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Identification and analysis of new Proteins involved in the DNA Damage Response network of Fanconi anemia and Bloom syndrome

Rong Guo^{1,#}, Dongyi Xu^{1,#}, and Weidong Wang^{1,*}

1 Laboratory of Genetics, National Institute on Aging, National Institutes of Health, NIH Biomedical Research Center Room 10B113, 251 Bayview Boulevard, Baltimore, Maryland 21224

Abstract

The use of co-immunoprecipitation (co-IP) to purify multi-protein complexes has contributed greatly to our understanding of the DNA damage response network associated with Fanconi anemia (FA), Bloom syndrome (BS) and breast cancer. Four new FA genes and two new protein partners for the Bloom syndrome gene product have been identified by co-IP. Here, we discuss our experience in using co-IP and other techniques to isolate and characterize new FA and BS-related proteins.

1. Introduction

Bloom syndrome is a human genetic disease characterized by growth retardation, immunodeficiency, cancer predisposition, and genomic instability (1). The disease is caused by mutations in BLM, a helicase that plays a key role in homologous recombination-dependent DNA repair (2). Fanconi anemia (FA), another human genetic disease, shares several features with Bloom syndrome, including cancer predisposition and genome instability (3). However, unlike Bloom syndrome which is always caused by mutations in a single gene, FA can result from lesions in any of multiple genes. To date, thirteen FA genes have been identified. All of their products function in a DNA damage response network that includes BLM. Three FA genes FANCD1, FANCN, and FANCI are breast cancer susceptibility (BRCA) genes BRCA2, PALB2, and BACH1, making FA an attractive model to investigate the mechanism of action by BRCA proteins (4).

Research on Bloom syndrome and FA has been greatly accelerated using immunoprecipitation to isolate their associated multiprotein complexes and subsequent identification of their components (5). The basic principle of this strategy is simple: components of the BLM or FA complexes will most likely have important functions similar to those of the disease proteins. Moreover, defects in these components may cause disease conditions similar to Bloom syndrome or FA. Indeed, two newly identified BLM complex components, RMI1 (BLAP75) and RMI2 (BLAP18), have been found to form a novel multi-OB-fold complex that has a critical role in protecting genome stability (6,7). Moreover, each of three novel FA core complex components have been found to be mutated to cause some cases of FA (FANCL,

*Correspondence should be addressed to WW. Telephone: 410-558-8334, Fax: 410-558-8331, Email: E-mail: wangw@grc.nia.nih.gov.

#These authors made equal contribution to this work.

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FANCB and FANCM) (8–10). Furthermore, another FA gene, FANCN (PALB2), was first identified as a component of the FANCD1 (BRCA2) complex (11). Interested readers can find references to these discoveries in other reviews (4).

Here we discuss our experience using co-IP to immunopurify BLM and FA protein complexes. We also describe several approaches to verify and analyze the newly identified proteins.

2. Developing a high-quality antibody

Crucial to our success in immunopurification of the FA core complex and the BLM complex has been the generation of highly specific antibodies against these disease proteins. In our experience, a good antibody is critical for the success of purification. One can find detailed protocols of developing antibodies in a previously published method paper (12). We usually make rabbit polyclonal antibodies against maltose-binding protein (MBP; New England Biolabs) fused to different regions of the target protein. We also use the same MBP-fusion protein to affinity-purify antibodies. The purified antibodies are always more specific and bring down fewer contaminants than crude anti-serum. These antibodies allowed us to co-IP cognate target complexes from HeLa nuclear extract in a single step (5). This is consistent with our experience in immunopurification of several chromatin-remodeling complexes (SWI/SNF, NURD, and ATRX complexes), all of which have been isolated using highly specific antibodies against the endogenous proteins.

3. Immunopurification of protein complexes and identification of their components

3.1 Preparation of nuclear extract (NE)

We normally start purification by determining the distribution of the target protein in the cellular extracts. We typically prepare cytosolic and nuclear extract, and determine the location of the target protein. If the nuclear extract contains the target, we can further prepare soluble and chromatin sub-fractions to further localize the target protein. Immunoprecipitation using subcellular fractions rather than whole cell lysates can reduce co-purification of contaminating proteins. Since FA and BLM complexes function in nuclear or chromatin fractions, we normally prepare extracts from these sources as starting material. It should be mentioned, however, that certain FA proteins are present in the cytosol, and FA subcomplexes have been isolated from this source (13).

We often use human HeLa cells as the starting material for purification. One advantage of using this cell line is that it contains higher levels of proteins that protect genome stability than other lines (such as transformed lymphoblastoid and fibroblast cells). Moreover, the cells can be purchased in large quantity from the National Cell Culture Center.

Nuclear extract from human HeLa cells is prepared essentially as described (14). To avoid degradation of proteins, all procedures were performed at 4°C.

- a. Cell pellets were washed twice with phosphate-buffered saline (PBS) and once with the hypotonic buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂) supplemented with 0.5 mM PMSF, 1 mM DTT and proteinase inhibitor cocktail tablet (Roche). They were collected by low-speed centrifugation (3000rpm, BECKMAN Allegra 6KR Centrifuge).
- b. Two volumes of buffer A were then added to the cell pellet. The pellet was homogenized about 10 times using a dounce homogenizer, and the nuclei were collected by centrifugation. Cells should be completely lysed in this buffer, which

can be tested by staining with dyes such as Trypan Blue. The supernatant is saved as the cytosol fraction.

- c. The nuclei were washed once with 5–10 volumes of buffer A to remove contaminating cytosol proteins, and collected by centrifugation.
- d. The nuclear extract was prepared by extracting the nuclei two consecutive times using two volumes of buffer C each time (20 mM HEPES pH 7.9, 0.42 M NaCl, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, supplemented with 0.5 mM PMSF, 1 mM DTT and cocktail). Because the nuclei are in one volume of Buffer A, the final salt concentration in the mixture is lower than that of Buffer C: the salt concentration of the first nuclear extract (NE1) is about 0.28 M, and that of the second extract (NE2) is about 0.37 M. This differential salt concentration results in differential extractions of the proteins: those that loosely associate with chromatin are enriched in the first extract (NE1), whereas those tightly bound to chromatin are in the second extract (NE2). We found that the full FA core complex is mainly present in NE2, whereas FA subcomplexes are largely present in NE1.
- e. The nuclei were homogenized about 10 times in to improve the efficiency of extraction. We usually rotate the nuclei in Buffer C for 30 min at 4°C, followed by centrifugation at high speed (15,000 rpm) for 15 min. The supernatant is saved as the nuclear extract. Its protein concentration should be between 6–8 mg/ml. One could perform immunoblotting analysis of the nuclear extract to ensure that the target protein is not significantly degraded. The nuclear pellet should also be saved as it contains many proteins that tightly associate with chromatin and can be used as a source for additional purification.

