PREPARATION AND CHARACTERIZATION OF BOVINE MEAT AND MILK FACTOR VESICLES/PARTICLES & IDENTIFICATION OF MSBI1 REP INTERACTING PROTEINS

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ABSTRACT:

Introduction: Bovine meat and milk factors (BMMF) frequently isolated from bovine meat and milk, are discussed as putative causative agents for breast, colon and possibly other cancers. The hypothesis was formulated based on earlier correlative studies indicating that BMMFs could indirectly trigger inflammation, accumulate mutational events via reactive oxygen and nitrogen species production which eventually provokes tumor formation.

Purpose: The study aimed to prepare and characterize BMMF particles or vesicles for future characterization and visualization, and identify possible interaction partners of BMMF1 Rep protein with human proteins from related carcinoma cell lines to better understand BMMF functions within the human host.

Methods: A plasmid-based overexpression of MSBI1.176 Rep C-HIS as representative of the intensively studied group of BMMF1 Reps was chosen for particle or vesicle preparations based on Cesium Chloride density gradient centrifugation. Co-immunoprecipitation experiments to detect the interaction of different BMMF1 and BMMF2 proteins were employed to identify possible helper or suppressor effects of different BMMF isolates and the correlated proteins which might be crucial for efficient production of BMMF particles/vesicles. Additional plasmids allowing codon-optimized Rep expression with specific tags were produced through cloning and verified by sequencing analysis. Protein interaction assays were conducted to analyze possible interaction partners of the MBSI1.176 Rep within the human host by pull-down assay based on five different cell lines: monocytes, MCF-7, RKO, Colo678, and HEK293TT cells.

Results: Particle/vesicle preparation experiments indicated that although MSBI1 specific DNA and protein were detected in fractions of high-molecular-weight, no evidence was found that the desired protein is initiating a specific formation of capsomeres or vesicles. Two new plasmids (for overexpression of N- or C-terminally Flag-tagged codon-optimized MSBI1 Rep) were designed and successfully cloned within the study. The newly designed cloned plasmids were suitable for Immunoprecipitation (IP) and Co-immunoprecipitation (Co-IP) experiments indicating the interaction of the MSBI1.176 Rep proteins with each other as a hint for the formation of Rep dimers, oligomers or aggregates. IP/ Co-IP results were optimized by modification of different lysis and washing buffer conditions allowing conduction of future experiments using combinations proteins of isolates to analyze the interaction of proteins of the BMMF1 and BMMF2 group. Importantly, protein interaction experiments based on MSBI1.176 Rep pulldown followed by mass spectrometry for verification of putative Rep interaction partners identified the two proteins RFWD2 and CEP83 as putative Rep interaction partners within all five cell systems tested. The current result indicates the interaction of MSBI1 protein with host proteins, which might have a role in cancer induction. More detailed experiments will have to follow to more specifically determine the BMMF functions crucial for formulation and understanding of preventive strategies of BMMF infection.

Keywords: Bovine meat and milk factors; multiple sclerosis; immunoprecipitation & co-immunoprecipitation; protein dimers; mass spectrometry; immunodetection; cesium chloride density gradient centrifugation; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; vesicle; particle; interaction partner; pull-down assay.

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Popular Summary:

What might be the consequence of Bovine Meat and Milk Consumption?

Commonly, bovine milk and meat are one of the most consumed food sources in most of the parts of the world. Recently, circular single-stranded DNA molecules have been isolated from dairy products, cow's serum and human tissues and serum of multiple sclerosis patients. Based on their frequent isolation from bovine source material those factors were named Bovine Meat and Milk Factors, shortly BMMFs. The epidemiologic analysis is linking the consumption of bovine meat and milk and the incidence of colon and breast cancer. Based on those findings and a possible involvement, a hypothesis was made that BMMFs can specifically trigger inflammation leading to increased levels of local radical formation. This, in turn, might induce random DNA mutations within the nucleic acids of replicating cells. Upon enrichment of mutations in several known cancer driver genes, those cells might transform into cancer progenitor cells inducing e.g. polyps as precursors for colon cancer. BMMFs might function as indirect carcinogens in colon and breast cancer and possible in a large set of additional chronic diseases linked to chronic inflammatory processes.

BMMFs might represent a new class of zoonotic infectious agents, which might be classified somewhere between bacterial plasmids and viruses. It is relatively unlikely, that a naked DNA is entering and circulating within the body as the bare genetic material. Instead, the BMMFs might be coated with associated protein. Exposure of humans towards BMMF DNA and proteins has been shown, but an association of BMMF DNA and proteins need to be investigated specifically. Therefore, traditional and established virus-like vesicle or particle preparations through the CsCl density gradient centrifugation method were performed. Unfortunately, we could not verify the specific formation of BMMF particles or vesicles due to high structural diversity and in general, very low levels of protein and DNA were detected. As the formation of infectious particles/vesicles might rely on the interaction of several BMMF proteins for the construction of larger functional assemblies, interaction studies of different BMMF proteins were performed.

It is known that a majority of pathogens rely on the interaction of the pathogenic agent with host proteins thus affecting or modulating specific host regulatory mechanisms like e.g. signaling or defense pathways. Protein pull-down assays for the identification of putative Rep interaction partners were performed within several human cell systems. Once we find any interaction and help-effect from Immunoprecipitation and Co-immunoprecipitation experiments, complementation experiments can be performed to generate the production of biologically active, infectious particles or vesicles preparation. The identification of interacting protein partners from Rep pulldown and Mass Spectrometry data will help to unravel the possible function of BMMF protein within the host to better understand the possible role of BMMFs in cancer induction.

The biggest challenge of this study was the optimization of biomolecular protocols. Different options were tested for the detection of BMMF proteins using different Flag, HIS epitope tags at both termini of the cloned BMMF proteins. In this approach, we considered the Multiple Sclerosis Bovine Isolate, shortly MSBI1.176 within BMMF1 group 1 out of more than 100

different BMMF samples, because MSBI1.176 showed active replication, transcription as well as productive protein translation in human cells, before. The MSBI1.176-encoded Rep is characterized as a putative replication initiator protein with the potential of initiating replication amongst additional other roles. The MSBI1.176 genome itself was isolated from the brain of Multiple sclerosis patients but was recently also isolated from the peritumoral tissue of colon cancer patients.

Upon examining the underlying protein interactions, we would have a more complete view on physiologically prevalent putative vesicle- or particle-like BMMF structures. This will further expand our knowledge on the role of BMMFs in neurodegeneration and cancer induction. Most interestingly, we identified a significant interaction of two cellular proteins (RFWD2 and CEP83) with MSBI1.176 Rep protein observed within different human cell lines within protein-protein interaction assay based on Rep-protein pulldown. Such systemic interactions might hint for a specific function of the BMMF protein and might play a crucial role in cancer induction.

We aim to further modify our screenings of BMMF protein-interactions using the IP & Co-IP method. Upon identification of further BMMF or host cell interaction partners, it might be more realistic to prepare infectious viral particles or vesicles. This research project might significantly contribute to a deeper understanding of potential BMMF activity and BMMF infection.

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INTRODUCTION

Bovine milk and meat factors (BMMFs)

Cattle constitute the greatest portion of animal husbandry worldwide due to the high consumption of bovine milk and meat. All over the world, the steady growth of the global population and the income level has generated a higher demand in the consumption of milk and meat as the source of protein, vitamins, and micro-nutrients (Speedy, A. W., 2003). In general, infants depend on the milk as the primary source of nutrients until their digestive system develops to uptake other food sources. After a certain period of nursing, the human child is subjected mostly to consume bovine milk directly or in a powdered form for nutrition. Recently, replication-competent episomal circular single-stranded DNA molecules named Bovine Meat and Milk Factors (BMMFs) were isolated from cow milk, blood and Multiple Sclerosis (MS) tissue samples and might represent a new group of possible infectious pathogens (zur Hausen, H., Bund, T., & de Villiers, E.-M., 2018). Episome is an extrachromosomal genetic element that can integrate with chromosomal DNA. Most of the BMMFs have shown high sequence identity with bacterial plasmids *(Acinetobacter baumanii)* so that BMMF might represent a new class of agents with both bacterial as well as viral characteristics. All identified BMMF genomes contain a conserved gene encoding a putative replication protein (Rep) (de Villiers et al, E.-M., 2019). The Rep gene has a very high sequence identity to bacterial replication initiation genes (de Villiers et al, E.-M., 2019). The protein structure of a part of the Rep of the BMMF1 plasmid MSBI1.176 was resolved experimentally and showed a high structure similarity to replication initiator proteins of different bacterial origins (Kilic T., 2019; Villiers, E-M, et al., 2019). Some of BMMFs contain additional open reading frames.

Epidemiological data showed that colon, breast, prostate and a set of additional cancers incidences are increased with higher levels of red meat and dairy products consumption (Alisson-Silva F, Kawanishi K, Varki A, 2016; zur Hausen, H, Bund, T, Villiers, E-M, 2017). The group of Prof. Harald Zur Hausen hypothesized a specific relationship of these types of cancer to infections through meat and milk consumption obtained from Aurochs-derived Eurasian dairy cattle. The global epidemiological patterns of colon and breast cancer incidence point to a close relation to the consumption of products originating from these specific breeds of cattle (zur Hausen, H, Villiers, E-M., 2015). It is postulated that a zoonotic infectious agent might be causatively associated with the pathogenesis of around 30-50% of all cancer cases worldwide.

The epidemiological associations stimulated the initiation of experiments to identify such infectious agents within bovine material. This led to the identification of virus-like circular single-stranded DNA isolates, which are potentially playing a role in the pathogenesis of chronic neurodegenerative disorders and certain cancers by indirect cancerogenesis. The group of Prof. H. zur Hausen identified a set of isolates which were found in a dairy products (milk, yogurt and crème fraiche), cattle serum and cancer/multiple sclerosis tissue samples (Whitley C al.,2014; Funk et al.,2014; Gunst, K et al., 2014; Lamberto, I et al.,2014; Falida, K et al., 2017). Sequence analysis of BMMFs revealed a high DNA sequence identity with the transmissible spongiform encephalopathy-associated circular single-stranded DNA isolates Sphinx 1.76 and Sphinx 2.36. Based on these data the individual BMMFs were classified into four groups – starting from BMMF1 to BMMF4. Interestingly, the RepA-coding sequence of Sphinx 2.36 has an absolute sequence identity to the double-stranded origin of replication (DSO) of *A. baumanii* (Longkumer T. et al, 2013). All members of BMMFs are small single-

stranded DNAs with a size range of 1500 to 3000 nucleotides. Some representative isolates of BMMF1, for example, includes Multiple Sclerosis biopsy isolates 1 and 2 (MSBI1.176 and MSBI2.176, correspondingly) and Cow Milk Isolate 1 (CMI1.252). For BMMF2, some of the representative isolates include Healthy Cow Blood Isolate 1/ 2 and 7 (HCBI1.225, HCBI2.170 and HCBI7.228, correspondingly). Interestingly, BMMF3 isolates share similarity with Gemycircularviruses which include Multiple Sclerosis Serum Isolate 2 (MSSI2.225) and Healthy Cow Blood Isolate 8/ 9 (HCBI8.215, HCBI9.212, correspondingly). BMMF group 4 consists of an isolate MSSI1.162 isolated from Multiple Sclerosis serum and featured similarity to a Psychrobacter plasmid. Even though initially there were only around 20 different BMMFs isolated, in total, now the number increased to more than 100 different isolates (Villiers, E-M, et al., 2019).

All BMMF1 genomes include an open reading frame encoding a replication initiator protein and an iteron-like tandem repeat region. Moreover, BMMF1 isolates constitute a 12 nucleotides conserved core palindromic sequence 5′-TAAATGCTTTTA-3′ located 41-52 nucleotide upstream of the repeat region, which functions as the putative origin of replication and might have the role in replication initiation (Eilebrecht, S et al., 2018). The replication proteins identified within BMMF1 isolates are similar to the replication initiator proteins of specific bacterial plasmids (zur Hausen, H, Bund, T, Villiers, E-M. 2017). The iteron-like repeat region might encompass binding sites for the replication initiator protein for controlling plasmid copy number (Chattoraj, 2000).

