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The Structure and Functions of NPM1/Nucleophsmin/B23, a Multifunctional Nucleolar Acidic Protein

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NPM1/Nucleophosmin/B23, also termed NO38 or numatrin, is an acidic nucleolar protein that plays multiple roles in cell growth and proliferation. In general, the expression level of B23 is proportional to the cell growth rate, suggesting that it plays a positive role(s) in cell growth and proliferation. It is important to note that the deletion of the *B23* gene and expression of an aberrant type of this gene—caused by gene conversion via translocation or reading-frame shift via nucleotides insertion—have been observed in diverse haematopoietic malignancies. Thus, it is important to understand the function of B23 in the regulation of cell growth and proliferation. In addition, B23 has been reported to undergo a variety of post-translational modifications such as phosphorylation, ubiquitination, SUMOylation, acetylation and poly-(ADP-ribosyl)ation. In this review, the basic structure and functions of B23 as well as the regulation of these functions are summarized.

Key words: histone chaperone, nucleoplasmin, nucleolus, phosphorylation, RNA.

NPM1/Nucleophosmin/B23 was originally identified as a phosphoprotein almost 30 years ago (*1*, *2*). It was found to be more abundantly expressed in proliferating cells than in resting cells. B23 is a member of the nucleoplasmin (NPM) family of proteins that includes nucleoplasmin/NPM2. Nucleoplasmin—identified from *Xenopus* egg extracts as a histone-binding factor that mediates nucleosome formation *in vitro* (*3*)—is the founding member of the NPM family. Nucleoplasmin plays a major role in sperm chromatin decondensation and chromatin assembly in fertilized eggs. The term 'chaperone' was first used to describe nucleoplasmin. Chaperones are molecules that associate with their target proteins to prevent misfolding and aggregation, but are not found in the final products comprising the target proteins. NPM family proteins share a similar N-terminal core structure that is required for oligomerization. In addition, domains rich in acidic amino acids that play a crucial role in histone-chaperone activity protrude from the N-terminal core domain. Three NPM family proteins, NPM1, NPM2 and NPM3, have been identified in mammals (*4*) (Fig. 1). NPM1 and NPM3 are expressed ubiquitously, whereas NPM2—suggested to be required for proper decondensation and re-organization of male and female gametes—is expressed mainly in the ovary (*5*). NPM3 is also suggested to play a role in chromatin remodelling in fertilized eggs (*6*). In this article, the term 'B23' is used instead of NPM1, nucleophosmin, NO38 or numatrin. Two splicing variants of B23, namely, B23.1 and B23.2, are expressed in growing cells. B23.1 and B23.2 have the same amino acid sequences except for 35 amino acids at the C-terminal end (Fig. 1). B23.1 is localized mainly at the nucleolus, and a minor fraction of B23.1 was found to be localized at the centrosome. The C-terminal domain present only in B23.1 is essential for its nucleolar localization. Moreover, several different types of genetic disorders with regard to the *B23* gene locus are implicated in haematological malignancies [many review articles have been published, for example see (*7*–*9*)]. For instance, the *B23* gene locus is a target for chromosome translocation that leads to the expression of oncogenic fusion proteins, frame shift mutations at the C-terminal region of B23 observed in acute myeloid leukaemia, and deletions observed in myelodysplastic syndrome. Therefore, clarifying the cellular function of B23 is vital in order to understand the molecular mechanisms underlying the development of cancer. However, since B23 is a multifunctional protein, the exact relationship between the *B23* gene mutations and cancer has not been described. Thus far, biochemical analyses have established that B23 associates with DNA and RNA (*10*), processes RNA (*11*), prevents misfolding and aggregation of target proteins as a molecular chaperone (*12*) and mediates chromatin assembly and disassembly as a histone chaperone (*13*). The biological significance of B23 in ribosome biogenesis and transport (*14*), anti-apoptotic activity (*15*), the regulation of centrosome duplication (*16*) and the regulation of tumour suppressors such as ARF and p53 have also been documented. In addition, it has been observed that B23 associates with various partner proteins including