3.2 Preparing chromatin fractions

Chromatin fractions can be used for purification of proteins that bind chromatin so tightly that they cannot be extracted efficiently by Buffer C using the above protocol. To extract these proteins, the chromatin can be digested with DNase I as described previously (15).

- a. Cells were first washed with PBS, and then incubated for 3 min at 4°C in cytoskeleton buffer (CSK): 10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, supplemented with 0.5 mM PMSF, 1 mM DTT, cocktail and 0.5% Triton X-100.
- b. The cytoskeletal proteins and membranes were removed from the soluble protein fractions by centrifugation at 5,000 g for 3 min.
- c. The pellet, which contains chromatin, was solubilized by digestion with RNase-free DNase I (Roche, 100 units/ml) in CSK buffer supplemented with protease inhibitors for 15 min at 37°C.
- d. Ammonium sulfate (from 1M stock) was then added to the mixture to make final concentration of 0.25 M. The mixture was incubated for 5 min at 4°C, and then centrifuged. The supernatant is the chromatin fraction containing solubilized chromatin-bound proteins (and DNA).
- e. The pellet was further extracted with 2 M NaCl in CSK buffer for 5 min at 4°C, and then centrifuged. The remaining pellet is solubilized in urea buffer and is considered as the nuclear matrix-containing fraction.

3.3 Co-immunoprecipitation (co-IP)

Co-IP has been widely used to isolate a target protein and its associated complexes from cell extracts. This method is based on the highly specific interactions between the antibody and the

antigen (i.e., the target protein). Such specific interactions often allow about 1000-fold purification of the target protein from cell lysate in a single step, often a purity sufficient for identification purpose. However, the success of IP often depends on the abundance of protein in the extract, the quality of the antibody, and the accessibility of the epitope on the target protein to the antibody. As the concentration of each protein in the lysate and their antibody qualities vary, it may be necessary to optimize conditions for each IP. For example, if the concentration of a target protein is too low in the extract, one may need to fractionate the extract to enrich the target. If the antibody is not of good quality, one may need to affinity-purify the antibody to improve its specificity and titer. If the epitope is not accessible to the antibody, or if the antibody cannot recognize the native form of the protein, one may have to develop a different antibody against a different region of the protein.

The basic IP protocol includes incubation of the antibody with the cell lysate to allow formation of the antibody-antigen complex. This complex is then captured on Protein A or Protein G Sepharose beads. The unbound proteins are removed by washing with various buffers. Finally, the target protein and its associated polypeptides are eluted off the beads with reagents that disrupt the antibody-antigen interactions. Our lab usually combines the first two steps by incubating antibody, cell lysate and Protein A/G beads together. Here is one procedure commonly used in our group for co-IP:

- a. HeLa nuclear extract (0.2–0.5 ml) was diluted 4-fold with IP buffer (20 mM HEPES [pH 7.9], 200 mM NaCl, 1 mM DTT, 0.2 mM PMSF, 10% glycerol).
- b. The diluted extract was centrifuged at 100,000 rpm using BECKMAN Optima TLX Ultracentrifuge. This step removes the insoluble membrane and cytoskeletal structures that non-specifically associate with protein A beads. This step is critical for purity of the complex, and can be repeated to ensure that the extract is clean of the any insoluble debris.
- c. After ultracentrifugation, the supernatant was carefully transferred to another tube containing the antibody and protein A-beads (Amersham Pharmacia). No insoluble debris should be transferred as this will contaminate the IP. The amounts of the antibody and the protein A beads should be determined experimentally for each individual protein. For small scale IP (less than 0.2 ml of nuclear extract), one can use 100–1000 ng of antibody (rabbit IgG) and 10 μ l of Protein A beads as a starting point.
- d. The mixture was rotated in the cold room on a rotator for 2 to 12 hours. The longer incubation may increase the yield of the IP, as well as the amount of contaminating polypeptides. One may need to balance these factors in deciding how long to incubate the antibody with the lysate.
- e. The immunoprecipitate on the beads was collected by centrifugation (1000 rpm, 1 min). The supernatant should be saved for immunoblotting analysis to determine how efficient the IP was, and whether the target protein was depleted from the extract by IP. If the target protein is completely depleted, one may consider increasing the amount of the extract.
- f. The beads were washed four times with the IP buffer (at least 10 volumes each time). The target protein and its associated complex on the beads were eluted from the beads by using 1 to 2 volumes of 100 mM glycine-HCl buffer (pH 2.5). The eluate should be neutralized immediately by adding 1/10th volume of Tris.HCl (1M, pH 8.0). It can then be analyzed by SDS-PAGE and immunoblotting.

We often perform the following procedures to improve IP. First, we often include negative controls by performing a mock IP with Protein A/G beads alone (without adding antibody to

the extract). This is because that Protein A/G beads can sometimes non-specifically pull down cellular proteins (actin, myosin and tubulin, etc) especially when the IP conditions are not optimal. These non-specific proteins, if present at high levels, can render identification of true target proteins difficult. One way to reduce contamination is to pre-incubate the cellular lysate with protein A/G-beads for 1–2 hours prior to IP. Proteins that bind non-specifically to the beads can be removed by centrifugation. Second, we sometimes optimize IP conditions by adjusting salt concentrations in the mixture. A high salt concentration may disrupt the interaction between the target and its associated complex, whereas low salt concentration may increase non-specific protein interactions and contamination. We normally use 200 mM NaCl for most IP. However, if the target protein complex is highly stable, we often use higher salt concentration (0.3 or 0.5 M NaCl) to reduce non-specific interactions. Third, one limitation of the above IP protocol is that the immunoprecipitate often contains immunoglobulin heavy and light chains (IgG-H and IgG-L), which may overlap with components of the target protein complex on the SDS gel, hindering their identification. To solve this problem, dimethylpimelidate (DMP) can be used to covalently crosslink antibody to Protein A beads, so that IgG will not be eluted by the elution buffer (see Figure 1A and 1B as examples). However, DMP crosslinking often reduces or sometimes inactivates activity of an antibody, so that it may require the use of 3 to 10 –fold more antibody than the standard IP protocol. The increased amount of antibody may yield more non-specific proteins in the IP. However, because this method can significantly reduce the level of IgG in the IP, we use it frequently. We follow a published protocol to crosslink antibody to Protein A beads (16). Briefly:

- a. Protein A beads (100 μ l) was incubated with 10–30 μ g of rabbit polyclonal antibody in 500 μ l of PBS at room temperature for 30 min by rotating. This allows binding of antibody to Protein A beads.
- b. The beads were washed 3 times with PBS, at least 10 volumes each time, to remove unbound antibody. They were then washed twice with 0.1 M sodium Borate, pH 9.0.
- c. The beads were resuspended in 1 ml of 0.1 M sodium Borate, pH 9.0 and 5.2 mg DMP (Pierce), and the mixture was rotated at room temperature for 45 min. This allows crosslinking of antibody to Protein A beads.
- d. The beads were washed once with 1M Ethanolamine, pH 8.0, and then incubated overnight at 4°C or 2 hours at room temperature in the same solution on a rotator to block the remaining reactive DMP, so that it does not crosslink cellular proteins during IP.
- e. The beads were then washed 4 times with glycine (0.1M, pH 2.5), and twice with PBS and kept at 4°C.

3.4 Mass spectrometry analysis

For mass spectrometric analysis, the co-immunoprecipitated proteins were visualized by staining with Coomassie blue or silver. They were then excised from the gel, digested with trypsin, and analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and/or liquid chromatography-mass spectrometry (LC/MS) analyses. Proteins that are visible by Coomassie staining are usually in sufficient quantity for identification by mass spectrometry, whereas those that can only be detected by silver-staining may not be identified consistently.

The entire immunoprecipitate can also be analyzed by mass spectrometry without fractionation by SDS-PAGE. This alternative has some advantages. For example, it may reveal all polypeptides that associate with the target protein in a single experiment. This feature is particularly desirable for some polypeptides that are difficult to stain. However, one disadvantage of the method is that it cannot conclusively identify individual polypeptides on

the SDS-gel. In addition, if the immunoprecipitate is contaminated by other polypeptides, they may obscure identification of the target protein and its associated complex. Furthermore, if the amount of target protein in the mixture is too high, which usually happens for epitope-tagged protein, the identification of low-abundance associated proteins would be difficult.

It should be emphasized that all antibodies have some degree of cross-reactivity, and they frequently co-immunoprecipitate cross-reactive polypeptides in IP. In our hands, a number of polypeptides frequently contaminate IP from HeLa nuclear extract (Table 1). These contaminants not only include cytoskeletal proteins (actin, myosin, and tubulin), but also protein chaperones, RNA-interacting proteins, ribosomal proteins, and polypeptides involved in transcription (BCLAF1, TAF15, MYCBP, NONO), RNA splicing, chromatin-remodeling (PRMT5), and DNA repair (TOP2A, PRKDC, RIF1). If one finds proteins listed in Table 1 in an IP, one should proceed carefully and perform additional experiments to determine whether these proteins are not contaminants. It should be emphasized that proteins on this list could nevertheless be components of a protein complex. For example, actin and actin-related proteins are components of SWI/SNF and other chromatin remodeling complexes. One should not overlook these proteins just because they are common contaminants in IP with many different antibodies.

3.5 Superose 6 gel-filtration chromatography

Superose 6 gel-filtration chromatography can be used both in analysis and in purification of a target protein and its associated complexes. This method fractionates proteins based on their size, and has been widely used to estimate the molecular weight of the endogenous globular proteins. We normally fractionate HeLa extracts before IP to determine if the target protein in the extract fractionates as part of a high molecular weight complex. For example, the calculated molecular weight of BLM is about 180 kDa, whereas its endogenous form in HeLa extract fractionates as a complex of 1 MDa by Superose 6 column (Fig. 2A), suggesting that BLM is likely present in a large complex. In contrast, if the calculated molecular weight matches that determined by Superose 6 column, the protein may not form a stable complex with other proteins in HeLa extract. In the latter case, one is unlikely to find a complex associating with the target protein by standard IP.

Superose 6 chromatography can also reveal whether a target protein is in one or several complexes. If the target protein fractionates in several peaks, the protein may be present in multiple complexes, with each peak corresponding to a unique complex. To purify each complex, one may need to do IP using each peak fraction. For example, RMI1, RMI2 and topoisomerase 3a in HeLa extract fractionate in two major peaks, suggesting that they are present in two different complexes (Fig. 2A). Co-IP from each peak obtained two complexes, one containing BLM and the other one not (Fig. 2B).

Superose 6 chromatography can also be used as a purification step to improve IP when direct IP from nuclear extract fails to achieve desired purity. The purity of IP should be considered as unsatisfactory when silver-staining analysis of the immunoprecipitate reveals the presence of hundreds of polypeptides, or when the target protein is a minor polypeptide. In this regard, Gel-filtration columns have an advantage over other columns (Ion exchange, for example) because they can be run using mild salt and detergent buffers, so that the potential target complex remains intact.

Superose 6 columns can be used to verify if two proteins are components of the same complex. If the column profiles of two proteins are coincident, the two proteins may well be components of the same complex. In contrast, if peak fractions of the two proteins are completely separated on the Superose 6, the majority of the two proteins are certainly not present in the same complex.

Here is a common protocol that we use for Superose 6 column fractionation of nuclear extract.

We control the Superose 6 column with an FPLC system (AKTA Purifier, Amersham/GE). The column should be calibrated with proteins of known molecular weight to ensure that it works properly and has good resolution. For purification purpose, the HeLa nuclear extract (up to 2 ml) was cleaned of debris by ultracentrifugation and filtering. It was then applied to a Superose-6 column (HR 16/50; Amersham) equilibrated with the IP buffer described above at 0.3–0.5ml/min. Fractions were collected (1.5 ml each) and analyzed by SDS-PAGE and immunoblotting. The peak fractions containing the target protein were collected, and used for immunoprecipitation as described above. For analytical purpose, one can fractionate HeLa nuclear extract (up to 200 μ l) through a smaller Superose 6 column (HR 10/30). Fractions (0.5 ml) were collected and analyzed by immunoblotting.

3.6 Co-IP using epitope-tagged protein

Sometimes one cannot generate high quality antibodies against a target protein for IP purpose. One way to circumvent this problem is to link the target protein with one or more epitope tags. One can then express the tagged protein in mammalian cell lines (such as HeLa), and use the antibody against the tag to purify the target complex. One can find detailed information regarding expression vectors and protocols in a previous publication (12).