In packaging proteins, capsid conformation-dependent Rep-capsid interactions may play a crucial role. Adeno-associated viral Rep proteins encoded Rep gene is found to have an additional role in encapsidation and gene expression (Chejanovsky, N et al., 1989; Muzyczka, N et al., 2001). In the report of King J.A et al (2001), it was proved that helicase activity of the small Rep proteins is needed for efficient packaging of single-stranded Adeno-associated Virus-2 DNA genome. Current project is aimed to reveal whether BMMF Rep proteins are associated with encapsidation of the BMMF Rep genome. Once identified, it would be further possible to know about the mechanism of Rep proteins in DNA packaging function.

Bioactivity of BMMFs

To test the exposure of humans towards BMMFs with the concomitant production of antibodies, BMMF blood tests were performed. The serological assays of human serum from different individuals showed significant titers of Rep-specific antibodies reacting with the BMMF1 Rep antigen. This indicates the exposure of human individuals to BMMFs and subsequent immune reaction inducing production of BMMF-specific antibodies. In asymptomatic healthy donors, broad seropositivity and high titers of anti-Rep reactive antibodies were measured which suggests that BMMFs may infect the majority of the human population (Eilebrecht, S et al., 2018).

The bioactivity of BMMFs in human cells was verified experimentally by showing BMMF replication as well as RNA transcription and protein expression of BMMF genes in a set of human cell lines. Based on BMMF transfections cloned as plasmids in human embryonic kidney cells (HEK293TT), it was shown that MSBI1.176 together with CMI3.168 had the highest transcription rates within the BMMF1 group. Replication of MSBI1 genome over periods of several days was observed for MSBI1.176-transfected HEK293TT cells in several repetitive experiments (Eilebrecht, S et al., 2018).

Detection of BMMFs in cancer Tissue

In several studies, the consumption of bovine meat and milk is reported to be associated with cancers like colon and breast cancers (Lippi et al., 2016; Farvid et al., 2015; Zur Hausen et al, E.-M., 2017). This inspired a set of experiments based on human cancer samples. For the detection of BMMF Rep protein in tissue samples from cancer patients, mouse monoclonal antibodies were designed and tested in several applications like e.g. Enzyme-Linked Immunosorbent Assay (ELISA), Western Blot assay (WB), Immunoprecipitation (IP), Immunohistochemistry (IHC) and Immunofluorescence (IF). Positive staining has been observed for a set of peritumoral and tumoral tissue sections from colon cancer tissue based on immunohistochemistry (Bund, T, et al., unpublished).
Based on these correlations and proofs, an indirect cancer hypothesis is being formulated.

Indirect Cancer Hypothesis

BMMFs, most likely taken up very early in life, are supposed to persist for a longer period within the colon interstitium (*lamina propria*) between the colon crypts under conditions of immunotolerance. Localization of BMMF proteins and/ or DNA within macrophages was shown (de Villiers et al. 2019) and is hypothesized to induce increased macrophage levels within large colonic interstitial areas contributing to local chronic inflammation. This might finally result in higher production rates and accumulation of reactive oxygen species (ROS) and nitrogen-oxygen species (NOS). ROS/NOS is known to induce oxidative changes in protein, DNA and RNA within the tissue cells, which, upon diffusion, might also induce an increased level of DNA mutations within replicating cells, which might be represented by epithelial stem cells and early daughter cells within the colon crypts (zur Hausen et al. 2018). This oxidative stress might fuel further inflammation within a feed-back loop (Canli et al. 2017 Cancer Cell). The replicating crypt cells might acquire more and more random DNA mutations within a stochastic process. At some point, when random mutations also target known cancer driver genes, these cells might transform into progenitor cells for polyp formation as precursors for cancer. Hence, BMMFs persisting within the interstitium might represent the specific trigger for chronic inflammations which induce cancer by induction of random mutation events in replicating epithelial cells as precursor cells for cancer (zur Hausen et al. 2018).

BMMF latency→ inflammation→ ROS/NOS → higher mutational rates→ susceptibility to carcinogenesis of replicating cells.

Density ultracentrifugation of particles and vesicles

Viral genomes can be packaged by capsid proteins forming stable virus particles, which can be additionally enveloped by specific envelope proteins. Typically, viral particle present as single infectious agent. But for trafficking in populations, virions can bud as membrane-bound carriers known as extracellular vesicles. In vesicles, virions are packaged by endosomal sorting complex for transporting (ESCRT). Unlike particle, the membranes do not surround with structured capsid core inside vesicles. (Alter-Bonnet N., 2016; Raab-Trau N., 2017).

The correlative evidence urge to characterize and visualize possible BMMF particles or vesicles containing BMMF agents, which might be functionally involved in the pathogenesis and accessible to characterization within infection experiments. Therefore, the traditional way of preparing virus-like particles by ultra-gradient centrifugation was proposed. In biochemical and virology research, ultra-centrifugation is one of the widely used methods where the

suspended particles separate according to their molecular weight while subjecting to high centrifugal force (Karp G, 2005). In particular, the preparative ultra-centrifugation method aims to isolate and purify particles, vesicles, subcellular organelles or viruses. Either swinging bucket or angle fixed rotors are required depending on the purpose. The use of a swinging bucket rotor over a fixed angle rotor moves to a longer distance, allowing better separation without disturbing the pellet. Additionally, the example of preparative ultra-centrifugation includes density gradient centrifugation where the separation of the desired components followed either by rate zonal or by isopycnic scale. In rate zonal centrifugation, the particles sediment and separate in zones based on size, shape whereas in isopycnic centrifugation, separation is achieved according to the buoyant density differences with independent of time (Fisher WD, Walier J, 2010).

In isolation of plant viruses, sucrose with cesium salts is commonly used as gradient which is layered underneath the sample solution to be separated. Upon this denser gradient formation, each kind of particle sediments and distributes throughout the tube according to molecular weight and density. The density of the centrifuge tube containing solution progressively increases from top to bottom (Hull, R, 2014). Following the prolonged and high-speed centrifugation, the oldest method is to puncture the tubes using the special syringe and the separated fractions collected in order of decreasing the sedimentation rate.

Density gradient centrifugation-based particle preparation protocols allow separating pure fraction of interest which can be used for downstream applications such as Electron Microscopy (EM), Immunogold Electron Microscopy visualization and infection analysis. Indeed, visualizing the prepared viral particles/vesicles would pave the way for a deeper understanding of these infectious agents in causing diseases. Electron microscopy is a suitable approach to determine the presence of a virus-like particle from infection experiment/assay. To increase the specificity of BMMF detection, Rep-specific colloidal gold particles were commercially prepared for Immunogold Labelling. This technique will allow performing the highly specific detection of BMMF1 isolates in prepared samples.

To get to know more about the particular physiological appearance of BMMF, the presence of either vesicle or protein-containing capsomeres structures needs to be addressed since it is unlikely that naked BMMF can specifically enter and circulate within the body freely.

Observation of helper or suppressor effect

MSBI1.176 and CMI1.252 have similar coding regions except that CMI1.252 has an additional genomic region, so two ORF exists in CMI1.252 BMMF isolate. In one of the co-infection studies, the replication of smaller genome MSBI1.176 was induced with the presence of a larger CMI1.252 genome, even though the replication of CMI1.252 was being unchanged (Eilebrecht, S et al, 2018). Besides, immunofluorescent analysis showed the co-localization of MSBI1.176 Rep and CMI1.252 ORF2 proteins after overexpression of these genomes together in HEK293TT cells (unpublished results). Henceforth, the previous examples showed the need to analyze combinations of isolated BMMF DNAs transfected in HEK293TT cells. This should allow the characterization of possible helper or suppressor effects of different BMMFs. Moreover, the level of Rep protein translation can be determined.

Immunoprecipitation and Co-immunoprecipitation assay

Previous results showed that different BMMFs might help each other in terms of replication (Eilebrecht S et al., 2018). The concept of IP and Co-IP started from the idea to extend data of these experiments and to explore protein-protein interactions and possible helper/suppressor effects between Rep-proteins of BMMF group's members. It is proposed that virus-virus interaction supports the persistence of individual classes of viruses by inducing a symbiotic/synergistic type of relation with a possible pathogenic function (zur Hausen, H., & de Villiers, E.-M., 2014). One of the popular virus-virus interactions includes the helper effect of Epstein Barr Virus and Hepatitis C virus. EBV acts as a helper virus for HCV replication and contrariwise there was a proof of Hepatitis C virus infection inducing reactivation of EBV in B cells (Shimozuma Y et al., 2010). Previously, it was also found that the larger virus (Epstein Barr Virus) is helping the smaller viruses (Torque Teno Virus) to amplify while the replication of the helper virus was halted (Borkosky S.S et al., 2012).

Immunoprecipitation/co-immunoprecipitation, and pull-down assays provide useful information about protein-protein interactions. In these biochemical methods, protein A/G affinity tag-based beads immobilize the antibody-antigen complexes used as bait to attract putative interaction partners (prey). The complex of bait and prey, still immobilized on the beads, is washed to get rid of background proteins. Finally, the bait and prey proteins are identified. In immunoprecipitation, the bait protein is captured, Co-IP captures the bait-reactive interaction partner. A pull-down experiment, in this context, is similar to IP/Co-IP but utilizes affinity tag at the bait protein and affinity tag-based beads immobilize the stable combination of immobilization matrix (beads) and bait protein.

Immunoprecipitation and Co-immunoprecipitation experiments are powerful tools to explore a potential helper-effect of proteins from different BMMF genomes to form multi-composite infectious particles/vesicles.

Dimers of Rep protein

In neurodegenerative diseases, abnormal protein misfolding is one of the common phenomena where aggregates tend to form. Strikingly, some of the BMMF isolates were isolated from patients with neurodegenerative diseases for which observing protein dimers/oligomers were subject of interest. Rep proteins are a group of proteins which were found much earlier in a large group of organisms. Rep protein might be involved in several processes apart from its direct DNA replication initiation function. The conformation of Rep protein structure is critical because it might determine the plasmid copy number. Rep protein, possessing an N-terminal and C-terminal winged-helix domain, exists in the dimeric form in solution. In plasmid replication the dimeric Rep protein dissociates to active monomeric subunit by binding to iteron or chaperones; proceed to nucleoprotein pre-initiating assembly. Upon dissociation of dimers, a conformational change takes place affecting the N-terminal dimerization domain (Giraldo R et al., 2003).

Potentially, dimers are the formulations of self-associated proteins which increase stability by conferring functional and structural advantages. However, non-native and unwanted dimers of proteins may be associated with pathogenic structure by creating misfolded/unfolded protein aggregates. In parallel, homodimers can form when the proteins have an overlapping binding

site which has a role in diverse biological processes (Marianayagam, N. J., Sunde, M., & Matthews, J. M., 2004).

Different BMMF sequences share similar palindromic sequences for which the formation of homodimers might be expected. (Bowers, S. R.et al., 2010) The interaction of Rep proteins may mediate homodimerization which can modulate the co-operative binding sites to regulatory elements (Li D et al., 2007). Indeed, homodimerization demonstrated an essential function in the amyloidogenesis of amyloid precursor protein (Khalifa, N.B., 2010). Amyloidogenesis is an inflammatory disorder where extracellular fibrillar proteins get aggregated and cause tissue damage. Misfolded proteins become insoluble and deposit as fibrils in extracellular tissue. The deposits of beta-amyloid peptide play a key role in the pathogenesis of multiple sclerosis and Alzheimer's disease (Chandra, 2015).

In the scientific report of Moreno-del Álamo (2015), the group observed the binding of dsDNA at the N-terminal dimerization (WH1) triggers to form amyloid fibers by expanding from single to double filaments. It was also found that the assembling of Rep filaments is prionoid and bacteriotoxic. Since Rep proteins can form dimers or oligomers, the Flag and HIS tagged Rep protein will influence in the formation of dimers through rep-rep interaction (Fernández et al, 2016). This was one of the reasons for conducting Immunoprecipitation and Co-Immunoprecipitation experiments.

The underlying mechanism of shifting towards monomeric forms in the N-terminal dimerization domain is still obscure. The dimeric or monomeric form of BMMF Rep will give overall insights about the active or inactive form for plasmid replication along with protein fibrillation in amyloidogenesis.

