in co-immunoprecipitation experiments. As shown in Fig. 3F, all of the above described proteins, excepting the double mutant, interacted with Ubc9. From these studies, we conclude that the proline-rich and C-terminal domains of HMGA1b interact identically with Ubc9 both in yeast and mammalian cells.

**Functional Consequences of HMGA1b Mutant Protein Expression**—The foregoing experiments established that two genetically separable domains in HMGA1a/b were capable of associating independently with Ubc9. This redundancy suggested that distinct outcomes might arise as a consequence of each interaction and that Ubc9 might serve to integrate HMGA1 protein functions. To explore this in more depth, we focused initially on HMGA1b-mediated transformation. Each of the Rat1a cell lines depicted in Fig. 3C was thus examined for its ability to form anchorage-independent colonies in soft agar. As expected, cells expressing unmodified, wild-type HMGA1b were highly clonogenic (14, 26), whereas those expressing the double or triple P → A point mutations were devoid of transforming potential (Fig. 4A). In marked contrast, cells expressing the acidic domain deletion of HMGA1b were not only more clonogenic than those expressing the wild-type protein but produced colonies of markedly larger average size (Fig. 4B). The combination mutation 3P → A/1–77 eliminated entirely the ability of the protein to confer
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A.

| Protein | Western Blot |
|---------|--------------|
| HMGA1b  | Moderate     |
| Ubc9    | Low          |
| Tubulin | Strong       |

B.

![Graph showing the modulation of HMGA1b function by Ubc9](image)

C.

![Western blots showing endogenous Ubc9 knockdown](image)

D.

![Graph showing cell number over time](image)

FIGURE 5. Consequences of endogenous Ubc9 knockdown in HMGA1b-expressing Rat1a cell lines. Control Rat1a cells or Rat1a-HMGA1b cells were stably transfected with a puromycin-selectable retroviral vector expressing a short hairpin RNA directed against Ubc9 or with a control vector. Puromycin-resistant clones from all cell lines were then pooled for subsequent studies. A, immunoblotting to establish the degree of endogenous Ubc9 knockdown. B, soft agar colony formation. The indicated cell lines were plated as described for Fig. 4. The values shown represent the average colony numbers for triplicate cultures ± S.E. C, representative photos of soft agar colonies from each culture. D, growth of Rat1a cell lines under reduced serum conditions (1% FBS). Each of the indicated cell lines was plated and maintained as described in the legend to the preceding figure. Each time point represents the average of triplicate determinations ± S.E.

Their ability to form colonies in soft agar. The results of these studies indicated that HMGA1b-overexpressing cells with reduced levels of endogenous Ubc9 showed a 2–3-fold increase in colony formation compared with cells with normal levels of Ubc9 (Fig. 5, B, compare third and fourth lanes, and C). A comparable reduction in Ubc9 levels had little effect on the already negligible clonogenicity of control cells (first and second lanes).

Although the suppression of endogenous Ubc9 alone did not noticeably affect the clonogenicity of Rat1a cells in soft agar, it did affect their rate of anchorage-dependent proliferation under reduced serum conditions. As seen in Fig. 5D, these cells grew somewhat more rapidly than control cells (~2-fold). Similarly, cells with a combination of Ubc9 reduction and HMGA1b overexpression grew modestly faster than those that overexpressed HMGA1b only. From these experiments, we conclude that endogenous Ubc9 can exert a suppressive effect on cell growth and that whereas it can also modulate clonogenicity, it does so only when HMGA1b expression is concurrently deregulated.