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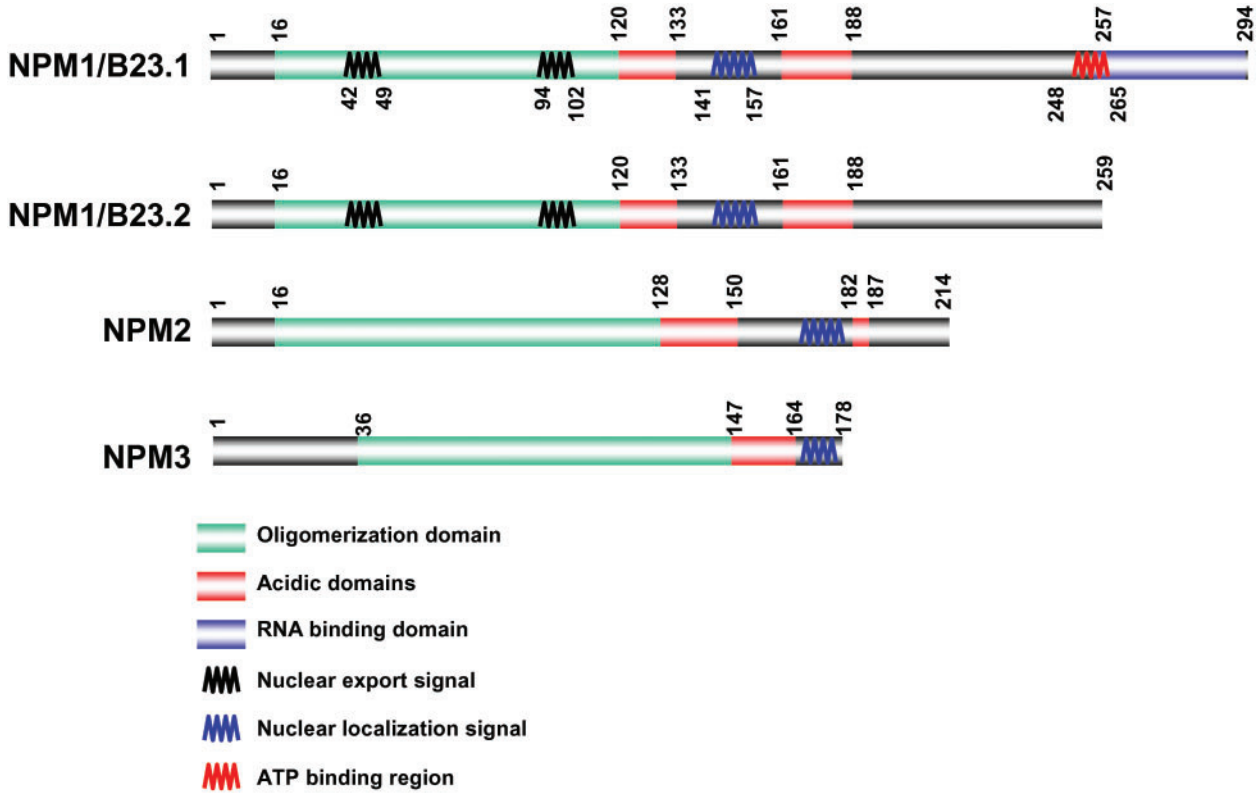

Fig. 1. **Schematic representations of the human NPM family proteins.** Human NPM1, NPM2 and NPM3 are schematically represented. The NPM family proteins share a conserved structure responsible for oligomer formation at their N-terminal regions. Of these, NPM1/B23.1 is the only protein that has the unique RNA-binding domain at its C-terminal end.

transcription factors. Given that mutation of the *B23* gene is closely related to a variety of haematological malignancies and inactivation of this gene leads to embryonic lethality (*17*), B23 is an important molecule in embryonic development and proper cell growth. Recently, a number of excellent reviews regarding B23 and cancer (*8*, *9*), the function of B23 in the ARF-p53 pathway (*18*), and centrosome duplication (*19*) have been published. In this review, the basic structure and functions of B23 as well as the regulatory mechanisms of the B23 functions will be discussed.