There are many affinity tags to choose from, including Myc, Flag, His (6xHistidine), TAP, and HA. Our lab frequently uses Flag and HA tags, which are small and less likely to interfere with assembly of a target protein into a complex. Both antibodies are commercially available (Sigma and Upstate). We sometimes link two tags on a target protein (Flag and 6xHis), which allows affinity purification of the target protein using both tags, resulting in better purification than that with a single tag (Ling et al. 2007).

One example of purification by Flag antibody is shown in Figure 1C. In this case, antibody against RMI2 is not specific enough for affinity purification purpose (data now shown). We therefore linked RMI2 to Flag and 6xHistidine tags, and stably expressed the tagged RMI2 in HeLa cells. IP with the Flag antibody obtained Flag-tagged RMI2, BLM, Topoisomerase 3a, and RMI1. All these proteins are identified by mass spectrometry and immunoblotting (data not shown). We also included a mock IP from a HeLa extract that lacks tagged RMI2. This allows us to distinguish the BLM complex components from contaminating proteins that were isolated due to cross reactivity with the Flag antibody.

Compared to the BLM complex immunoprecipitated by using antibodies against endogenous proteins, the complex isolated by the Flag antibody is missing RPA (compare Fig. 1B and 1C; data not shown). Therefore, one may not be able to obtain the entire complex using the tagged-protein approach.

4. Verification of protein association

After the target protein and its co-immunoprecipitated polypeptides are identified by mass spectrometry, it is imperative to perform subsequent experiments to verify if their association is real and functionally relevant. This is because non-specific proteins can sometimes be co-immunoprecipitated, due to antibody crossreactivity. We suggest the following experiments for verification purpose. First, we often perform additional co-IP experiments using antibodies against different regions of the target protein. If different antibodies isolate the same protein (s), such proteins are less likely to be contaminants. Second, we always perform reciprocal IP—immunoprecipitation using antibody against newly identified proteins. If the reciprocal IP yields the original target protein, based on silver-staining and mass spectrometry analysis, the data provide compelling evidence that these proteins are components of a single complex.

Third, we often perform Superose 6 gel-filtration chromatography to analyze if the newly identified proteins co-fractionate with the original target protein in the extract (see Section 3.5 above). Fourth, we often do co-IP in the presence of ethidium bromide (100 $\mu\text{g/ml}$) to rule out the possibility that association between the proteins is due to their interactions with DNA.

5. Functional analysis of newly identified components

The experiments above can only provide evidence for physical association between these proteins. Functional studies are necessary to determine if such association occurs in cells, and whether it is biologically relevant.

5.1 Study protein co-stabilization—an indicator for protein association in vivo

In cells, proteins that constitute the same complex often depend on one another for their stability. When one component of the complex is removed, either by siRNA depletion or by genetic mutation, its partners often lose their stability and show reduced levels in cells, which can be detected by immunoblotting. One reason could be that proteins need to interact with their partners within the complex for proper folding and compartmentalization. Without their partners, proteins may fold improperly and become targets for rapid degradation by proteosomes. This feature of protein co-stabilization can be used to judge whether two or more proteins are components of the same complex in vivo. For example, when the RMI2 subunit of the BLM complex is depleted by siRNA or inactivated by knockout in chicken DT40 cells, the other components of the BLM complex (BLM, RMI1, and Topo 3 α) show reduced levels in cells as measured by immunoblotting (7). Similarly, when FANCB component of the FA core complex is depleted by siRNA or by genetic mutations, some of the other FA core complex components (FANCL and FAAP100) show reduced stability (9,13). The data provide further evidence for association between these proteins in vivo.

As siRNA depletion is easier and faster than gene knockout, it is preferable to investigate protein co-stabilization by siRNA. We normally choose ON-TARGET $plus$ SMARTpool siRNA oligos (Dharmacon Inc.), which have reduced off-target effects and satisfactory depletion efficiency. The siRNA knockdown procedure using Oligofectamine follows manufacturer's instructions (Invitrogen) with minor modification:

- a. HeLa cells (about 4×10^5) were plated in 6 cm cell culture dish one day prior to siRNA transfection. The cells should reach 20–30% confluency at the time of transfection.
- b. Oligofectamine (8 μl) was mixed with of Opti-MEM I medium (22 μl), and incubated at room temperature for 5–10 min.
- c. Control or gene-targeting siRNA oligos (400 pmol) were added to 370 μl of Opti-MEM I.
- d. The mixtures in b and c were combined and incubated for 15–20 min at room temperature to allow formation of complexes between siRNA oligoes and Oligofectamine.
- e. The cells were removed of its medium, washed once with 2 ml of Opti-MEM I. Opti-MEM I medium (1.6 ml) was then added to the cells.
- f. The siRNA mixture in d (400 μl) was added to each dish (the final concentration of siRNA is 200 nM), and incubated with cells for 4 hours at 37°C. New medium (1 ml) containing 30% FBS was then added to cells.
- g. Cells were grown for 48 to 72 hours before harvest. Cells were lysed either by SDS-gel loading buffer or 1x RIPA buffer. The cell lysate was then analyzed by

immunoblotting to determine the efficiency of depletion. We normally achieve 80–90% of depletion of the target protein.

5.2 DNA-damage drug sensitivity assay

Cells deficient in BLM and FA proteins show hypersensitivity to several DNA damaging drugs: BLM-deficient cells are sensitive to MMS, whereas FA cells are highly sensitive to DNA-crosslinking reagents, such as MMC (mitomycin C), cisplatin and DEB (diepoxybutane). In fact, MMC-sensitivity assay has been used as a diagnostic test for FA patients. Thus, a quick way to assess if a protein is involved in the FA-associated DNA damage response pathway is to perform MMC sensitivity assay for HeLa cells depleted of the target protein (10). Briefly,

- a. HeLa cells were transfected with either siRNA target oligo or a control oligo, and grown for 60 hours.
- b. The cells were then plated at a density of 500 cells/10 cm dish and grown for 8 hours.
- c. DNA damaging drugs were then added to the cells at different concentration either in duplicates or triplicates. We normally use MMC at final concentration up to 1000 ng/ml, and cisplatin up to 10 μ M.
- d. Cells were grown in drug-containing media for 2 hours. The media was then washed away with PBS, followed by addition of fresh medium.
- e. Cells were grown for 9 to 12 days until the colonies are visible. The colonies are then washed with PBS and stained with Crystal Violet solution (Sigma).