Mass spectrometry

Mass Spectrometry (MS) is one of the most valuable and widespread techniques in the field of proteomics for the identification of proteins. MS routinely used to identify unknown protein interaction partners prepared by pulldown or IP/Co-IP experiments. It can be used to easily access the full host interactome of putative pathogenic protein like e.g. the BMMF Rep by measuring the relative abundance of Rep-captured host proteins. At first, the liquid pull-down preparations including the protein interaction complexes are turned into ions and then this electrically charged particle deflected by the magnetic field in a beamline. The proteins are ionized following either electrospray ionization (ESI) or matrix-associated laser desorption/ionization (MALDI) method. In MALDI, the matrix is used and proteins are ionized through the pulse of laser light whereas, in the ESI method, multiple charged ions are created directly from proteins in solution. Then, the intact ions are subjected to mass analyzer where the proteins can be analyzed through following any of the three approaches which include top-down, bottom-up and middle down mass spectrometry-based on the purposes. In the bottom-up strategy, a protease enzyme is used to digest the proteins into smaller fragments that are preferred over others for its higher accuracy level (Chait, B. T., 2011). Upon this spectrum-based detection, the digested peptides are departed based on mass to charge ratio which is being analyzed through peak positions via computational methods (BLASTp hit analyses, domain sweep data analysis).

The desired proteins were purified from the crude lysates following the newly established pull-down assay using HIS beads. In this approach, affinity purification epitope-tag is used which allows the fusion proteins to be pulled down (Chang et al., 2006). This

comprehensive pulling down co-purifies or purifies the interactors along with the expected protein.

Conclusion

The presence of reactive Rep specific antibodies in a wide set of human serum along with the bioactivity of BMMFs in human cells and specific detection of BMMF proteins in cancer tissue further provokes us to know more about BMMFs and in which molecular shape they exist and how they might enter the organism. Preparation of BMMF particles/vesicles by ultra-gradient centrifugation might represent the basis for future experiments on BMMF visualization by electron microscopy and infection assays to explore the characteristics of these agents.

Infection with purified fractions of BMMF viral particles in continuous and primary cell cultures will lead us to clarify the mechanisms of entry, replication, and transcriptional activity. Furthermore, the interaction with desired molecules and cell response can be observed from infection analysis.

In terms of the hypothesis, BMMFs have a role in carcinogenesis and other chronic infections. Protein-protein networking has always played a dynamic role in altering chemical signaling pathways. To observe the BMMF Rep protein interaction with the proteins of carcinoma cell lines and macrophages, the interactome study was proposed using a pull-down assay. Pull-down assay is one of the widespread and useful approaches where a combination of desired BMMF1 protein and interacting protein from cell-lysates can be immobilized using the affinity tag-based beads. Moreover, characterization by Mass spectrometry will identify the BMMF Rep interacting proteins from the different cell lines. Therefore, the possible interactome involving cancer induction pathways can be investigated.

Dimerization is valuable and important for BMMFs Rep to observe the Rep-Rep interaction. To screen and characterize the insights of protein dimerization, Immunodetection followed by IP & Co-IP would be a useful approach. No doubt, this will further help us to gain an understanding of the structural basis of BMMF N- or C-terminal Reps in amyloid assembly.

AIM

It has been hypothesized that the BMMF infection within the human host might lead to neurological disorders and certain cancers (Zur Hausen, 2018). The objectives of this part of the thesis project are:

1. BMMF particle/vesicle preparation
2. Identification of BMMF1 Rep interacting proteins.
3. Characterization of Rep-Rep interaction

This project, in combination with a basic analysis of BMMF protein overexpression, will expand our understanding of BMMF physiology and enable detailed studies on a possible BMMF infection within the human host. The results might allow for the identification of specific BMMF functions, propose strategical BMMF mutations for modulation of BMMF function for future experiments and allow setting up new strategies for prevention and modulation of BMMF infection.

2. Materials and methods

2.1. Cell culture work:

2.1.1 Cell cultivation

All work was performed inside the Biosafety class II cabinet (SAFE 2020, ThermoScientific Biological Safety Cabinet). Cells were incubated at 37 °C and 5% CO_2. HEK293TT cells were confluently grown in single layer flasks in Dulbecco Modified Eagle Medium (DMEM) (Sigma-Aldrich, D5796-6X500ml) supplemented with Glutamax, penicillin-streptomycin (Sigma, P0781-100ml), non-essential amino acids and 10% (v/v) fetal calf serum (FCS).

Before subculturing, the medium was poured off and the cells were washed with 1xPBS (Phosphate Buffer Saline) (Gibco,70013-065) to remove serum and dead cells. Upon washing, 0,05% Trypsin (Sigma, T3924-100ml) was spread and then the flask was kept at 37 ℃ incubator for 3 minutes so that the adherent cells on the surface get detached. Then, the cells were resuspended and mixed well in 10x volume of fresh medium containing DMEM and 10% FCS for trypsin deactivation. While $1/5^{th}$ of cell suspension was kept and rest of the approximate full DMEM media was added. Lastly, 400 µg/ml of Hygromycin B (Invitrogen, 10687010) was put for the selective growth of the cells as well as stimulating small and large antigen of HEK293TT cells for replication.

2.1.2 Preparation of frozen cell aliquots

For the preparation of HEK293TT cells stocks, freshly cultivated cells from 70-80% confluent T175 flask was centrifuged. The cell pellet was resuspended in pre-cooled 90% FCS and 10% DMSO (Dimethyl Sulfoxide). Dimethyl Sulfoxide is used to freeze the cells without forming any ice-crystals. Then, 1 ml aliquots were transferred into 2 ml cryotubes and incubated over 72 h at -80 °C in freeze boxes containing isopropanol. Finally, for long term storage, the cryo-tubes were stored in a tank of liquid nitrogen.

2.2 DNA transfection

With the aim to choose transfection reagent with the highest transfection efficacy three reagents had used before proceeding to further experiments. Lipofectamine 2000, Effectene and PEI were tried for transfection. At first, HEK293TT cells were preincubated for 24 h prior to transfection procedure to obtain 70-80% confluent monolayer cell culture as described above.

2.2.1 Effectene protocol: (QIAGEN GmbH, 301425)

0.5 µg of BMMF1 DNA (pc DNA 3.1 (-) Msbi1 Rep cod opt C-HIS / N-Flag Msbi1 Rep p3xFlag CMV 7.1), Buffer EC and Enhancer were preincubated in a proportion offered by the manufacturer according to standard protocol. Then, the mixture was incubated for 5 minutes for the optimum condensation of the DNA. Afterward, Effectene was added as a non-liposomal reagent in order to coat the DNA with lipid. Later, the mixture was kept for 10 minutes before applying to the cells. Before incubation at 37℃ incubator, the plates were shaken gently horizontally and vertically for even distribution.

2.2.2 Lipofectamine 2000: (LIFE Technologies, 11668019)

Lipofectamine 2000 reagent encloses the DNA plasmid through forming liposomes. 1µg of BMMF1 DNA (pc DNA 3.1 (-) MSBI1 Rep cod opt C-HIS / N-Flag MSBI1 Rep p3xFlag CMV 7.1) was added with pure DMEM medium in one Falcon tube. In the other tube, Lipofectamine 2000 was added with DMEM medium in a certain amount provided by standard

protocol. Later, these two solutions were mixed together and incubated for 5 mins and then dropwise applied to the cells.

2.2.3 PEI protocol: (Sigma, 408727-100ML)

Polyethylenimine (PEI) condenses the DNA into positively charged molecules for adhering with the cell surfaces. For transfection with PEI, two Eppendorf tubes of solutions were prepared. One Eppendorf with PEI (B) and pure DMEM and the other containing 2 µg DNA along with pure DMEM (A).

Once the DNAs were added to solution A then it was incubated for 5minutes. After incubation, the second Eppendorf with PEI and pure DMEM were added. These two solutions were mixed and the mixture was incubated for 20 minutes before applying to the plates.

2.2.4 Transfection with GFP:

Cells could be transfected with GFP plasmid for direct detection. In order to calculate the transfection efficiency by visualizing under Light field microscopy, Green Fluorescence protein (GFP) plasmid was transfected with the usage of all three reagents (Effectene, Lipofectamine 2000, PEI). The imaging of GFP protein was performed on a Zeiss Cell Observer and a color CCD digital camera AxioCam after 48h and 72h of incubation.

2.3 Protein detection/measurement

2.3.1 Ponceau S staining

To get a first measure of the protein amount and to have a look at the location of the protein bands, the blot membranes were stained and incubated with Ponceau S for 40-60 s. This particular rapid and reversible staining gives reddish-pink bands without any deleterious effect. Then, the membrane was rinsed with distilled water until the background is clear. After taking the image, the stain was completely removed by continue washing with distilled water and 1xPBS.

2.3.2 Coomassie staining:

Coomassie staining was conducted to observe the separated specific and non-specific protein bands for further confirmation of protein presence. In coomassie staining, five different concentrations of BSA controls were loaded to observe and compare with the protein of interest. After SDS-PAGE, the gel is soaked and dissolved in the coomassie-directRed stain in order to permeate and fix the protein in the gel. Once the gel gets stained, it is destained with the acetic acid and ethanol solvent so that the separated protein can be visible as the brown colored bands.

First, the loading gel was prepared. After protein separation, the gel was immersed into a coomassie solution for 2 hours. This special kind of blue staining solution, coomassie with direct red stains all the specific and non-specific bands. After incubation, destaining was followed with 10% acetic acid and 10% EtOH for around 3 hours. In every 20 minutes, the destain solution was changed and the tray was heated for 10 seconds. Destaining was done on the shaker.

2.3.3 Silver staining:

To detect the least amount of protein product along with protein degradation or impurities. Silver staining was conducted using Pierce Silver Stain Kit (ThermoScientific, QB210810A) according to the manufacturer manual where the detection limits ranging from 0.5-5 ng.

In silver staining, silver is incorporated into the protein bands to visualize the trace amount of separated protein. Upon fixation, the background molecules are removed. The sensitizer and enhancer reagents specify the silver ions binding to separated proteins. In addition, the silver reduction stain reduces ionic silver to metallic silver for which metallic silver gets deposited at the site of location. The developer solution develops images, resulting in brown-black color.

2.3.4 Bradford Assay:

Bradford assay was followed in order to know the concentration of the proteins which are measured by absorbance shift from reddish to the blue complex through binding with Coomassie Brilliant dye. Upon binding with the dye, the protein converts to anionic or more stable bound state with the ionic interaction of the lone pair of dye. In this UV/visible Spectrophotometer instrument (Ultrospec 3000pro), the O.D.(Optical density) value, the absorbance was measured at 595nm wavelength. The samples were prepared using six different serial diluted Bovine Serum Albumin(Sigma, A7030-100G)controls where the dilutions were in 6.4, 3.2, 1.6, 0.8, 0.4, 0.2 and 0.1 µg/µl. For each sample, duplicates were measured for confirmation. 200µl of Bradford reagent was added with 800µl of autoclaved water. The special labeling cuvettes were vortexed carefully and incubated for 10 minutes before measurement.

2.3.5 Measuring protein concentration using Nanodrop:

Nanodrop 2000 Spectrophotometer (ThermoScientific) instrument was used for measuring the protein concentration with purity value. For calibration, 1µl CsCl, Tris standard, MgCl, Ammonium Sulfate, NaCl buffer was put for blank measurement. After cleaning, 1µl of each of the samples containing protein product were quantified.

2.4 SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis)

2.4.1 Western blot preparation

For protein characterization through immunodetection, the proteins have to be separated according to their molecular weight. The SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) gel run was performed to denature the proteins and separating them mainly based on their size, molecular weight. For standard SDS-PAGE protein separation 4-12% TruPAGE Precast 12 well (Sigma, PCG2004-10EA) or 17 well gel (Sigma, PCG2008-10EA) with 1x SDS Running Buffer (Sigma, PCG3001) were used.

To prepare protein solution, cells were mixed with 2.5x or 5x SDS Loading Buffer with a reducing agent (with ß-ME) and heated for 5-10 min at 95 °C. The protein solutions were loaded into separation SDS-PAGE gels and run for 2 hours at 35 mA. After SDS-PAGE, the gels were subjected to transfer blotting.