Interaction with Proteins Other than Ubc9 May Be Required for the Proline-rich Domain of HMGA1b, but Not the C Terminus, to Modulate Transformation—Our finding that transformation by HMGA1b is modulated by two Ubc9-interacting regions of the protein does not necessarily imply that these regions interact with no other regulators. To examine this in more detail, we created a new set of vectors that expressed in-frame fusions between Ubc9 and either wild-type HMGA1b or each of the mutations shown in Fig. 3B. These were then stably transfected into Rat1a cells, and the arising G418-resistant clones were pooled. As seen in Fig. 6A, all four fusion proteins were expressed at comparable levels. When plated in soft agar, cells expressing the wild-type fusion protein formed numerous, large colonies similar to those shown in Fig. 4B for the nonfused HMGA1b protein (Fig. 6B). In contrast, despite its now forced interaction with Ubc9, the HMGA1b(3P→A)-Ubc9 fusion protein was unable to restore anchorage-independent growth. Although the HMGA1b (1–77)-Ubc9 fusion protein was able to transform Rat1a cells, it did so no better than the wild-type HMGA1b-Ubc9 fusion protein, and the colony sizes in both cases were comparable (not shown). Finally, fusion of Ubc9 to the double HMGA1b mutant, HMGA1b(3P→A)/(1–77), behaved similarly to its unfused counterpart in that it was completely lacking in transformation. These studies are consistent with the idea that the proline-rich domain, which positively regulates transformation, might also interact with
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FIGURE 6. Transformation of Rat1a cells by HMGA1b-Ubc9 fusion protein. The HMGA1b proteins depicted in Fig. 3B were expressed as C-terminal fusions with full-length Ubc9 in the pcDNA3.1 + vector. Following transfection, G-418-resistant clones were pooled for subsequent studies. Control cells were transfected with the empty vector only. A, immunoblotting was performed with a mAb directed against Ubc9 to detect the appropriate fusion proteins. Endogenous Ubc9 was simultaneously detected at equivalent levels in all cell lines (lower band). B, soft agar colony formation by each of the cell line was assessed in triplicate 12–14 days after plating. WT, wild type.

FIGURE 7. Model to explain the dual role of Ubc9 in integrating proliferative and transforming signals of HMGA1 proteins. The thick solid line represents the HMGA1b protein with the Ubc9-interacting proline-rich and C-terminal acidic segments being represented by gray and checkered boxes, respectively. Each of these is capable of interacting independently with Ubc9 (dashed arrows). The proline-rich segment exerts potent positive effects on transformation and proliferation (thick solid arrow), whereas the C-terminal acidic segment exerts weaker, negative effects on these same properties (thin solid line). Under typical conditions, the phenotype resulting from HMGA1 protein overexpression integrates both sets of signals leading to an overall transforming specifically on amino acids Glu110–113, Asp127, Ala129, Glu130, and Glu132 (47). Because the interaction of this region with HMGA1 proteins does not require the active Cys93 residue of Ubc9, which is otherwise mandatory for target protein SUMOylation (Fig. 2), it lends additional strong support to the notion that substrate recognition by Ubc9 and its subsequent SUMOylation are independent and separable events. Indeed, our finding that HMGA1 proteins neither contain consensus lysine recognition sites for constitutive or phosphorylation-dependent SUMO conjugation (48, 49) nor demonstrate altered mobilities in conjunction with SUMO overexpression3 is consistent with the idea that they belong to the small subset of Ubc9-interacting proteins that are not SUMOylated such as Vsx-1, p19ARF, and Sox4 (36, 38, 39, 46).

It is clear from both our yeast and mammalian co-expression studies (Figs. 1 and 3) that the singular elimination or mutation of the proline-rich domain or the C-terminal acidic domain of HMGA1b is insufficient to abrogate its association with Ubc9. The abnormal interaction that persists, however, is associated with profoundly different outcomes depending on the identity of the remaining HMGA1 domain. Thus, Ubc9 interaction with only the proline-rich HMGA1b domain results in a reduced serum requirement for Rat1a cells, an increase in their anchorage-independent clonogenic fraction, and an increase in average colony size (Fig. 4). In contrast, Ubc9 interaction with only the C terminus of HMGA1b results in a complete loss of clonogenic capacity and no effect on growth compared with control cells. Together, these findings support the contention that Ubc9 is a bi-functional regulator that operates via its obligate and bipartite interaction with HMGA1 proteins to integrate both positive and negative signals for proliferation and transformation (Fig. 7). The recent unexpected finding that HMGA1 proteins may be positive regulators of senescence in some circumstances lends further support to the idea that they can function in such a dual capacity (50).

We also addressed the possibility that, in addition to their interaction with Ubc9, the above two regulatory domains of HMGA1b might serve as sites of interaction for one or more proteins other than Ubc9. In contrast, the ability of fused Ubc9 to prevent the otherwise more highly transforming yHMGA1b (1–77) deletion from forming more and larger colonies than wild-type HMGA1b suggests that the extreme C terminus of HMGA1b does not require an interaction with proteins other than Ubc9 for this negative regulatory role to be manifested.