5.3 Sister-chromatid exchange (SCE) assay

The hallmark feature of Bloom syndrome cells is elevated SCE levels, typically about 10-fold higher than those in normal cells (1,17). Increased SCE levels have also been observed in HeLa cells depleted of RMI1 and chicken DT40 cells inactivated of RMI2, suggesting that the entire BLM complex is involved in suppressing SCE (6,7). Although DNA damage hypersensitivity has been observed in some BLM-deficient cells, this feature is absent in BLM mutant cells from some species. Thus, the SCE assay is the most reliable way to determine whether a new protein is important for BLM function.

Although SCE assays have been performed in HeLa cells depleted of BLM or RMI1, only a two-fold increase of SCE level has been observed. This is in contrast to a 10-fold increase of SCE in BLM patient cells, and 6-fold increase in chicken DT40 cells with inactivated RMI2. This difference is likely due to the inability of siRNA to completely remove endogenous BLM or RMI1; the residual amount of protein can partially suppresses SCE. Therefore, we recommend performing this assay using cells in which the candidate gene is inactivated. The chicken DT40 cell line is particularly suitable for this purpose, because one can readily knock out the candidate gene and examine its effect on the SCE level. Both BLM and Topoisomerase 3a have been inactivated in this cell line, and the analyses of their phenotypes have provided important insight to the function of the BLM complex. Our group has inactivated RMI2 in this cell line, and found that RMI2 suppresses SCE through the same pathway as BLM. Protocols for inactivating genes in DT40 cells can be found elsewhere (18). Here we describe the SCE assay used in our group for DT40 cells, which is modified based on previous publications (19,20). One can also adapt this protocol to other cells.

- a. DT40 cells were cultured in T25 flask containing RPMI medium supplemented with 10 % FBS, 1% chicken serum, 10 mM Hepes and 1% Penicillin/Streptomycin. Cells were grown at 39.5°C, in 5% CO₂, until reaching density of 0.5–1 million cells/ml.

- b.** One million cells were incubated in 10 ml fresh medium containing 10 μ M BrdU for 2 cell cycles (about 16 to 18 hours for wild type cells). The time of incubation is critical, as longer growth period causes insufficient staining of sister chromatids, whereas shorter period results in over-staining of both sister chromatids, both of which will prevent distinction of SCE.
- c.** Cells were pulsed by adding colcemid (0.1 μ g/ml) to the medium and allowed to grow for the last 2 hours of BrdU treatment.
- d.** Cells were collected by centrifugation (1000 rpm, 5 min), resuspended in 2 ml of KCl (75 mM), and incubated for 15–30 min at room temperature.
- e.** Cells were fixed by adding 5 ml of fixing solution (methanol/acetic acid mixture, ratio 3:1), and collected by centrifugation. They were then resuspended in 5 ml of new fixing buffer, and incubated for 30 min at room temperature.
- f.** Cells were collected by centrifugation, and resuspended in 100–200 μ l of fixing buffer, and dropped onto 50% ethanol pre-wet slide from 1 feet height. The slides were aged for 1–2 days before staining.
- g.** The slides were soaked for 5 min in 50 mM phosphate buffer (pH 6.8), and stained for 20 min at room temperature using Hoechst 33258 dye (10 μ g/ml in 50 mM phosphate buffer, pH 6.8)
- h.** The slides were then rinsed in Macllvaine solution (164 mM Na_2HPO_4 , pH 7.0 and 16 mM citric acid), covered with coverslips, and exposed to UV light (UVP, 15watt, 365 nm) for 30–60 min at a distance approximately 1 inch from the bulb. The time of exposure is critical, as shorter time makes sister chromatids indistinguishable, whereas longer exposure prevents one sister chromatid from staining by Giemsa.
- i.** The slides were removed of their coverslips and incubated in 2 \times SSC solution (0.3M NaCl, 0.03M Sodium Citrate) at 62°C for 1 hour. They were rinsed once with 50 mM phosphate buffer (pH 6.8), and stained with 3% Giemsa in 50mM phosphate solution (pH 6.8) for 20 min. Afterward, they were rinsed with water and air dried.
- j.** The slides were mounted in EUKITT mounting fluid and then can be viewed with a microscope (100 \times objective lens). As SCE is counted by individual scientist, the results are subject to human errors. To reduce human bias, we strongly recommend doing this experiment in a double-blind way: one researcher marks the slides with codes (to hide the genotypes of cells), whereas the second researcher counts the SCEs. At least 50 metaphase cells should be counted.

6. Concluding remarks

Co-IP has been successfully used in several groups to identify components of the BLM and Fanconi anemia complexes. Several components of these complexes have been shown to play critical roles in mediating function of BLM and FA proteins in DNA damage response. A few of them have also been found mutated in patients with the same disease. Therefore, this approach is a powerful way to study DNA damage response pathways, and could be applied to study proteins involved in other vital cellular processes. However, co-IP may not be able to isolate and identify all the proteins that mediate BLM and FA protein functions particularly those that have transient and weak interactions. Other methods, such as 2-hybrid screening or crosslinking associated proteins prior to co-IP by protein crosslinkers (for example DSP), may be used to identify such proteins.

Identification of proteins by co-IP is just the first step in elucidating their mechanism. Further experiments (reciprocal-IP and Superose 6 chromatography) are necessary to exclude the

possibility that they are contaminants in the co-IP. Functional analysis (by siRNA or by genetic knockout) can provide evidence of whether these proteins act in the same pathway as BLM and FA proteins. Immunofluorescence analyses can reveal whether these proteins co-localize with BLM or FA proteins in DNA damage sites in cells (focus formation). And biochemical experiments can show whether these proteins can stimulate the activity of BLM and FA proteins in vitro. A range of approaches (some of them not described here) can facilitate our understanding of the mechanism of action of BLM and FA complexes.