STRUCTURAL AND FUNCTIONAL DOMAINS OF NUCLEOPHOSMIN/B23

As described earlier, B23 belongs to the NPM family and has a conserved N-terminal core domain. This domain is important for the formation of a pentamer in which five wedge-shaped monomers are linked by significant apolar interactions (*20*) (Fig. 1). Pentamer formation via apolar interactions confers thermostable properties on NPM family proteins including B23; these properties are critical for the molecular chaperone activity of B23. It has been well established that B23 can suppress the misfolding and aggregation of target proteins at high temperatures, and it was found that the N-terminal core domain of B23 is required for this activity (*21*). In addition to the oligomerization properties of the N-terminal core domain of B23, it should be noted that this domain contains two leucine-rich nuclear export signals (NES) that are targeted by the nuclear export receptor CRM1. Nuclear-cytoplasmic shuttling of B23 is regulated by these NES sequences and a classic nuclear localization signal (NLS), which was first characterized in nucleoplasmin (*22*), located between the 2 acidic regions (Figs 1 and 2). B23 has been implicated in the transport of pre-ribosome subunits from the nucleus to the cytoplasm (*14*). It was recently reported that B23 directly associates with the ribosomal protein L5, a 5S rRNA chaperone, in a NES sequence (amino acids 42–49)-dependent manner, and the complex is thereby transported to the cytoplasm (*23*). On the other hand, another NES sequence (amino acids 94–102) is required for the nuclear export of B23 and its centrosome localization that ensures proper centrosome duplication (*24*). The usage of the NES sequences in B23 may be regulated by association with partner molecules and/or post-translational modifications.

The N-terminal core region of B23 is followed by the two highly acidic regions that are required for efficient histone binding and nucleosome assembly (*25*). The biological significance of the acidic domains has not been established; however, the acidic nature of these domains could play a crucial role in buffering the positive charge of basic proteins such as histones and ribosomal proteins in the nucleolus. We recently demonstrated that in adenovirus (Ad)-infected cell extracts, B23 associates with Ad basic histone-like proteins via the acidic domains (our unpublished results and (*26*)). B23 could play a crucial role in buffering newly synthesized viral basic proteins for the packaging of the Ad genome DNA into the virion.