DISCUSSION

The studies presented here were predicated upon our initial finding that Ubc9 and HMGA1 proteins strongly interact in a conventional yeast two-hybrid assay. In the case of the latter protein, this association is mediated by a bi-partite domain consisting of a proline-rich segment between AT hooks 1 and 2 and a highly acidic C-terminal segment.

It is of interest that the HMGA1-binding domain of Ubc9 (amino acids 110–134) corresponds precisely to a previously described substrate recognition site for the p53 tumor suppressor and the c-Jun oncprotein (47). Based on NMR studies, this region has a high degree of conformational flexibility, centering specifically on amino acids Glu110–113, Asp127, Ala129, Glu130, and Glu132 (47). Because the interaction of this region with HMGA1 proteins does not require the active Cys93 residue of Ubc9, which is otherwise mandatory for target protein SUMOylation (Fig. 2), it lends additional strong support to the notion that substrate recognition by Ubc9 and its subsequent SUMOylation are independent and separable events. Indeed, our finding that HMGA1 proteins neither contain consensus lysine recognition sites for constitutive or phosphorylation-dependent SUMO conjugation (48, 49) nor demonstrate altered mobilities in conjunction with SUMO overexpression is consistent with the idea that they belong to the small subset of Ubc9-interacting proteins that are not SUMOylated such as Vsx-1, p19ARF, and Sox4 (36, 38, 39, 46).

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additional proteins. By generating fusions between Ubc9 and mutant forms of HMGA1b, we created an association that was, at best, only partially intact and, at worst, nonexistent in the case of the nonfused mutant proteins. In this fused state, the wild-type HMGA1b moiety was quite effective at imparting anchorage-independent growth to Rat1a cells, thus indicating that it was functionally little changed from its nonfused counterpart. That the Ubc9 fusion to the HMGA1b (3P→A) mutant did not restore transformation indicated that, even when a more complete association was restored, the HMGA1b protein nonetheless still required an intact proline-rich domain. In contrast, fusion of Ubc9 to the HMGA1b (1→77) mutant was sufficient to prevent the increase in soft agar colony number and size that is associated with this truncation. Together, these studies suggest that one or more proteins other than Ubc9 need to interact with the proline-rich domain, whereas Ubc9 interaction with the C terminus of HMGA1b is both necessary and sufficient for the modulation of transformation (Fig. 7). Despite its being consistent with all observations, however, this conclusion must remain qualified because it remains possible that differences in the nature of the association between Ubc9 and HMGA1b in their fused and free states can explain our findings without necessarily evoking the existence of other interacting factors.

The identification of the acidic C terminus of HMGA1b as a negative regulator of proliferation and transformation in Rat1a fibroblasts is reminiscent of the role it has been proposed to play in 3T3-L1 adipocyte proliferation (15). A similar function for the acidic domain of the HMGA2 protein, which is 40% identical to the acidic domain of HMGA1a/b has also been proposed (51). Interestingly, the C terminus of HMGA2 is often deleted in lipomas as a consequence of gene translocation (reviewed in Ref. 52). It will be of interest to determine whether Ubc9 is able to modulate the biological functions of HMGA2 as it does for the proteins described here.

A role for Ubc9 and/or Ubc9-mediated SUMOylation in tumor suppression has been suggested as a result of several independent finding. For example, SUMOylation of p53 has been shown to enhance its transcriptional activation of target genes (53). SUMOylation can also stabilize and thus cause the accumulation of the SMAD tumor suppressor (45). Although Ubc9 does not directly SUMOylate the p14ARF tumor suppressor, the association of these two proteins promotes the SUMOylation of p14ARF targets such as hdm2, with a subsequent strong growth inhibitory effect mediated by both p53-dependent and p53-independent pathways (38, 46). The SUMOylation of downstream targets is abrogated by naturally occurring, melanoma-associated mutations in p14ARF (38). On the other hand Mo et al. (28) have identified high levels of Ubc9 in some ovarian cancers and have shown that its enforced overexpression confers an in vivo growth advantage to MCF-7 breast cancer cells. Given the large number of its known targets, both SUMOylated and otherwise (27), it is perhaps not surprising that Ubc9 might function in such a dual oncogenic and tumor suppressor capacity. The relative dominance of each function may thus be determined by the abundance and identities of the specific oncopgenes and tumor suppressors that serve as Ubc9 targets in a particular context. The results presented here provide a molecular framework within which to consider and investigate such opposing functions.

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