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References

1. German J. *Medicine* (Baltimore) 1993;72:393–406. [PubMed: 8231788]
2. Ellis NA, Groden J, Ye TZ, Straughen J, Lennon DJ, Ciocci S, Proytcheva M, German J. *Cell* 1995;83:655–66. [PubMed: 7585968]
3. Joenje H, Patel KJ. *Nat Rev Genet* 2001;2:446–57. [PubMed: 11389461]
4. Wang W. *Nat Rev Genet* 2007;8:735–48. [PubMed: 17768402]
5. Meetei AR, Sechi S, Wallisch M, Yang D, Young MK, Joenje H, Hoatlin ME, Wang W. *Mol Cell Biol* 2003;23:3417–26. [PubMed: 12724401]
6. Yin J, Soback A, Xu C, Meetei AR, Hoatlin M, Li L, Wang W. *EMBO J* 2005;24:1465–76. [PubMed: 15775963]
7. Xu D, Guo R, Soback A, Bachrati CZ, Yang J, Enomoto T, Brown GW, Hoatlin ME, Hickson ID, Wang W. *Genes Dev* 2008;22:2843–55. [PubMed: 18923082]
8. Meetei AR, de Winter JP, Medhurst AL, Wallisch M, Waisfisz Q, van de Vrugt HJ, Oostra AB, Yan Z, Ling C, Bishop CE, Hoatlin ME, Joenje H, Wang W. *Nat Genet* 2003;35:165–70. [PubMed: 12973351]
9. Meetei AR, Levitus M, Xue Y, Medhurst AL, Zwaan M, Ling C, Rooimans MA, Bier P, Hoatlin M, Pals G, de Winter JP, Wang W, Joenje H. *Nat Genet* 2004;36:1219–24. [PubMed: 15502827]
10. Meetei AR, Medhurst AL, Ling C, Xue Y, Singh TR, Bier P, Steltenpool J, Stone S, Dokal I, Mathew CG, Hoatlin M, Joenje H, de Winter JP, Wang W. *Nat Genet* 2005;37:958–63. [PubMed: 16116422]
11. Xia B, Sheng Q, Nakanishi K, Ohashi A, Wu J, Christ N, Liu X, Jasin M, Couch FJ, Livingston DM. *Mol Cell* 2006;22:719–29. [PubMed: 16793542]
12. Chi T, Yan Z, Xue Y, Wang W. *Methods Enzymol* 2004;377:299–316. [PubMed: 14979033]
13. Ling C, Ishiai M, Ali AM, Medhurst AL, Neveling K, Kalb R, Yan Z, Xue Y, Oostra AB, Auerbach AD, Hoatlin ME, Schindler D, Joenje H, de Winter JP, Takata M, Meetei AR, Wang W. *EMBO J* 2007;26:2104–14. [PubMed: 17396147]
14. Dignam JD, Martin PL, Shastray BS, Roeder RG. *Methods Enzymol* 1983;101:582–98. [PubMed: 6888276]
15. Reyes JC, Muchardt C, Yaniv M. *J Cell Biol* 1997;137:263–74. [PubMed: 9128241]
16. Harlow, E.; Lane, D. Cold Spring Harbor Laboratory; New York: 1988.
17. Chaganti RS, Schonberg S, German J. *Proc Natl Acad Sci U S A* 1974;71:4508–12. [PubMed: 4140506]
18. Lahti JM. *Methods* 1999;17:305–12. [PubMed: 10196101]
19. Wang W, Seki M, Narita Y, Sonoda E, Takeda S, Yamada K, Masuko T, Katada T, Enomoto T. *EMBO J* 2000;19:3428–35. [PubMed: 10880455]

20. Seki M, Nakagawa T, Seki T, Kato G, Tada S, Takahashi Y, Yoshimura A, Kobayashi T, Aoki A, Otsuki M, Habermann FA, Tanabe H, Ishii Y, Enomoto T. *Mol Cell Biol* 2006;26:6299–307. [PubMed: 16880537]

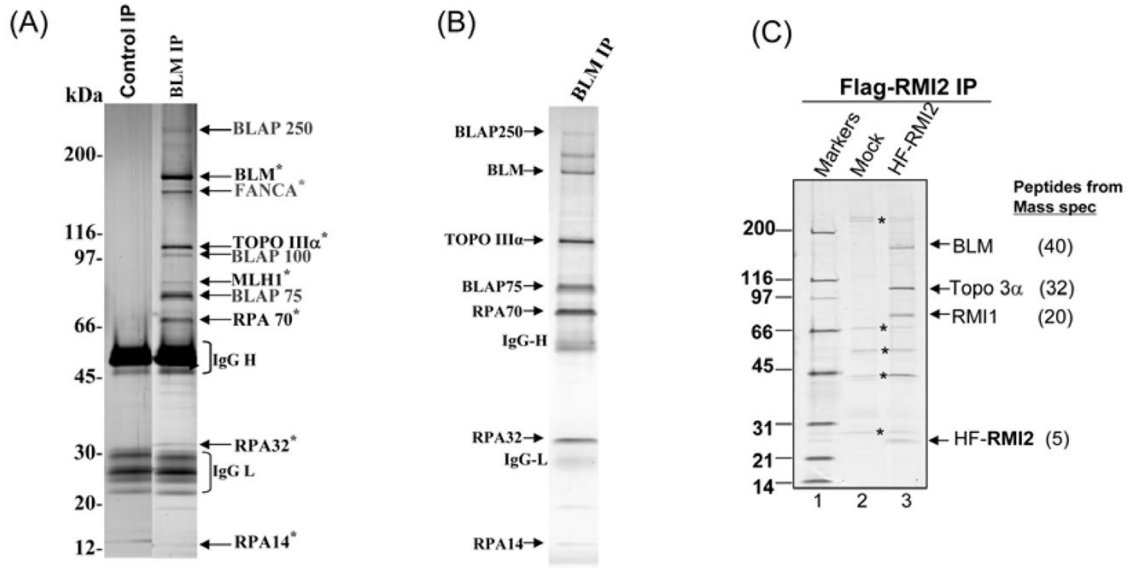


Figure 1. Immunoprecipitation of the BLM complex by different methods

(A)(B) silver-stained SDS gels showing the BLM complex immunoprecipitated by a polyclonal BLM antibody that was uncrosslinked (A) or crosslinked (B) to protein A beads by DMP. The complex was eluted from beads using 0.1 M Glycine (pH 2.5). Different BLM complex components are indicated by arrows. Notably, the IgG heavy and light chains are present in large quantity in A but not B. (C) A silver-stained SDS-gel showing polypeptides immunoprecipitated by Flag antibody against RMI2 that is linked to both Flag and 6xHistidine tags. A mock IP was performed to show contaminating polypeptides isolated due to crossreactivity with Flag antibody. Notably, RPA was missing in the co-IP, suggesting that the tagged approach may not isolate the full BLM complex. These figures were all reproduced from previous publications: Fig. 1A was from Figure 1A of reference (5); Fig. 1B was from Figure 1A, lane 1, of reference (6); and Fig. 1C was from Fig. 1C of reference (7).