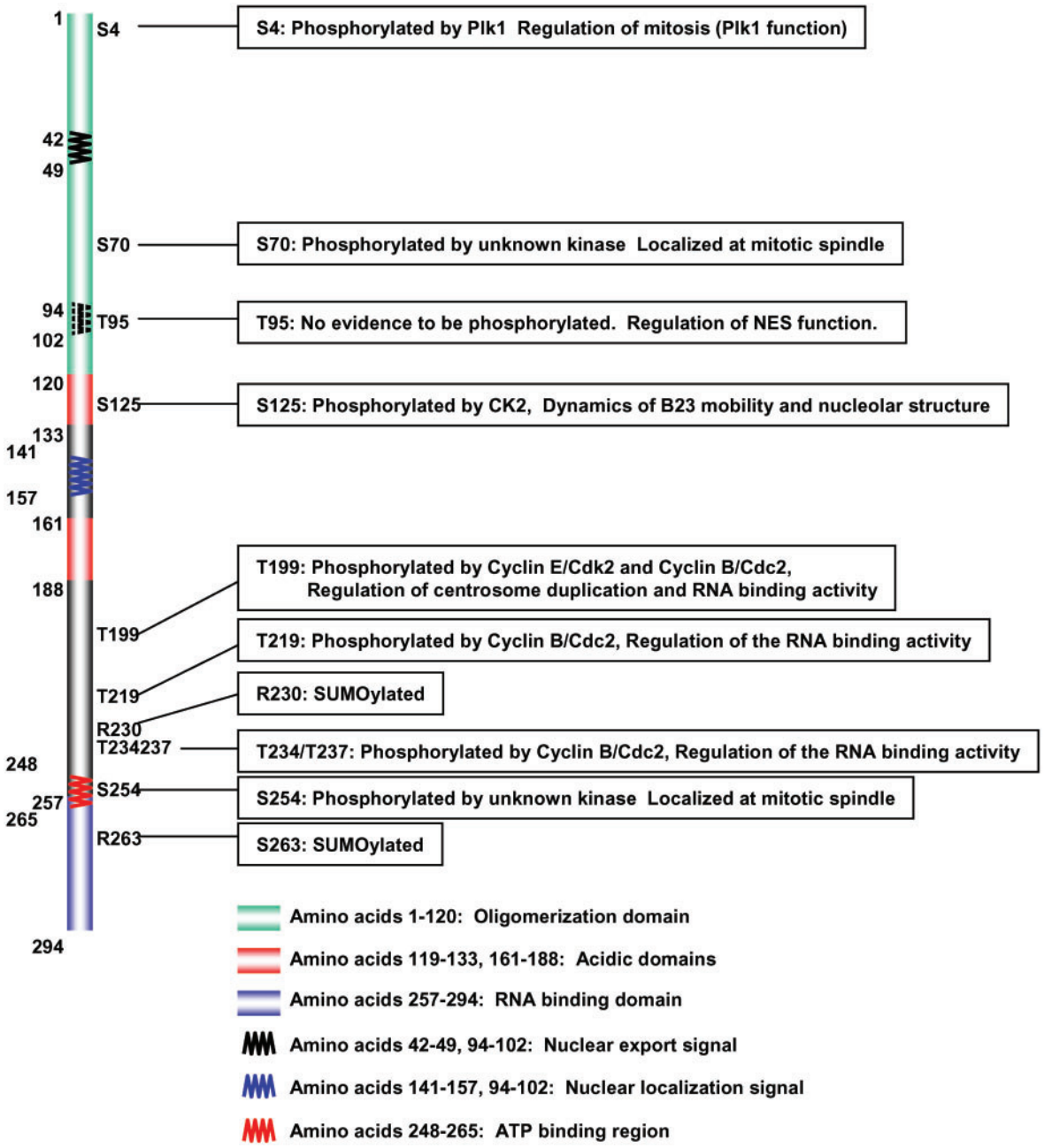

Fig. 2. **A schematic representation of the NPM1/Nucleophosmin/B23 structure.** Human NPM1/Nucleophosmin/B23 consists of 294 amino acids. The functional domains and target sites of post-translational modifications know thus far are shown at the left and right side of the protein structure, respectively. Although it is not mentioned in the main text, the position 248–265 is a consensus ATP-binding motif and B23 was found to bind to ATP *in vitro* (*54*). Biological significance of ATP binding of B23 remains to be established.

Another important domain involved in the functioning of B23 is the RNA-binding domain located at the C-terminal end of the protein. Since the RNA-binding activity of B23 is closely related to its nucleolar localization, it is suggested to be essential for ribosome biogenesis function of B23. Interestingly, two tryptophan residues, *i.e*. W288 and W290, within the RNA-binding domain are critical for its nucleolar localization (*27*). This RNA-binding domain is unique to B23 and is not found in other proteins. Thus, it is speculated that B23 has a specific RNA partner for its cellular functions. This idea is partly supported by the fact that recombinant B23.1 associates with 28S or 5.8S rRNAs but not with 18S rRNA *in vitro* (*28*). It is important to note that the RNA-binding domain alone cannot associate with the target RNA [our unpublished result and (*21*)]: proper folding and conformation of the RNA-binding domain in the context of the B23 oligomer are crucial for association with RNA. This is

consistent with our previous study, which demonstrated that oligomer formation is required for B23 to associate with RNA (*28*).