Table 1: Composition of SDS-PAGE protein loading dye (Laemmli buffer):

5xLaemmli Buffer recipe:
250 mM Tris HCl pH 6.8
8% (w/v) SDS
30% (v/v) glycerol
0.2% (w/v) bromphenol blue
10% (v/v β-mercaptoethanol)(Gibco,31350010)

2.4.2 Transfer blotting

For transfer blotting, the nitrocellulose membrane was used as the blotting paper because of its high protein binding affinity. The SDS-PAGE gel with the separated proteins was transferred onto the nitrocellulose membrane (Trans-blot Turbo mini size Transfer stacks L002043A) by following semi-dry transfer blotting. Carefully, the gel was placed on the blotting paper packed with two additional sponge blot stacks like the sandwich. Then, the transblot sandwich was put horizontally on the turbo chamber and was fixed in the machine. In the Trans-Blot Turbo machine (Bio-Rad laboratories, 170-4270), the gel along with nitrocellulose membrane was run for 10 minutes at 25 V and 1.3 A.

After transfer blotting, the nitrocellulose membrane was stained with Ponceau S for visualization of gel bands and was directly used for immunodetection.

2.4.3 Immunodetection

To characterize the protein of interest, immunodetection was followed.This method comprises incubation of two antibodies which are primary (target-specific) and secondary antibody. In certain cases of immunodetection, the secondary antibody can be coupled with horseradish peroxidase (HRP) for better detection. Briefly, with higher affinity, the secondary antibody binds with the primary antibody while the primary antibody recognizes and attaches to the specific antigen of the protein of interest. This eventually gives a higher detection level of the antigen. As the blocking agent, 5% skim milk in 1xPBS was used for reducing the non-specific bindings in between target protein of the membrane and the immobilized (target-specific) antibody. The blot membranes were incubated with the blocking agent for 1h at RT on a shaker. Later on, the incubation with primary antibodies was performed overnight at 4℃ whereas, the secondary antibody incubation for one hour at RT.

The nitrocellulose membranes were incubated with any of the four primary antibodies based on purpose. The list of primary antibodies presented in Table 2.

Table 2: List of primary antibodies with working dilutions used for SDS-PAGE analysis.

Mouse anti-FLAG M2 (1:5000) (SigmaAldrich, F1804)
Mouse anti-HIS (1:2000) (Qiagen, 34660)
Mouse Rep mix(381+441+523) (1: 1000 each) (in-house produced)
Mouse γ-Tubulin Ab (1:2000) (Life Technologies, MA 1850)

For all the above primary antibodies, an anti-mouse secondary antibody (1:5000) (Dianova, 115-035-062) was used.

In the case of immunoprecipitation with Flag Agarose beads, rat anti-mouse IgG HRP TrueBlot secondary antibody (1:1000) was put on the membrane. This true blot specific antibody only detects the target primary antibody, not the heavy and light chain of primary antibody dissociations. Every incubation was followed on a shaker and in the meanwhile of every incubation, the membrane was washed with 0.05%/ 0.1% 1xPBS-Tween (MP Biomedicals, Tween 201) 3 times for 10 mins. Afterward, the Clarity Western ECL Select (Amersham, Bio-RAD, 170-5060)/ was used as the chemiluminescent detection reagent to visualize the protein signal in the Chemidoc machine (Bio-Rad).

Data were analyzed using Image Lab software 5.2.1. After detection, the membranes can be used further by continuous washing with 1xPBS and using Restore plus Stripping Buffer (Life Technologies, 11668019).

2.5. BMMF1 particle preparation:

In this specific method, all the reagents and chemical compounds used so that the DNA can be provided by the factors to form and purify infectious capsomere. Therefore, the plasmid acting as vector with the insert of rep sequence from BMMF isolate had been produced by transformation and maxi/mini prep. This vectors were proved to be efficient as the gene transfer vehicles.

2.5.1 Sample or Isolate description:

One of the representatives from group BMMF1, Multiple Sclerosis Biopsy Isolate MSBI1.176 Rep Cod Opt C-HIS was used in the consecutive experiments. The isolate (MSBI1.176 Rep Cod Opt C-HIS) underwent PCR, ligation and then transformation. So, the dsDNA was transformed into a bacterial plasmid and then plasmid DNA was purified through Maxi-prep and kept at -20℃ prior to transfection in HEK293TT cells/ further analysis.

2.5.2. DNA preparation:

Several plasmids for further experiments were prepared according to the following protocol. Transformed bacteria were grown in, 400ml LB medium (10g tryptone, 10g sodium chloride, 5g yeast extract and 900ml of distilled water), 4ml Ampicillin and 200µl from the plasmid stock in the 2L conical flask. For the bacteria to grow, the flask was incubated overnight at 37℃ with on 130rpm shaker.

Plasmid DNA purification: NucleoBond® Xtra Midi / Maxi

Maxi DNA kit allow to prepare 1000µg plasmid DNA. After overnight incubation, cells were pelleted by centrifugation at 6000g for 15minutes, 4℃. The supernatant was discarded and the cell pellet was used for further procedures. Plasmid DNA was purified using NucleoBond® Xtra Midi / Maxi kit (Macharey-Nagel, 740410; 50) according to the manufacturer manual.

Miniprep: GeneJet Plasmid Miniprep Kit

Picked colonies were grown overnight in 2ml of LB Amp media at 37℃ 130rpm shaker. In Miniprep, the bacterial culture was harvested through higher centrifugation at 8000rpm and three different solutions were added to the pellets. These Resuspension, Lysis and Neutralization buffer solutions help to isolate the plasmid DNA. Then the supernatant containing the desired DNA was bind to the Thermo Scientific GeneJet Spin Column. After that, the column was washed out with washing buffer and at the last step, 60 µl of elution buffer was added to elute out the DNA solution from the column.

2.5.3Control digestion:

Digestion was proceeded to check whether the insert had been taken by the bacterial plasmid. In order to visualize the band lanes properly the plasmid with insert was cut by two restriction enzymes. 20 µg of prepared by MaxiPrep DNA was mixed with 40µl Buffer for BamHI, 5 µl KpnI and 5 µl BamHI enzymes, and double-distilled water in a total volume of 400 µl. This digested DNA solution was then incubated for 8 h at 37℃ water bath. Digestion products were separated on 1.2% agarose gel for 1 h at 100 A. The separated DNA bands were observed on a Gel documentation system (Bio-RAD, laboratories GmbH) under Ultraviolet light.

2.6 Density gradient centrifugation:

The traditional way of preparing virus-like particles by gradient ultra-centrifugation described in C. Buck paper (Buck C.B., 2007) with modifications was used. The protocol requires several steps as follows: cell pellet preparation, cell lysate clarification, and gradient ultra-centrifugation. This method separates the particles in terms of size and shape by molecular weight in a relatively dense solution along a density gradient. This method refines the virus particle through yielding the purified material by density gradient centrifugation. It further let us know about the associated host cell, virus protein and genome in BMMF infected HEK293TT cell.

80% confluent HEK293TT cells were transfected with pcDNA3.1 (−) MSBI1 Rep cod opt C-HIS using PEI-based technique as described above. After 72 h incubation cells were harvested on ice and washed 3 times in 1xPBS. The supernatant-free cell pellet was stored at -80 °C.

Particle fraction was characterized by gradient ultra-centrifugation. In order to get the cleaner and purer protein fraction prior to detection of capsids from the protein of interest gradient CF in Cesium chloride (CsCl) solution were performed with or without PEG8000 precipitation.

2.6.1 Cell lysis and PEG precipitation:

To prepare the cell lysate, the cell pellets underwent freeze-thaw cycles which were applied 5 times as follows: liquid nitrogen for 20 seconds followed by 37°C water bath incubation for 60 seconds.

CsCl solution in VTA Buffer (50mM Tris-HCl pH 7.5, 0.5 mM EDTA), adjusted with a refractometer to RI=1.372, and 1M Saccharose solution in VTE Buffer was prepared prior to centrifugation.

The steps for cell lysis prior to CsCl gradient centrifugation as followed:

Samples for centrifugation were prepared by mixing the different amount of chemicals and detergents with the aim to prevent protein degradation and allow particle formation. Approximately, 1.4 pellet volume of Tris standard/9.5 mM $MgCl_2$ was mixed with a 1x protease inhibitor cocktail. Then, 1/40th of the actual volume of 10% Triton X-100 and 1 M ammonium sulfate (pH 9, sterile filtered) was added. Afterward, this mixture was transferred to protein low bind tubes (Eppendorf, 10708704), at this step the cells were suspended slightly with the cut tip so that no DNA shearing took place. Later, in order to degrade DNA 1% Benzonase (25 U/µl) (Merck, 70664-3) and to protect protein shielded circular DNAs 0.1% Plasmid Safe (Biozym, E3101K) was added. Benzonase endonuclease cut down any DNA except for protective environment of capsid. After that, the cell lysates were incubated at 37℃ for 2 hours allowing particle formation. Before and during incubation, the tube was mixed shortly. All the cell debris and less denser proteins and biological macromolecules are cleared out by using this chemicals and detergents. In order to sink the particle, purification is done through salting out by reducing the volume. This will eventually clears out other biological macromolecules.

After 2 h of incubation, the cell lysates including the overexpressed Rep protein were chilled on ice for 5 min. The salt concentration was brought to 850 mM (0.17 V of 5 M NaCl) and the lysate was kept on ice for additional 10 min. Then, the lysates were clarified by centrifuging at 5000 g for 5 minutes at 4℃. Small amount of supernatant with loose pellets was seen. The clarified supernatant was transferred into a fresh siliconized tube which was then labeled as supernatant 1. After collecting supernatant, the 2X pellet volume of Tris/0.8 M NaCl

was added to the pellet. Then, the tubes were resuspended by flicking and re-centrifuged at 5000 g for 5 min, 4℃. After centrifugation, the supernatant was put into another tube and labeled as Supernatant 2 and the pellets were stored after lysis at−80℃. Later, supernatant 1 and 2 were combined and re-clarified the pooled supernatants for 5mins at 5000 g, 4℃. Afterward, high-speed centrifugation was followed at 18000 g for 5 min, 4℃ and 0.22µm filtration as final steps of lysis clarification before loading onto the gradient.

Polyethylene glycol, shortly PEG8000 was used to upconcentrate the virus particle. PEG8000 solution from PEG Virus Precipitation Kit (Biovision, K904-50-BV) was added to clarified cell lysate to the final concentration of 8% followed by 2 h incubation on ice.

Later on, four tubes were then weighed and balanced with the same measurements within metal centrifugation tubes including lids. For balancing, a VTE buffer was used. After that, the samples were run in the ultracentrifuge with the S50-ST rotor (Acceleration 1/Deceleration 1) at 253000 g/50000 rpm for 20h at 15℃.

2.6.2 Fraction collection:

The top of UZ tubes were covered with parafilm and the bottom of the tube was pricked using a 22G needle (Medoject, Luer-lock). Then, a big hole was made in parafilm and regulated slowly via thumb to avoid air bubbles. Twenty fractions were distributed into the protein low-binding tubes by dropping from the bottom of the UZ tube. Through pressing minimized, the fractions were collected by droppings. This allows to collect the fractions from UZ tube without disturbing the upward layers since the particle hides into the fraction based on density.

To specify the peak fraction SDS-PAGE was performed for all fractions as was described before. Since the transfected DNA was HIS-tagged, the primary monoclonal anti-mouse HIS antibodies were incubated with the membrane.

2.6.3 Dialysis:

The chosen fractions were filled in the Slide-A-Lyzer G2 Dialysis Cassettes (0.5 ml or 3 ml/10K MWCO, CAT. # 87729 or 87730). Firstly, dialyzed against 700 ml VTE buffer for 1hr at RT and afterward the VTE buffer was replaced with fresh 700 ml VTE buffer for another 1.5 hr at RT on the stirrer. Then, the membrane of the cassette was torn off using syringe at the bottom and the dialyzed samples were collected into the fresh Eppendorfs. Later on, the aliquots for DNA isolation, WB assay, density measurements and storage were prepared and stored.

2.6.4 Measurement of Refraction Index of fractions:

To prepare a refraction curve of the gradient fractions, 10 µl from each of the fractions was placed on the surface with the attached prism. For measuring the Refraction Index (RI) of each fraction from gradient centrifugation, the Refractometer (Refractometer AR-3) was used according to protocol.

There is a density measuring guideline book for the CsCl gradient where the density corresponding to the RI is mentioned. Therefore, after measuring the RI, the guideline book was looked at for knowing the exact density number. In the guideline book (dkfz facility), the range of RI for a certain density was specified.