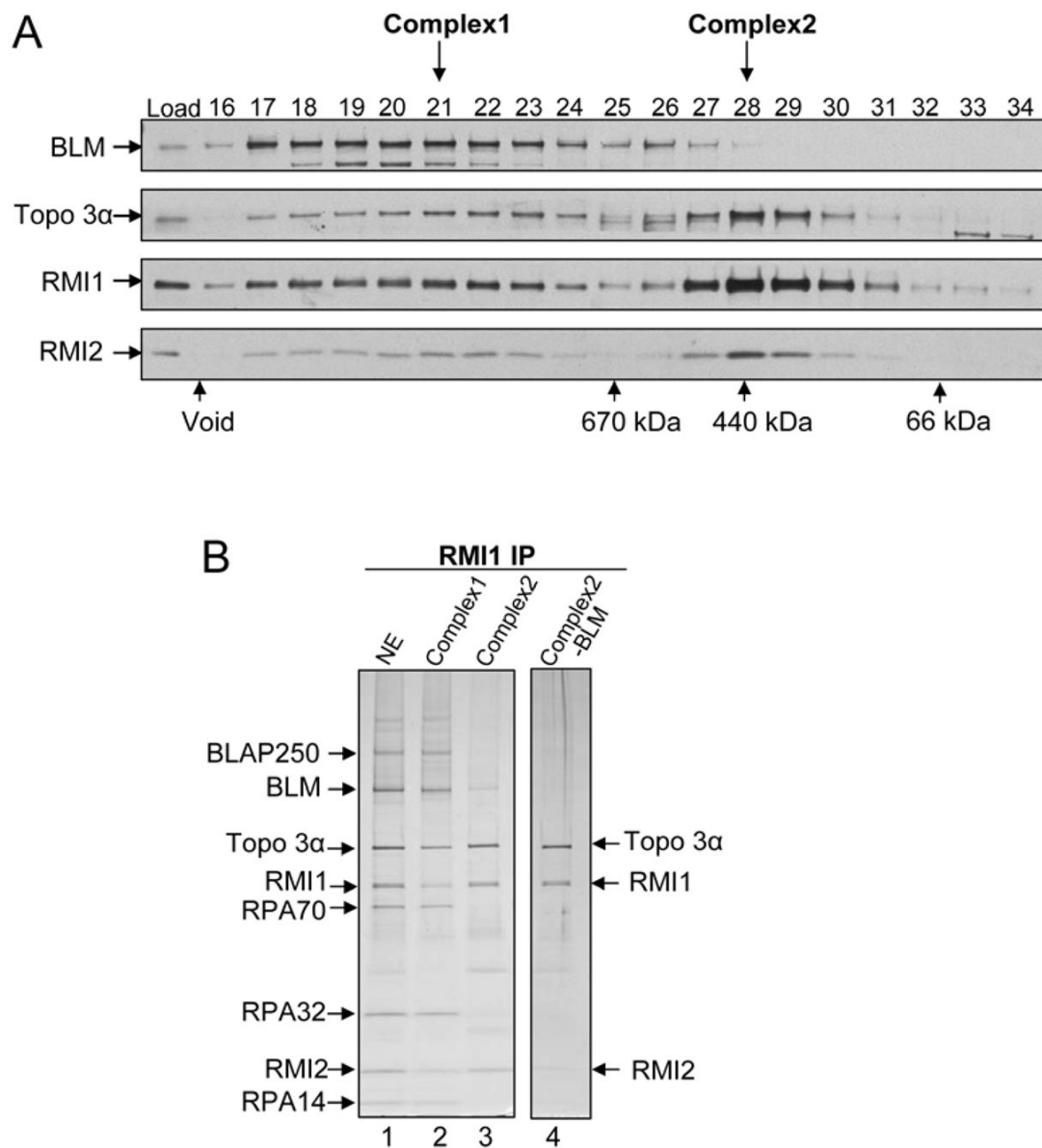


Figure 2. Superose 6 chromatography fractionates RMI into two complexes: one contains BLM and the other one does not

(A) Immunoblotting shows that RMI2 in HeLa nuclear extract fractionates in 2 major peaks by Superose 6 gel-filtration chromatography, which correspond to two complexes designated as Complex 1 and 2 (marked by arrows on top). Notably, the elution profiles of RMI2, RMI1 and Topo 3α are coincident, suggesting that they always associate in complexes. In addition, BLM overlaps with the peak of complex 1 but not complex 2, suggesting that BLM is absent in complex 2. (B) Silver-stained SDS gels show that while Complex 1 contains all BLM complex components, complex 2 consists of only Topo 3 α, RMI1 and RMI2.

Immunoprecipitation was done by using RMI1 antibody from crude nuclear extract (NE), Superose 6 fractions containing either Complex 1 or Complex 2, or Complex 2-containing fractions after immuno-depletion of BLM (Complex 2-BLM), as indicated on top of the figure. Notably, RMI1 co-immunoprecipitated with Topo 3 α and RMI2 but not BLM (lane 4 in B), indicating that the two RMI proteins and Topo 3α form a stable subcomplex. Figures 2A and

2B were reproduced from Supplementary Figures 1A and 1B, respectively, of a previous publication(7).

Table 1

Common contaminants in immunoprecipitation from HeLa nuclear extract

Proteins identified by mass spectrometry in the immunoprecipitate from HeLa nuclear extract by rabbit polyclonal antibodies against a variety unrelated proteins. Some of these (highlighted by bold characters) are also identified in the immunoprecipitate by Flag antibody from nuclear extract of HeLa cells that do not express any tagged proteins. We believe that these are common contaminants that interact non-specifically with antibodies.