REGULATION OF B23 FUNCTIONS THROUGH HETERO-OLIGOMER FORMATION WITH THE NPM FAMILY PROTEINS

Both B23.1 and B23.2 are expressed in growing cells and form oligomers, and B23.2 does not associate with RNA and is localized throughout the nucleus. These observations prompted us to speculate that B23.2 could negatively regulate the C-terminal function of B23.1 by forming hetero-oligomers (*28*). We found that B23.1 and B23.2 form hetero-oligomers in cell extracts and that B23.2 inhibits the RNA-binding activity of B23.1 by hetero-oligomer formation. However, since one B23.2 molecule in a B23 pentamer would not be adequate to completely abolish the RNA-binding activity of B23.1, the balance between the two proteins in the pentamer is a critical determinant of the B23.1 C-terminal function. Similarly, NPM3 that has the conserved N-terminal oligomerization domain of the NPM family proteins is suggested to regulate B23 function via hetero-oligomer formation with B23.1 (*29*). It is possible that localization of B23.1 is regulated by B23.2 and NPM3 because these proteins inhibit the RNA-binding activity of B23.1, as described earlier. Thus, the relative balance between the expression level of B23.1 and those of B23.2 and NPM3 is suggested to be important for B23 function. Moreover, it was recently demonstrated that the tumour suppressor ARF associates with the N-terminal core domain of B23 and induces the destabilization of B23 oligomer formation (*30*). The B23 monomer created by ARF binding is a target for ubiquitin–proteaosome-mediated protein degradation. Thus, it is suggested that B23-interacting proteins, which target the N-terminal core domain of B23, regulate B23 functions by destabilizing oligomer formation.

REGULATION OF B23 FUNCTIONS THROUGH POST-TRANSLATIONAL MODIFICATIONS

B23 was identified as a phosphoprotein, and many amino acid residues were found to be phosphorylated in cells. Typical cyclin-dependent kinase (cdk) consensus sites are distributed throughout the B23 sequence (S10, S70, T199, T219, T234 and T237). Of these, the four threonine residues T199, T219, T234 and T237 were shown to be phosphorylated by cyclin E/cdk2 or cyclin B/cdc2 *in vivo* and *in vitro* (*16*, *28*, *31*). These cdk consensus sites play crucial roles in the regulation of B23 functions. T199 of B23 localized at the centrosome was found to be phosphorylated by cyclin E/cdk2 during the G1 phase, permitting the majority of B23 to dissociate from the centrosome. It is suggested that B23 links to the paired centriole (*32*), and that dissociation of B23 is essential for the initiation of centrosome duplication. Thus, the phosphorylation of B23 at T199 could trigger the initiation of the centrosome duplication; however, the molecular mechanism of the suppression of this process by non-phosphorylated B23 remains to be clarified. By using a specific antibody, it is recently reported that B23.1 phosphorylated at T199 is localized at the nuclear speckles that contain splicing machineries such as SC35 (*33*); this suggests that the phosphorylation of T199 plays a role in the localization of B23.1 at the speckles. However, since phosphorylated B23.1 at T199 exhibited a higher affinity to RNA (*33*), it is unclear how T199 phosphorylation permits the localization of B23 at the speckles.