2.6.5 DNA isolation:

To check whether the dialyzed BMMF particles have the DNA or not, DNA isolation was followed according to the manufacturer's protocol (Qiagen, 51306). During DNA isolation, 1µg of carrier DNA was mixed which was from salmon sperm. An additional step was observed so that the least possible DNA can be recovered through isolation. For efficient lysis and further purification, Buffer AL was added. In consecutive steps, the samples were

cleaned and centrifuged out using QIAamp Spin Column and two washing Buffers, AW1 and AW2. Lastly, Elution Buffer AE was used to elute out the purified DNA out of the spin column.

2.6.6 Detection of DNA by Long PCR:

For efficient and optimum DNA amplification, Long Polymerase Chain Reaction was performed where the amplification can be up to the range of 40kb. After purification, it is needed to know whether the genome is present or not. Therefore, Long PCR was conducted to observe whether the DNAs were present or not in transfected control after viral particle preparation. After DNA isolation from CsCl gradient centrifugation, the samples underwent PCR reaction to observe whether the DNAs were present or not in transfected control after viral particle preparation. LPCR primers to detect pcDNA3.1 (-) MSBI1 Cod Opt HIS6 plasmid presented in Table 3.

The master mix calculation has shown in Table 4. Once the master mix was made through mixing with primers, PCR grade water, DNTP, GC-buffer, TaqPol and Template, the Eppendorfs were vortexed gently and spun down so that every droplet stays at the bottom of the microcentrifuge tube. Then LPCR was performed in 50 reactions containing 20ng of template DNA. As a positive control pcDNA3.1 (-) MSBI1 Rep cod opt HIS6 plasmid with the concentration of 1ng/reaction were used. The thermal cycling profile of LPCR is presented in Table 5.

Table 3: The primer sequence for LPCR

Forward primer	5´gcggatccgccatgagcgacctgatcgtgaaag 3´
Reverse primer	5´gcggtacctcactttggtctcttgtcagag 3´

Table 4: The calculation for 50 reactions of LPCR

Long PCR	1x, µl
dNTP	8
Primer mix	2
GC-buffer 1	25
TaqPol	0.5
Template 20ng	
water	6
Total	50µl

Table 5: Thermal cycling profile of LPCR

Steps	Temperature; Time
Denaturation	94°C; 30 sec
Annealing	56°C ;1 min
Extension	72°C ;1 min
Cycle numbers	25

Master Mix preparation was done in the separate hood before adding the sample DNAs and putting the tubes in BioRad PCR (Flexcycler^2 analytical Jena) analyzing machine.

2.6.7 Visualization of PCR product using agarose gel electrophoresis

The 1.2% agarose gel was prepared with mixing 100 ml buffer with 1.2 g Agarose powder (SigmaAldrich).After PCR reaction, the PCR products were run through Agarose gel electrophoresis. About 15 µl aliquot of the PCR product along with 5 µl of loading dye Orange G was mixed and loaded total 20µl into individual wells of the gel. While the gel was run in an electric field, the DNAs were to be separated according to the amplicon size and electric charge.

A ladder of size 1kb plus (ThermoScientific) was also added into the same gel for estimating the molecular weight of linear, double-stranded PCR products which were estimated to be within 1766 bp. Amplified PCR products were electrophoresed at 100 volts for around 40 minutes and then the gel was drenched with Ethidium Bromide (EtBr) for 20 minutes to have the fluorescence image. The separated DNA bands were observed on a Gel documentation system (Bio-RAD, laboratories GmbH) under Ultraviolet light. EtBr is an intercalating mutagen, so the gel should always be handled with double gloves and discarded every time ones get contacted.

2.7 Plasmid preparation: cloning of N-/C-terminal 3xFlag

2.7.1 Flag tag insertion by Polymerase Chain Reaction:

For amplification of pcDNA3.1 (-) MSBI1 Rep cod optimized plasmids, qPCR was conducted using two different primers. Both primers were Flag sequenced at both 5' and 3'terminus so that the produced insert can be with Flag sequence in N and C terminus. The qPCR primers to detect pcDNA3.1 (-) MSBI1 plasmids presented in Table 6.

Table 6: Primer list for attaching the specific tag in both (a) N and (b) C terminus of the desired product

(a) The primer sequence for N-Flag pcDNA3.1 (-) MSBI1 Rep Cod Opt

Forward primer (M1Repopt3xNflag5p)	GTGGATCCgccATGGACTACAAAGACCAtgACGgtgATTATAA AGATCATGACATCGATTACAAGGATGACGATGACAAGggag caAGCGACCTGATCGTGAAAGACAATGC
Reverse primer (M1Repopt3xNflag3p)	GAGGTACCTCAAAACACGACTCCAAACTCTTCCAGTTTAG

(b) The primer sequence for C-Flag pcDNA3.1 (-) MSBI1 Rep Cod Opt

Forward primer (M1Repopt3xCflag5p)	GTGGATCCgccATGAGCGACCTGATCGTGAAAGACAATGC
Reverse primer (M1Repopt3xCflag3p)	GAGGTACCTCACTTGTCATCGTCATCCTTGTAATCGATGT CATGATCTTTATAATcacCGTcaTGGTCTTTGTAGTCtgctccCT AAACTGGAAGAGTTTGGAGTCGTGTTT

The PCR mix was made through mixing with forward-reverse primers, PCR grade water, 2xPfu PCR master mix, and template DNA. Then, the Eppendorf was vortexed gently and spun down so that every droplet stays at the bottom of the microcentrifuge tube. After that, qPCR was performed in 50 reactions containing 100ng of template DNA. The master mix calculation and thermal cycling conditions have shown in Tables 7 and 8.

Table 7: The calculation for 50 reactions of qPCR where 100ng of DNA template was mixed with the master mix; in total 50µl were put in each tube

PCR precursors	Amount
2x Pfu PCR master mix	25µl
Primer 3p(1:10)	1µl
Primer 5p(1:10)	1µl
Template DNA	50-100ng(~1µl)
Water	22µl

Table 8: Thermal cycling profile of qPCR

Steps	Temperature; Time
Denaturation	95°C; 2min
Denaturation	95°C ;45sec
Annealing	58°C ;45sec
Elongation	72°C;2min
Extension	72°C;7min
Cycle numbers	35cycles

The PCR program (Flexcycler^2 analytical Jena) defining the timing with temperature which includes denaturation, annealing and elongation steps.

After PCR reaction, the PCR products were visualized through agarose gel electrophoresis by following the steps in section 2.6.7.

2.7.2 Gel Extraction:

The approximate size of the target band is 1000bp, therefore the target band was cut and extracted following the DNA extraction from agarose gel NucleoSpin® protocol (Macharey-Nagel, 740,609,250).

2.7.3 Restriction: Control digestion for both insert and vector

After gel extraction, the sample of interests was restricted with BamHI and KpnI restriction enzymes in order to cut the DNA at the recognition sequences of BamHI and KpnI. The digested reaction was incubated for 2hrs at 37℃.

The restriction target vector pcDNA3.1 (-), the original vector was also restricted following the same way mentioned above.Later the digested PCR samples were combined with 10 µl of the target vector restriction. Afterward, the DNA extraction was followed into 35µl of water.

2.7.4 Ligation:

In order to ligate the target vector with the desired samples, ligation was done using T4 ligase buffer and T4 ligase enzyme (ThermoScientific, EL0011). The ligated reaction was kept at room temperature for overnight.

2.7.5 Transformation:

Finally, around 22µl of DNA were transformed into DH5alpha *E.coli* cells. Before the transformation, the ligated samples were inactivated for 15mins at 65℃.

NEB 5-alpha Competent *Escherichia coli* (C2987H)

To insert the ligated PCR product within the target vector pcDNA3.1 (-), the transformation was followed after the ligation step. Transformation was performed using NEB 5-alpha Competent *Escherichia coli* (C2987H) according to the manufacturer manual.

2.7.6 Plating of the transformed solution onto the LB Amp agar plates:

Approximately, 150µl of the transformed solution was drop plated onto LB Amp plates for the selective Ampicillin resistant colonies of interest to pick and the plates were incubated overnight invertedly at 37℃ incubator. The differential single colonies were picked and incubated with LB Amp broth at 37℃ shaker.

2.7.7 Miniprep:

For plasmid preparation, Miniprep was followed as the section 2.5.2.

2.7.8 Transfection and harvesting:

The desired proteins were overexpressed based on transiently-transfected HEK293TT cells in 24well culture plates (cat no: 2511) using PEI as the transfection reagent. 72 hrs after transfection, cells were washed in 1xPBS and harvested in 200µl of Laemmlli Buffer, boiled at 95℃ for 10 minutes and stored at -20°C.

2.8 C-/N- Flag-tagged MSBI1 Rep protein detection by SDS-PAGE and Immunofluorescence

In order to look for the protein bands at the sample of interests, western blot was run by following the protocol in the previous section 2.3.

Firstly, the cells were plated on 8 well special mini plates (Neolab, 354118) where each well with 80,000 cells. To have a look at the protein production and distribution, 0.5µg of selected DNAs were transfected in HEK293TT cells using PEI as the transfection reagent on the plate. In three different time points of 24hrs, 48 hrs, and 72 hrs, the cells were fixed with two separate fixation solvent, Methanol and 4% PFA (Paraformaldehyde).

Three different mouse anti-Rep antibodies were used in combination with the guinea pig anti-Rep antibody. Different primary antibodies were used which is presented in Table 9.

Table 9: List of primary antibodies used in Immunofluorescence

Primary antibody
Mouse anti-Rep Ab #13 (1:500)
Mouse anti-Rep Ab #3-6(1:500)
Mouse anti-Rep Flag Ab(1:500)
Guinea pig anti-Rep GP (1:400)

To detect antibodies from both species, a secondary antibody mix was used where anti-mouse Alexa Fluorephore 488 and anti-GP Alexa Fluorophore 546 (both in 1:500) was mixed with Hoechst DNA dye (1:2000) for DNA staining.

In prior to see the speckles or distribution or any signals from the desired protein, the transfected cells were washed with PBS and then fixed using two different incubation periods. For methanol selection cells were fixed for 2 mins with ice-cold methanol whereas, for 4% PFA fixation, the plate was kept at room temperature for 15minutes. For removing the lipid contents from the cells and to make an entry for the protein, the cells were permeabilized for 3 mins with 0.5% Triton in 1xPBS at 4℃ on ice. Later the cells were washed thrice with 1xPBS and blocked with 1%BSA in 1xPBS. Afterward, the plates were incubated with primary antibodies for one

hour at RT and washed 3 times with 0.1% Tween in 1xPBS. Then, blocking with 1%BSA in 1xPBS was followed for 15mins and the secondary antibody mix was added for 1 hour at RT. Finally, the cells were was washed 3 times with 0.1% Tween in 1xPBS, mounted using mounting medium and dried in the dark for 10minutes. Until analysis, the slides were kept at 4℃ in the darkened slide chamber.

2.9 IP/Co-IP Experiment:

Immunoprecipitation was performed with tag-specific beads to capture the beads attached protein complex whereas, co-immunoprecipitation detect the interacting protein in captured protein-complex by western blot analysis.

2.9.1 Cell lysis:

For lysis, the frozen cell pellets were thawed on ice and 1.2 ml lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 1% (v/v) Triton X-100) was added before sonication was performed. At the time of the sonication step, the cell pellet with lysis buffer was put into the Falcon tube on ice with 30% output, 30% duty cycle for around 20 seconds. Then, the lysates were incubated for 20 minutes at 4℃ on an overhead rotator. Afterward, the lysate was cleared by centrifugation at 12000g for 15minutes and later, protein-specific pulldown experiments were conducted.

2.9.2 Immunoprecipitation with anti-Flag Antibody and Agarose beads or Flag-beads

The same samples which were prepared from cloning experiment (2.7 section) were used for immunoprecipitation and co-immunoprecipitation experiments. Specific primers were used with the HIS- and Flag- tag sequences for generation of the overexpression plasmids by PCR.

The lysates containing FLAG-tagged and HIS-tagged proteins of interest were incubated with 2.5 µg monoclonal anti- FLAG antibody for 1.5 h at 4 °C. Then, 50 µl Protein A/G-Agarose beads (Santa Cruz Biotechnologies, SC-2003) were added. Alternatively, 50µl magnetic Flag-beads (Sigma Aldrich, M8823) were added to the cell lysate. The mixture was incubated for 1.5 h at 4 °C. The beads were centrifuged for 3 min at 600 g at 4 °C and then washed with 500 µl washing buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% (v/v) Triton X-100, 0.05% (w/v) SDS). The washing step was repeated additional two times until the beads were finally incubated and boiled with 75 µl 2.5 x SDS loading dye without reducing agent (w/o ß-ME) for 10 mins at 95 °C. The samples were kept at -20℃ before proceeding to immunodetection.