Official Symbol	GeneID	Official Full Name	Official Symbol	GeneID	Official Full Name
ACTA1	58	actin, alpha 1, skeletal muscle	PRKDC	5591	protein kinase, DNA-activated, catalytic polypeptide
ACTA2	59	actin, alpha 2, smooth muscle, aorta	PRMT5	10419	protein arginine methyltransferase 5
ACTBL2	345651	actin, beta-like 2	PRPF31	26121	PRP31 pre-mRNA processing factor 31 homolog (<i>S. cerevisiae</i>)
ACTBL3	440915	actin, beta-like 3	RBM10	8241	RNA binding motif protein 10
ACTG1	71	actin, gamma 1	RBM39	9584	RNA binding motif protein 39
AHNAK	79026	AHNAK nucleoprotein	RBMX	27316	RNA binding motif protein, X-linked
ANXA2	302	annexin A2	RIF1	55183	RAP1 interacting factor homolog (yeast)
BAT2D1	23215	BAT2 domain containing 1	RPL13	6137	ribosomal protein L13
BCLAF1	9774	BCL2-associated transcription factor 1	RPL29	6159	ribosomal protein L29
C11orf84	144097	chromosome 11 open reading frame 84	RPL38	6169	ribosomal protein L38
C1QBP	708	complement component 1, q subcomponent binding protein	RPLP2	6181	ribosomal protein, large, P2
CAPZA1	829	capping protein (actin filament) muscle Z-line, alpha 1	RPS4Y1	6192	ribosomal protein S4, Y-linked 1
CCAR1	55749	cell division cycle and apoptosis regulator 1	RPS8	6202	ribosomal protein S8
CLK3	1198	CDC-like kinase 3	SF3B1	23451	splicing factor 3b, subunit 1, 155kDa
CLNS1A	1207	chloride channel, nucleotide-sensitive, 1A	SF3B2	10992	splicing factor 3b, subunit 2, 145kDa
CMBL	134147	carboxymethylglutaminolase homolog (<i>Pseudomonas</i>)	SF3B3	23450	splicing factor 3b, subunit 3, 130kDa
CORO1C	23603	coronin, actin binding protein, 1C	SF3B4	10262	splicing factor 3b, subunit 4, 49kDa
DDX17	10521	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	SFPQ	6421	splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)
DDX20	11218	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20	SFRS1	6426	splicing factor, arginine/serine-rich 1
DDX3X	1654	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	SFRS10	6434	splicing factor, arginine/serine-rich 10
DDX5	1655	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	SFRS3	6428	splicing factor, arginine/serine-rich 3
DHX15	1665	DEAH (Asp-Glu-Ala-His) box polypeptide 15	SFRS4	6429	splicing factor, arginine/serine-rich 4
DHX9	1660	DEAH (Asp-Glu-Ala-His) box polypeptide 9	SFRS5	6430	splicing factor, arginine/serine-rich 5
DNAJA1	3301	DnaJ (Hsp40) homolog, subfamily A, member 1	SFRS6	6431	splicing factor, arginine/serine-rich 6

Official Symbol	GeneID	Official Full Name	Official Symbol	GeneID	Official Full Name
EEF1A2	1917	eukaryotic translation elongation factor 1 alpha 2	SFRS7	6432	splicing factor, arginine/serine-rich 7, 35kDa
EEF1AL3	158078	eukaryotic translation elongation factor 1 alpha-like 3	SFRS9	8683	splicing factor, arginine/serine-rich 9
EIF4B	1975	eukaryotic translation initiation factor 4B	SLTM	79811	SAFB-like, transcription modulator
FLNA	2316	filamin A, alpha (actin binding protein 280)	SNRP70	6625	small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen)
FMR1	2332	fragile X mental retardation 1	SNRPB	6628	small nuclear ribonucleoprotein polypeptides B and B1
FUS	2521	fusion (involved in t(12;16) in malignant liposarcoma)	SNRPD1	6632	small nuclear ribonucleoprotein D1 polypeptide 16kDa
FUSIP1	10772	FUS interacting protein (serine/arginine-rich) 1	SNRPD3	6634	small nuclear ribonucleoprotein D3 polypeptide 18kDa
FXR1	8087	fragile X mental retardation, autosomal homolog 1	SPATS2	65244	spermatogenesis associated, serine-rich 2
FXR2	9513	fragile X mental retardation, autosomal homolog 2	SPIN1	10927	spindlin 1
GAPDH	2597	glyceraldehyde-3-phosphate dehydrogenase	SPTAN1	6709	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)
GEMIN4	50628	gem (nuclear organelle) associated protein 4	SPTB	6710	spectrin, beta, erythrocytic
HNRNPH1	3187	heterogeneous nuclear ribonucleoprotein H1 (H)	SPTBN1	6711	spectrin, beta, non-erythrocytic 1
HNRNPK	3190	heterogeneous nuclear ribonucleoprotein K	SR140	23350	U2-associated SR140 protein
HNRNPM	4670	heterogeneous nuclear ribonucleoprotein M	STK38	11329	serine/threonine kinase 38
HNRNPR	10236	heterogeneous nuclear ribonucleoprotein R	STK38L	23012	serine/threonine kinase 38 like
HNRNPU	3192	heterogeneous nuclear ribonucleoprotein U	SYNCRIP	10492	synaptotagmin binding, cytoplasmic RNA interacting protein
HSPA1A	3303	heat shock 70kDa protein 1A	TAF15	8148	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa
HSPAIL	3305	heat shock 70kDa protein 1-like	THRAP3	9967	thyroid hormone receptor associated protein 3
HSPA2	3306	heat shock 70kDa protein 2	TOP2A	7153	topoisomerase (DNA) II alpha 170kDa
HSPA5	3309	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	TPM1	7168	tropomyosin 1 (alpha)
HSPA6	3310	heat shock 70kDa protein 6 (HSP70B')	TRA2A	29896	transformer-2 alpha
HSPA8	3312	heat shock 70kDa protein 8	TUBA1C	84790	tubulin, alpha 1c
IGKV2-40	28916	immunoglobulin kappa variable 2-40	TUBA3C	7278	tubulin, alpha 3c
KHDRBS1	10657	KH domain containing, RNA binding, signal transduction associated 1	TUBB	203068	tubulin, beta
KIF11	3832	kinesin family member 11	TUBB1	81027	tubulin, beta 1

Official Symbol	GeneID	Official Full Name	Official Symbol	GeneID	Official Full Name
LMO7	4008	LIM domain 7	TUBB2C	10383	tubulin, beta 2C
MATR3	9782	matrin 3	TUBB4Q	56604	tubulin, beta polypeptide 4, member Q
MYCBP	26292	c-myc binding protein	TUBB6	84617	tubulin, beta 6
MYH10	4628	myosin, heavy chain 10, non-muscle	VIM	7431	vimentin
MYH11	4629	myosin, heavy chain 11, smooth muscle	WDHD1	11169	WD repeat and HMG-box DNA binding protein 1
MYH9	4627	myosin, heavy chain 9, non-muscle	WDR77	79084	WD repeat domain 77
NONO	4841	non-POU domain containing, octamer-binding	YBX1	4904	Y box binding protein 1
PABPC4	8761	poly(A) binding protein, cytoplasmic 4 (inducible form)	YLPM1	56252	YLP motif containing 1
PPP1CC	5501	protein phosphatase 1, catalytic subunit, gamma isoform			