T199 as well as T219, T234 and T237 is phosphorylated during mitosis. We found that the RNA-binding activity of B23.1 was abolished by cdc2-mediated phosphorylation of these four sites *in vitro* (*28*). Although the biological significance of the mitotic phosphorylation of B23 is obscure, the formation of a complex between RNA-binding proteins such as B23 and RNA molecules could be important for the association with the nucleolus. Therefore, disruption of the RNA–protein complexes during mitosis could contribute to disassembly of the nucleolus. During mitosis, the different sites of B23 were observed to be phosphorylated. B23 associates with Polo-like kinase 1 (Plk1) and is phosphorylated at S4 by it (*34*). Expression of a B23 mutant in which S4 is replaced by alanine induces multiple mitotic defects, suggesting that B23 plays a role in mitosis via Plk-mediated phosphorylation. Moreover, proteomic analysis of the mitotic spindle revealed that S70, S125 and S254 of B23 are phosphorylated (*35*), although the kinase(s) that phosphorylates these sites and the significance of these phosphorylation sites are unknown. Nek2a, a mitotic regulator, is a potential candidate kinase that phosphorylates S70 and S254, since it was shown that Nek2A associates directly with B23 and is essential for its localization at the mitotic spindle (*36*). During mitosis, the bulk of B23 phosphorylated by cyclin B/cdc2 at the four threonine residues is located throughout the cell, and a minor fraction of B23 is localized at the mitotic spindle. The distinct phosphorylation patterns of B23 could be associated with the regulation of B23 localization, partly via the association with Plk1 and/or Nek2A.

An additional relevant phosphorylation site of B23 is S125; it is phosphorylated during the interphase (*37*). This site is within a consensus sequence that is targeted by casein kinase 2 (CK2). It was demonstrated that the overexpression of a mutant in which S125 is replaced with alanine induces segregation of the nucleolar structure and reduces the ribosome biogenesis function (*38*). In addition, phosphorylation of S125 has been shown to regulate the dynamics of B23 (*39*). The localization of most nucleolar proteins involved in ribosome biogenesis, such as B23, is dynamic and they are rapidly exchanged between the nucleolus and nucleoplasm (*40*). Therefore, phosphorylation at S125 plays a role in the dynamic nature of B23 in the nucleolus that could be crucial for the maintenance of the nucleolar structure. It will be interesting to determine the function(s) of B23 that is affected by S125 phosphorylation and thus understand the regulation of the nucleolar structure and the dynamics of B23. The residential time of non-phosphorylated form of B23 in the nucleolus is longer than that of the phosphorylated form of B23 (*39*); considering this, phosphorylation of S125 may regulate the interaction between B23 and a component of the nucleolus. Since the RNA-binding activity of B23 is closely related to its nucleolar localization, it is possible that the phosphorylation of B23 at S125 regulates its