2.9.3 Pulldown with HIS-beads

For HIS-pulldown experiments, the lysates containing the proteins of interest were incubated with 50 µl HIS60 NiSuperflow Resin (pre-incubated in interaction buffer, Takara Bio Clonetech, 635659). After incubation for 1 h at 4 °C, the samples were centrifuged (3 min, 600 g, 4 °C) and the supernatant was discarded before 500 µl wash buffer (50 mM Tris pH 7.5,150 mM NaCl, 0.5% Triton X, 0.05% (w/v) SDS, 20 mM imidazole) was added. This step was repeated an additional two times. After the last centrifugation, the supernatant was discarded and the beads were boiled with 7.5 µl 2.5 x SDS loading dye with a reducing agent (with ß-ME) for 10 mins at 95℃. The samples were kept at -20℃ before proceeding to immunodetection.

For optimizing the pull-down protein detection with HIS and Agarose beads, different attempts of various Lysis Buffer and Washing Buffer condition was applied. The Lysis and Washing Buffer composition is mentioned in Tables 10 and 11.

Table 10: Composition of lysis buffers.

Lysis Buffer 1	Lysis Buffer 2/3
50 mM Tris pH 7.5	50 mM Tris pH 7.5
300 mM NaCl	300 mM NaCl
1% Triton X	1% Triton X
	5/10mM Imidazole

Table 11: Composition of washing buffers

Washing Buffer X	Washing Buffer Y	Washing Buffer Z/N	Washing Buffer Sigma
50 mM Tris pH 7.5	50 mM Tris pH 7.5	50 mM Tris pH 7.5	50 mM Tris pH 7.5
150 mM NaCl	150 mM NaCl	150 mM NaCl	150 mM NaCl
0.5% Triton X	0.5% Triton X	0.5% Triton X	0.5% Triton X
	0.05% SDS	0.05% SDS	1mM EDTA
		20/35 mM imidazole	10% Glycerol

2.10 Mass spectrometry analysis

Mass Spectrometry was followed to identify the protein interactomes, the combination of protein of interest with the lysates of different cell lines was fractionated by gel electrophoresis and western blot analysis.Five cell preparations were used to specifically search for interaction partners of MSBI1 Rep protein. From the colon carcinoma cell lines, rectal carcinoma cell line RKO and Colo 678 were chosen. Apart from referred Human embryonic kidney 293TT cells, breast cancer cell line MCF-7 and monocyte cells were used for experimental search of putative Rep interaction partners.

MSBI1 Rep protein was produced in SoluBL21 bacterial cells. The same transformation steps were followed for the transformation of pUC19 as a negative control (cat no: C700200-GL). After the transformation step, instead of plating, the cell mixture was cultivated in 400ml of LB Amp media and incubated overnight at 37℃ with 130rpm. On the next day, 2ml of Isopropyl β-D-1 thiogalactopyranoside, shortly IPTG lactose metabolizer (Roth) was added to induce protein expression before proceeding to cell lysis.

The cell pellets were obtained from human cell lines Colo 678, RKO, HEK293TT, MCF-7 and primary monocytes obtained from peripheral blood of healthy donors. The cell lines were harvested by scratching cells in 1xPBS + 1% Protease Inhibitor and centrifuged at 300g for 5mins at 4℃. Later, liquid-free cell pellets were stored at – 80℃.

MSBI1 Rep cod opt C-HIS protein from SoluBL21 bacterial cells was up concentrated by coupling with Ni-NTA HIS-beads. After thawing the cell pellets, 8ml lysis Buffer was added and sonified on ice with 30% duty cycle, output for 5x20s. Then, the lysate in 15ml Falcons was incubated for 20mins at 4℃ on shaker and centrifuged for 15mins at 20.000g at 4℃. Meanwhile, HIS-beads (HIS60 NiSuperflow Resin) was prepared by washing with 2ml water and 2ml Lysis Buffer. After adding the HIS-beads, the samples were also incubated with pre-equilibrated HIS-beads for another 30mins at 4°C and centrifuged at 20.000g over 20 minutes

at 4°C. Beads were resuspended in 400µl of Interaction Buffer and stored at 4°C for further procedures. The composition of Lysis and Wash Buffers mentioned in Table 12.

In parallel, whole lysate proteins from each of cell line pellets were precleared using HIS-beads. After thawing the cell pellets, 8ml Lysis Buffer was added and sonified on ice for 5x20s. The lysate was incubated for 20mins at 4℃ on the shaker and centrifuged for 15mins at 20.000g at 4℃. After adding the pre-equilibrated HIS-beads, the samples were also incubated for 30mins at 4°C followed by centrifugation at 20.000g over 20 minutes at 4°C.

For combining the specific proteins with the lysates from different cell lines, around 50µl Ni-NTA coupled MSBI1 Rep protein and Ni-NTA coupled pUC19 control were added separately with each 800µl of lysates. For optimum combination, the samples were incubated for 3h at 4℃ on the rotating wheel. To specify the more distinct protein bands, two different washing concentration was applied where one washing condition with 15mM Imidazole and the other with 30mM Imidazole. Three times washing steps were followed and in each washing step, the samples were centrifuged at 600g for 3minutes, after which the beads were boiled with 150µl of 2.5xSDS loading buffer and stored at -20°C for further analysis (Western blot and Mass Spectrometry).

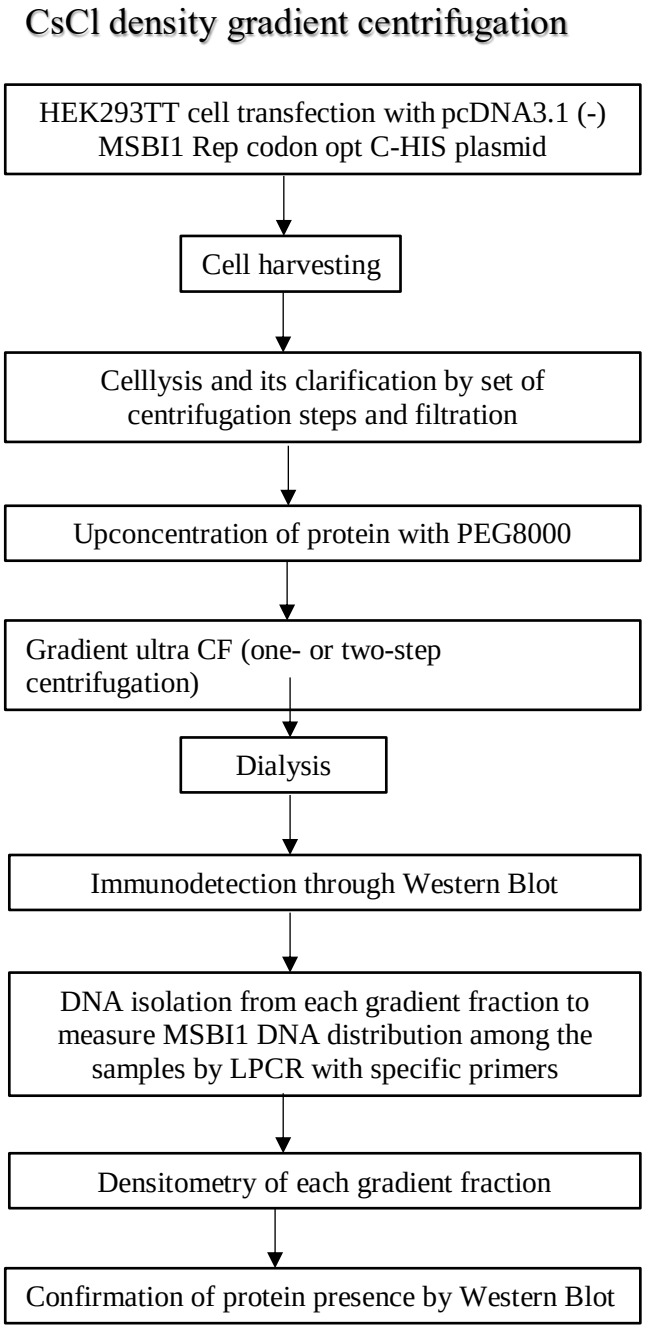
Qualitative and quantitative assessment of prepared samples was done by western blot analysis. All 20 samples were sent to the Core Facility of DKFZ for Mass Spectrometry.

Table 12: The composition of Lysis and Wash Buffers for Immunoprecipitation assay

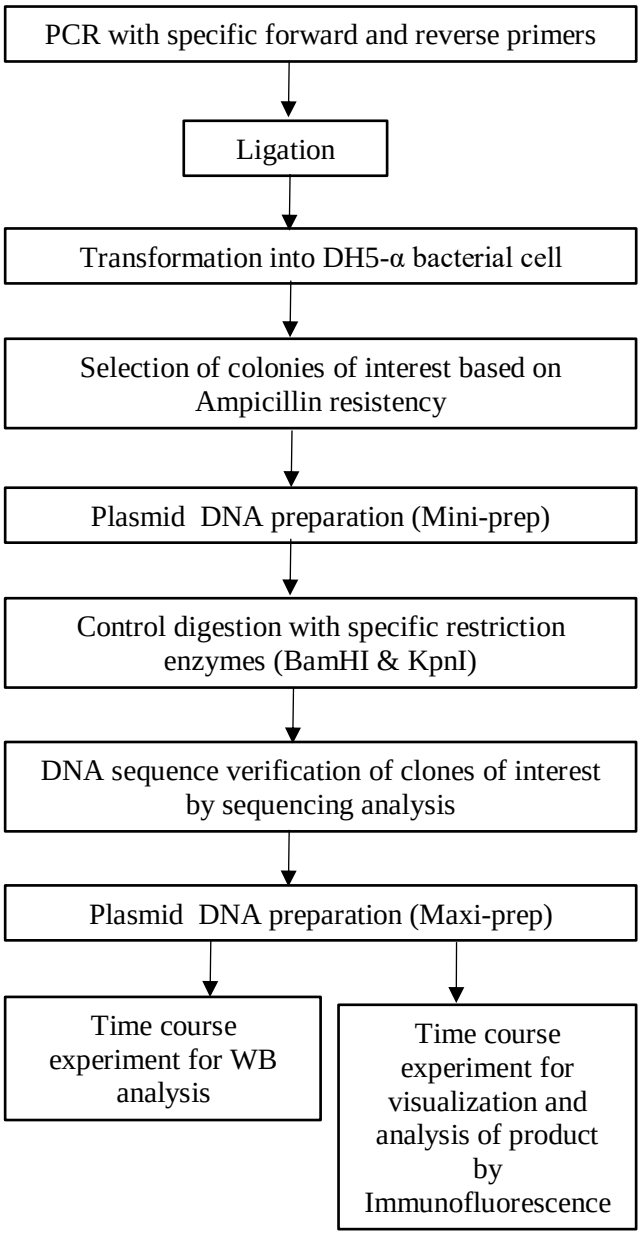
Native lysis buffer/ Interaction buffer
20mM Tris pH 8.0
500mM NaCl
5mM Imidazole
1%Protease Inhibitor

HIS wash buffer
20mM Tris pH 8.0
500mM NaCl
15/30mM Imidazole
1%Protease Inhibitor

Experimental chart representation:



Preparation and Characterization of C-Flag pcDNA3.1 (-) MSBI1 Rep Cod Optimized and N-Flag pcDNA3.1 (-) MSBI1 Rep Cod Optimized plasmids



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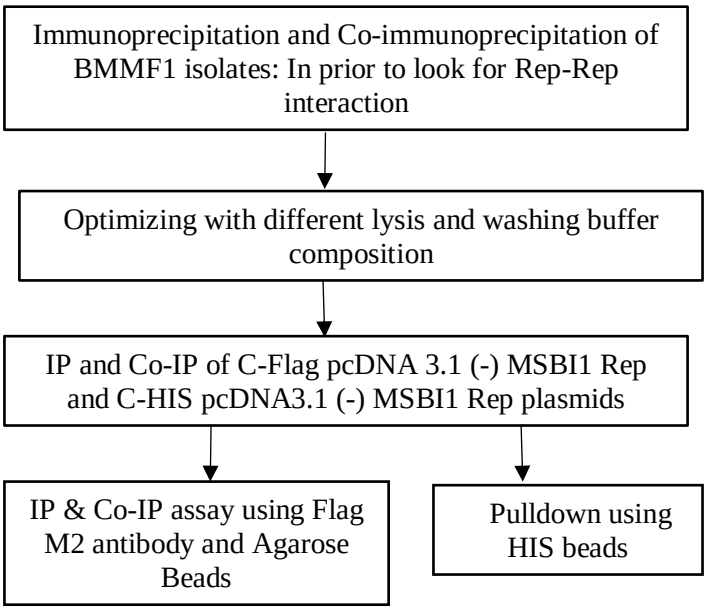
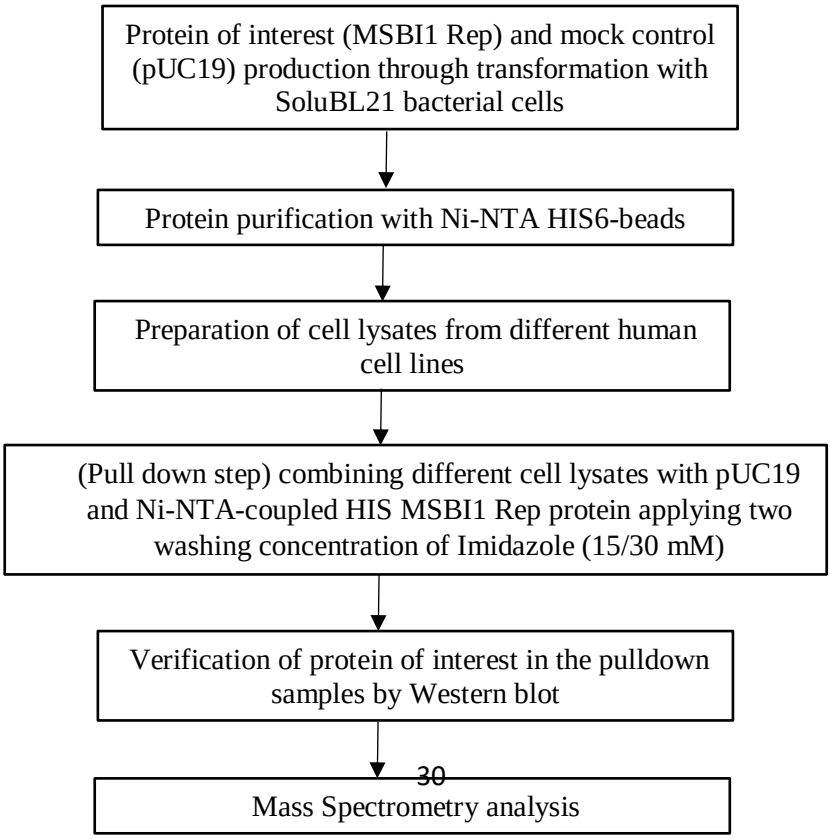
Immunoprecipitation & Co-immunoprecipitation experiment



Interaction partner experiment



3. Results

3.1 BMMF particle/vesicle preparation using the Cesium Chloride density gradient centrifugation

3.1.1. PEG8000 precipitation

Purification of particles or vesicles by the CsCl density gradient using ultracentrifugation was performed based on systematical optimization of clarification steps. Also, some modification steps were followed along with the original method which is described in C. Buck's (2007) reference paper (Buck, C, et al, 2007). In this specific method, the nontoxic density gradient medium was prepared with a CsCl solution. Simultaneously, this gradient-based strategy purifies the particle solution based on both velocity and buoyancy because of its distribution with high-density CsCl and high viscosity sucrose solution. As a result, the empty capsids and host protein and/or DNA impurities disassociate from the DNA containing capsid which migrates and distributes based on the gradient and buoyancy differences.

The sample was chosen to overexpress MSBI1 Rep from the BMMF1 group. The sample was pcDNA3.1 (-) MSBI1 Rep Codon Optimized C-HIS, originally isolated from the brain tissue of a patient with Multiple Sclerosis. To enhance eukaryotic expression, the rep gene which was cloned into the overexpression plasmid was codon-optimized.

On density gradient centrifugation, the particles sediment from the starting zone with velocities where the density of the particle is greater than the density of the gradient. Virus fractions have a higher density than host protein fractions because they include nucleic acid. So, all the particles will eventually form a pellet when it is centrifuged long enough.

Particle formation may initiate directly after sonication of the cell lysate, which represents a mix of the host and Rep proteins, lipids and nucleic acids. Assembly of many virus capsids require the assistance of scaffolding proteins or the viral Nucleic acid, which interact with the capsid subunits to promote and direct the process (Thuman-Commike, P. A et al.,1999). Once assembled, many capsids undergo a maturation reaction that involves covalent modification or conformational rearrangements, which may increase the stability of the particle. During preparative steps, maturation of 2 h was followed for the formation of a capsid or vesicle if it exists. To remove the debris and to obtain purer protein fractions in the liquid phase, two clarification steps of high-speed centrifugation and filtration through 0.8 µm filter were followed by ultracentrifugation. Then, precipitation was followed with and without Polyethylene glycol 8000, shortly PEG8000. The polymer compound up concentrates the particle suspensions. The production of infectious agents might be in lower entities which is unknown, so precipitation with PEG has also performed the test for higher yields. Here, a double ultracentrifugation protocol was performed to more specifically deplete human selectable proteins.

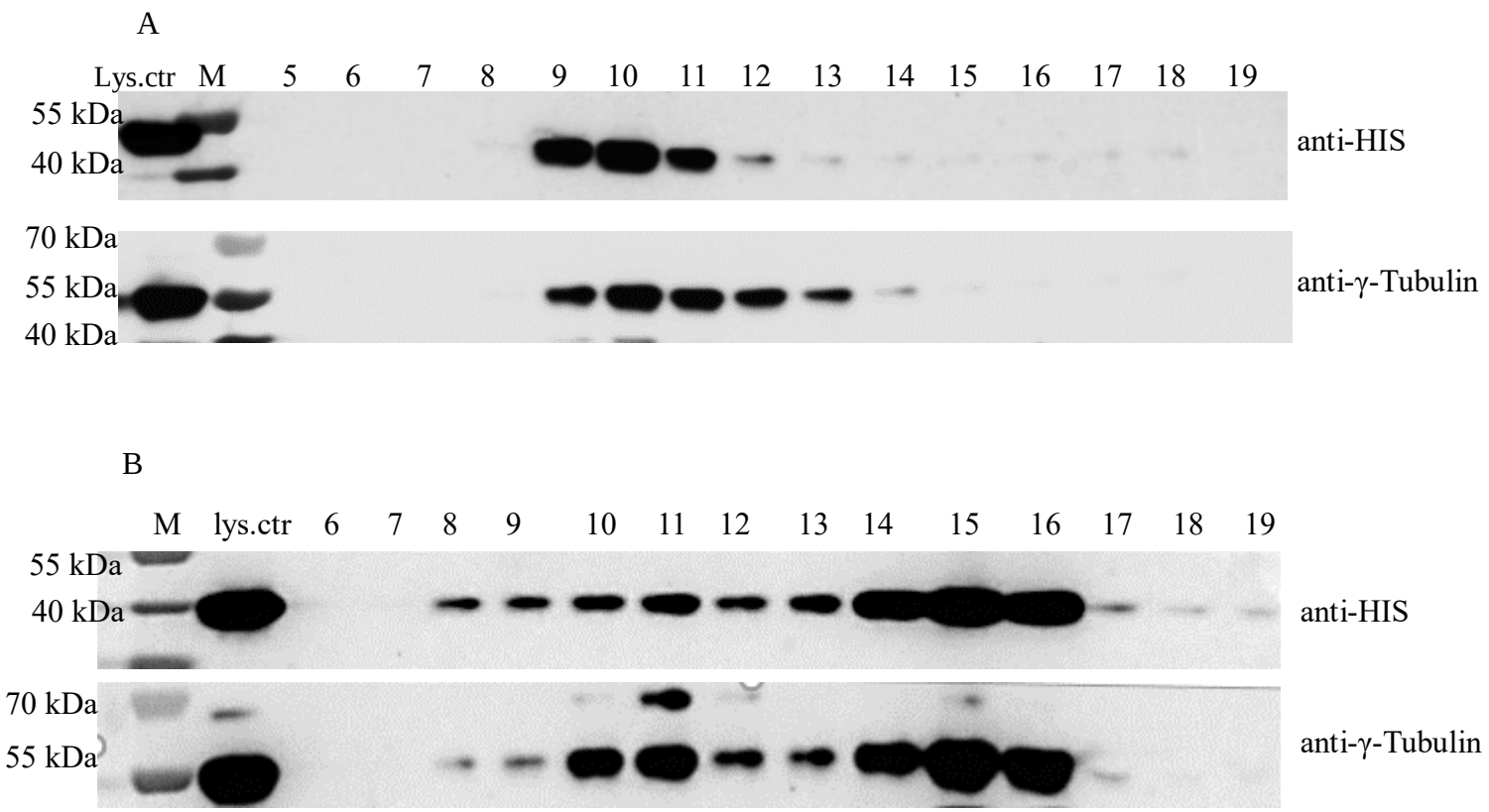


Fig 1: Western blot analysis of BMMF particle preparations without (A) and with (B) PEG8000 after SDS-PAGE. Immunodetection with anti-HIS and anti-γ-Tubulin antibodies performed for fractions after ultracentrifugation. Lys. ctr - a control fraction of BMMF preparation before loading into the CsCl gradient; fractions 5-19 – fractions of BMMF preparation after gradient centrifugation distributed from high to low density (fractions 5 to 19, respectively). The samples without PEG8000 precipitant gave rise to the band distribution from fractions 9 to 12 whereas the background proteins from cells were seen from the fraction 9 to 14. The samples with PEG8000 precipitant gave rise to the band distribution from fractions 8 to 16 with a similar pattern of distribution for the background proteins from cells.

In PEG8000 precipitated samples, more distribution of proteins was observed from fraction 8 until fraction 16 whereas, without PEG8000, the proteins were observed from fraction 9 to fraction 13. Based on western blot results PEG8000 precipitated samples showed higher HIS-tagged-protein load as well as lower penetration of the protein in gradient fraction. Based on this PEG precipitation compare result, the next density gradient sample was prepared with PEG precipitation.

3.1.2. Two-step gradient centrifugation

To get even the purer fractions, two steps of the gradient cycle was performed both with the speed of 50,000rpm for 20hours. After the first gradient, the immunodetected western blot result identified the desired protein from the fraction 7 to fraction 12. Then, the bands were paired up and combined for proceeding to the second gradient cycle. From the interesting fraction of the first gradient, the desired protein was detected from fraction 8 to 11. In both of the western blot results of the gradient, cycles showed the decrease of the density with the increase of fraction numbers which is as expected.

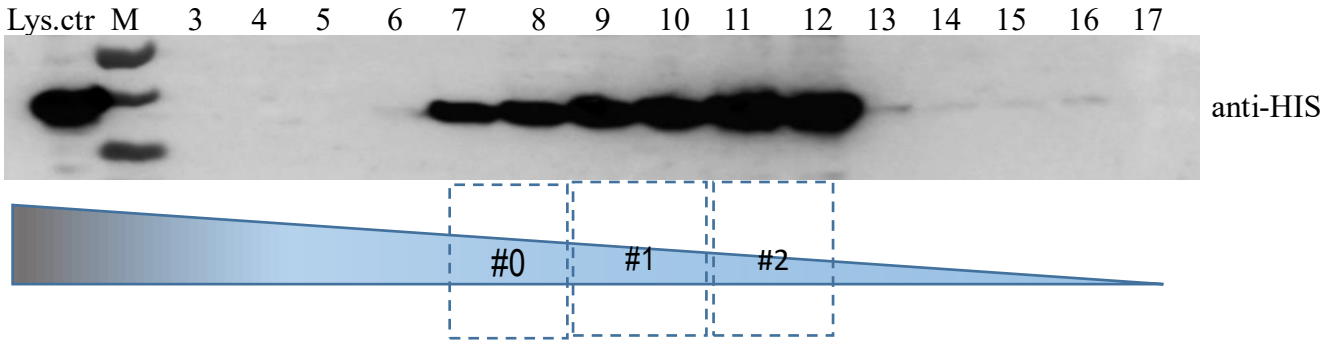


Fig 2: Western blot analysis of the fractions after the first gradient cycle. Immunodetection with the anti-HIS antibody. The results show the distribution of protein bands from the fraction 7 to 12. The fractions 7, 8 combined into fraction #0, fractions 9, 10 – into fraction #1 and fractions 11, 12 – into fraction #2 for the second gradient cycle. The triangle bar indicates the consistency from shifting the pattern from high density to low density.