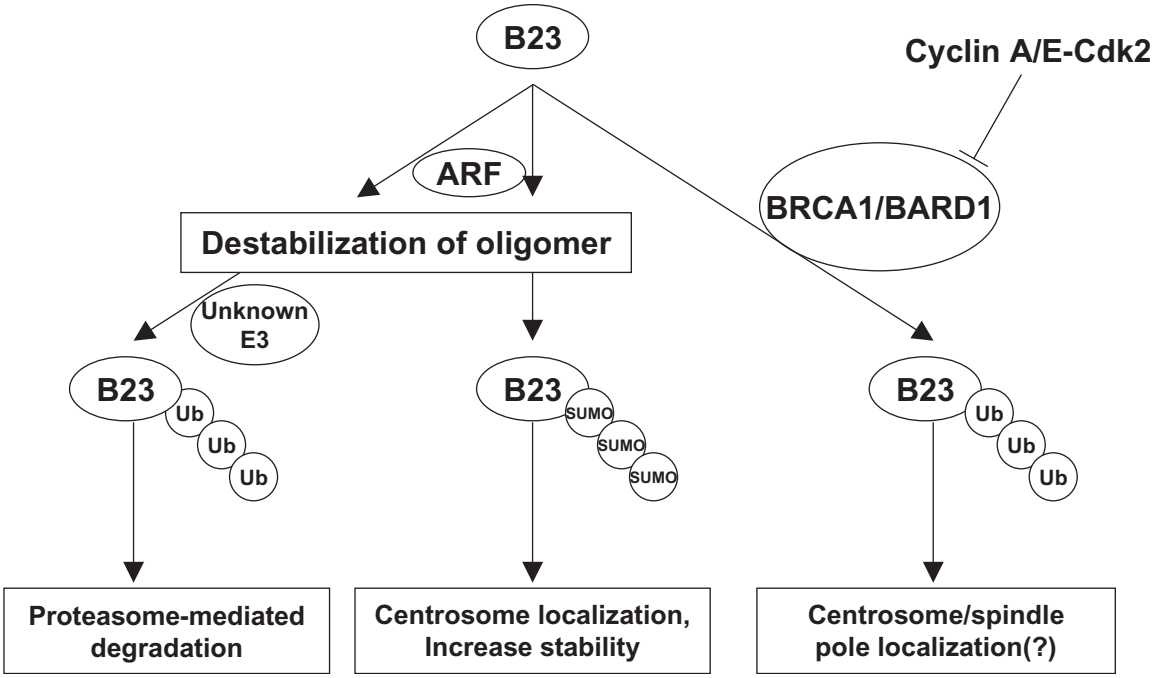

Fig. 3. **Regulation of the B23 function by ubiquitination and SUMOylation.** ARF induces destabilization of a B23 oligomer that could allow the ubiquitiantion or sumoylation system to access the target site of B23. Ubiquitination induces proteasome-mediated B23 degradation, whereas SUMOylation increases stability of B23. It is currently unknown how these two modifications are regulated to determine B23 stability. ARF induces the traditional lysine 48-linked poly-Ub chain addition, whereas BRCA1-BARD1 mediates the lysine 6-linked poly-Ub chain addition. BRCA1–BARD1-mediated ubiquitination occurs during mitosis, because its activity is down-regulated by cyclin A/E-cdk2 complexes during G1 to S phase.

RNA-binding activity. It is also reported that the molecular chaperone activity of B23 is regulated by CK2-mediated phosphorylation (*37*), although the biological relevance of the molecular chaperone activity of B23 has not yet been established.

T95 within the NES sequence (94–102) is suggested to be phosphorylated, although this has not been proven. It was observed that the CRM1-dependent export of B23 in which T95 was replaced with aspartic acid was abolished, and this induced unregulated centrosome duplication (*24*).

Besides phosphorylation, B23 was found to undergo poly-(ADP-ribosyl)ation (*41*), acetylation (*42*), SUM Oylation (*43*) and ubiquitiation (*44*, *45*). B23 was found to associate with the poly-(ADP-ribosyl)ation factors, PARP-1 and PARP-2 (*46*); however, the enzyme responsible for poly-(ADP-ribosyl)ation of B23 and the effect of poly-(ADP-ribosyl)ation on the B23 functions remain unknown. PARP-1 is a structural component of chromatin similar to linker histones and regulates the chromatin structure (*47*). Furthermore, B23 associates with PARP-1 via its DNA-binding domain (*46*). It is therefore possible that B23 is involved in chromatin association and dissociation of PARP-1. Acetylation of B23 is suggested to be involved in the chromatin regulation function of B23. Several lysine residues at the C-terminal half of B23 were found to be acetylated *in vivo* (*42*). Acetylation of B23 increases its affinity to histones. The effect of B23 acetylation as well as poly-(ADP ribosyl)ation on the B23 functions would be an interesting issue to be addressed in future.

Two different types of poly-ubiquitination of B23 have been reported. B23 is found to be a potential substrate of BRCA1 E3 ligase (*44*). BRCA1 complexed with BARD1 mediates the addition of an unconventional Lysine 6-linked poly-ubiquitin (Ub) chain to substrate proteins (*48*). It is known that proteins with this Lysine 6-linked poly-Ub chain are not substrates for proteasome-dependent degradation. This poly-Ub chain is believed to be a post-translational modification of substrate proteins. From this observation and the co-localization of B23 and BRCA1-BARD1 at the mitotic spindle, BRCA1–BARD1-mediated poly-ubiquitination is suggested to regulate the B23 function during mitosis. In addition, it was reported that inactivation of BRCA1 induces centrosome amplification, thereby genomic instability (*49*) and the E3 ligase activity of BRCA1-BARD1 is down-regulated by Cyclin A/E-cdk2 (*50*). Thus, it is suggested that B23 is a major downstream target of BRCA1-mediated ubiquitination during mitosis (Fig. 3). It would be interesting to understand the relationships among poly-ubiquitination, Plk1 and Nek2a association and phosphorylation of S70, S125 and S254 at the mitotic spindle. Another group reported that the tumor suppressor ARF directly associates with B23 and induces ubiquitin–proteasome-dependent degradation, thus regulating the stability of B23 (*45*). ARF expression also induces SUMOylation of B23 at K230 and K263 (*43*). Unlike ARF-mediated ubiquitination, SUMOylation of B23 is believed to affect its functions (*51*). Surprisingly, substitutions of lysine 230 or 263 to arginine abolish the localization of B23 at the nucleolus

and centrosome. Loss of B23 localization at the centrosome leads to premature centrosome duplication. In addition, K263R mutation decreases the stability of B23 by evading the caspase-3-mediated cleavage, indicating that SUMOylation of B23 could regulate the B23 stability. However, it is currently unclear whether or not these effects of substitution mutations on B23 functions actually depend on SUMOylation at these sites. We cannot exclude the possibility that the presence of arginine instead of lysine at these sites affects the functions of B23. Except the ARF-induced ubiquitination that is followed by proteasome-mediated degradation, the functional significance of the poly-ubiquitination or poly-SUMOylation of B23 has not been well understood. The most obvious consequence of B23 poly-uqbiquitination or SUMOylation defect is the hyper amplification of the centrosome as described earlier. The possible function of B23 at the centrosome is suggested to link two paired cetnrioles, because Cyclin E/cdk2-mediated phosphorylation at T199 of B23 is essential for splitting the paired centrioles (*16*). The important points to be clarified are how B23 is involved in establishment of two centrioles cohesion and what the target molecule to recruit B23 to the centorosome is. The interaction between B23 and a molecule(s) in the centrosome could be regulated by poly-Ubiquitination and poly-SUMOylaion.

CONCLUSIONS AND FUTURE DIRECTIONS

Since B23 is a multi-functional protein and associates with a variety of cellular proteins, it directly or indirectly plays crucial roles in the infection cycle of many viruses. Proteins expressed in cells infected with adenovirus, human immunodeficiency virus, adeno-associated virus, hepatitis C virus hepatitis D virus, retrovirus, Japanese encephalitis virus and influenza virus have been shown to interact with B23. In addition, B23 is suggested to regulate the interferon system through its association with the transcription factor, interferon regulatory factor-1 (*52*) and the double strand RNA-dependent kinase, PKR (*53*). Many viruses possess their own strategy to escape from the cellular interferon system, in order to produce progeny viruses. In infected cells, B23 may be exploited by viruses for its anti-interferon activity. Analyses of the B23 functions in virus-infected cells may lead to the identification of a novel and unexpected cellular function of B23. The *B23* gene is one of the most frequent targets of mutation, deletion, and chromosome translocation, leading to a variety of human cancers. Altered functions or loss of functions of B23 is suggested to cause un-regulated cell growth and proliferation. Thus, it is important to clarify the function of B23 and the underlying regulatory mechanisms. As described in this review, B23 function is suggested to be regulated by post-translational modifications such as phosphorylation, ubiquitination, SUMOylation, acetylation and poly-(ADP-ribosyl)ation. While attempting to clarify the function of B23, understanding the effects of these post-translational modifications and the combination of these modifications on B23 functions will definitely contribute to a better understanding of the tumourigenesis.

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