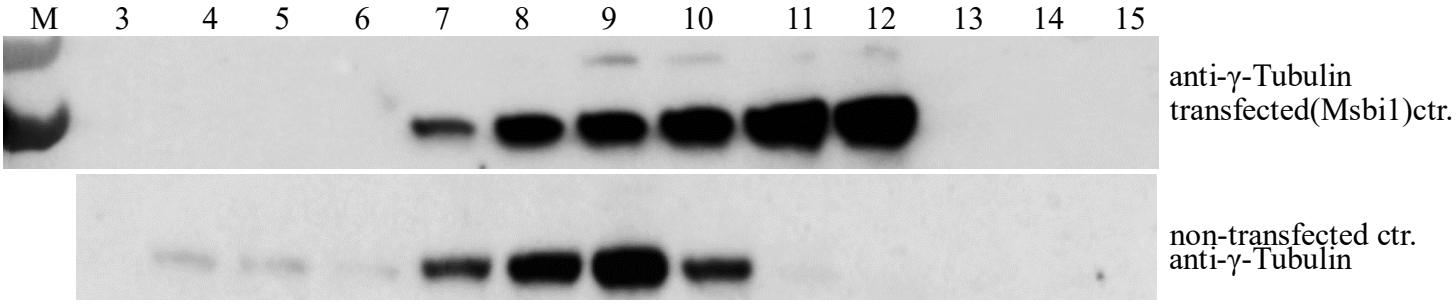


Fig 3: Immunodetection of the first gradient fractions of transfected and non-transfected controls when exposed to anti-γ-Tubulin. The Western blot image upon exposure to the anti-γ-Tubulin antibody for the transfected control samples (upper blot) showed a higher level of cellular background from fractions 7 to 12 whereas non-transfected control (bottom blot) detected lower level from fraction 7 to 10.

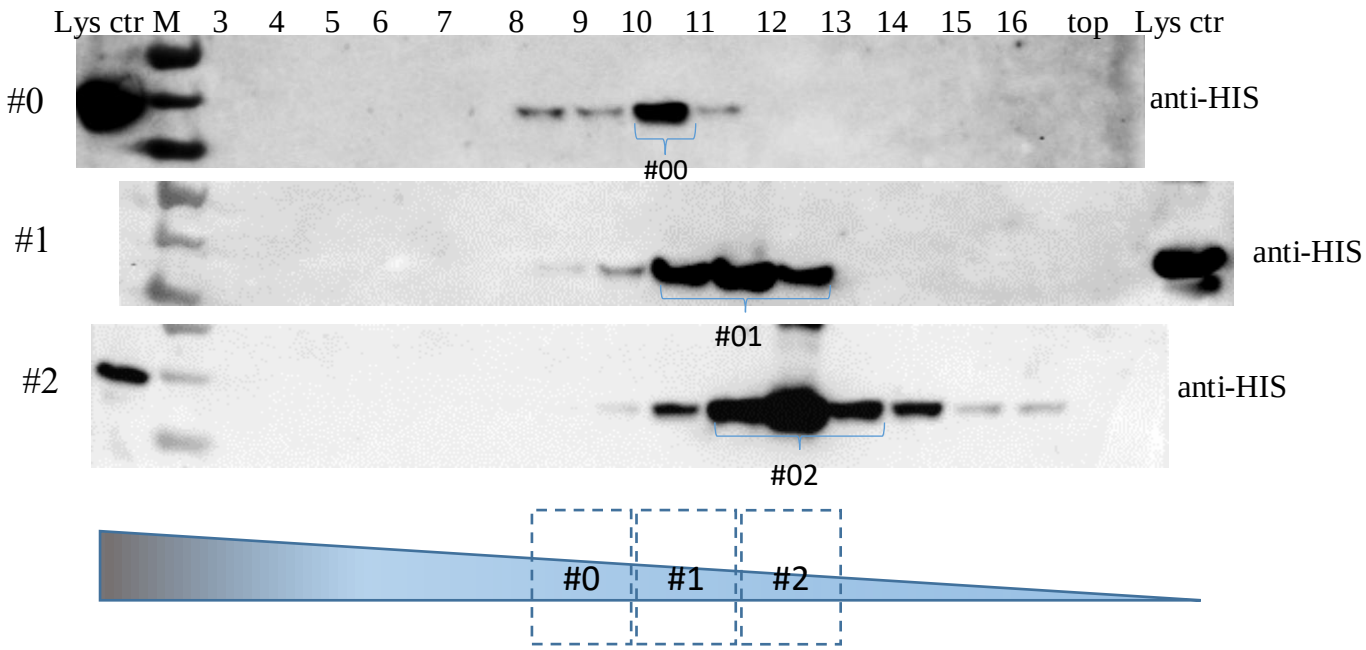


Fig 4: Western blot analysis of the collected fractions after the second gradient cycle. Western blot result with anti-HIS antibody showed the distribution of protein bands for all the #0, #1, #2 fractions of the first gradient cycle. The fraction 10 from #0, fraction 10, 11, 12 from #1 and fraction 11, 12, 13 from #2 samples of the first gradient were similarly combined and separated as #00, #01 and #02 for the second gradient. The triangle bar indicates the consistency from shifting the pattern from high density to low density.

Based on the western blot results, samples containing high and low-density fractions were pooled separately and dialyzed using the G2 dialysis cassette. After collecting the fractions, protein detection through western blot was followed to ensure the presence of the MSBI1 protein. The blot was also exposed to the gamma-tubulin antibody where gamma-tubulin is universally expressed and acts as the loading control allowing quantification of the cellular background.

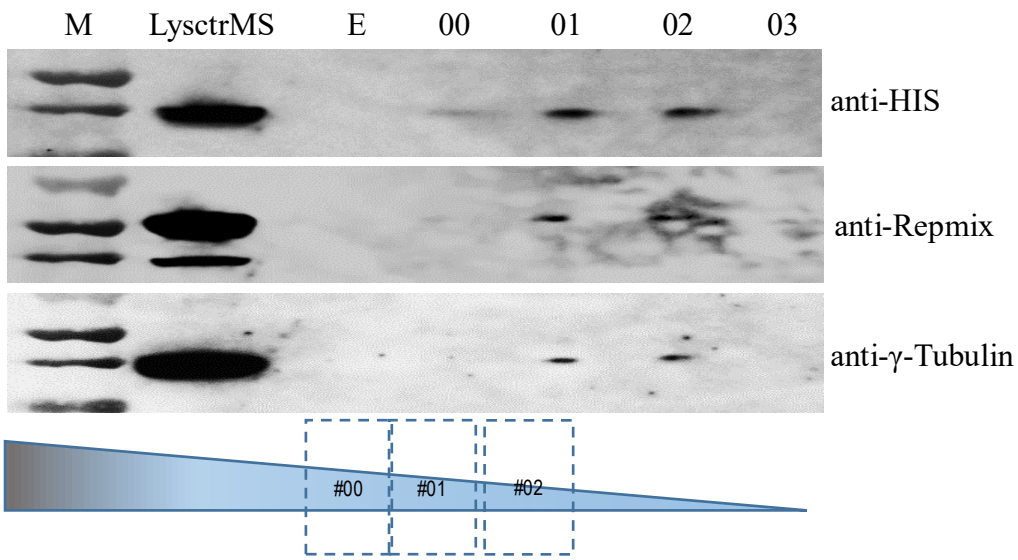


Fig 5: Western blot analysis for validation of MSBI1 Rep protein products following two-step density gradient centrifugation. SDS-PAGE analysis using the same blot with anti-HIS, anti-Rep mix and anti-γ-Tubulin antibodies performed for pooled fractions after dialysis. Lys. Ctr - a control fraction of BMMF preparation before loading into the CsCl gradient cycles. The second gradient samples showed protein bands for the transfected 00, 01 and 02 controls but at comparatively low levels. Here, fraction 03 was the non-transfected control (prepared from cells without transfected DNA) fraction of the second gradient cycle. With anti-HIS antibodies, protein bands showed up for all the positive controls even though the fraction 00 showed very faint band. While exposed to anti-Rep mix antibodies, protein bands showed up only for 01 and 02. With anti- γ -Tubulin exposure very less amount of cellular background showed up for the transfected control fraction 01 and 02.

The differences in protein detection upon exposure to different antibodies (anti-HIS vs anti-Rep) is due to the sensitivity and specificity of the proteins for immunodetection. But the presence of MSBI1 is confirmed in the transfected controls.

3.1.3. Density measurement through Refractometry

To measure the density of fractions after gradient centrifugation assessment of Refraction Index (RI) was applied to all fractions before dialysis.

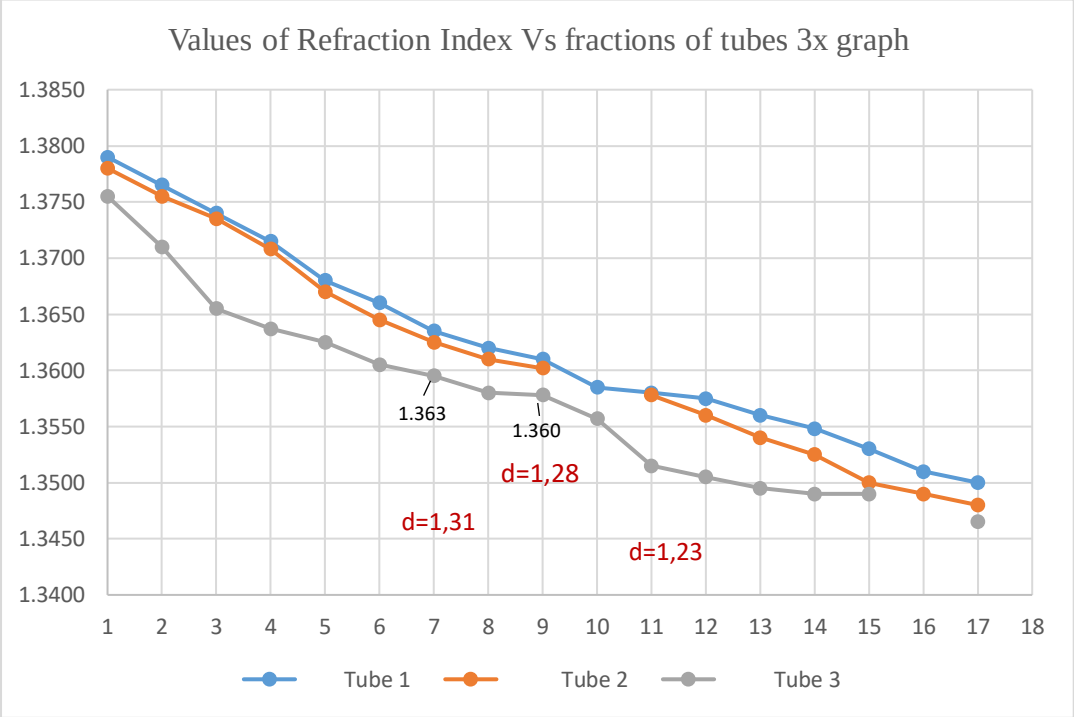


Fig 6: Graphical representation of density measurement from the refraction index of the fractions. Tube 1, 2 and 3 define the samples of transfected control after the second gradient cycle. Tube 1, 2 and 3 correspond to all the fractions starting from 1 to 17 for each of the second gradient samples #00, #01 and #02.

The Graphical representation of density measurement showed a decrease of density with the increase of the number of fractions. The refraction index varied from 1,363 to 1,351 in the fraction 7 to 12, respectively. The RI of the lowest fraction displaying the protein of interest (8 and 9) corresponds to a density of 1, 31.

3.1.4 Long PCR

To detect the specific full-length MSBI1 product, long Polymerase Chain Reaction (LPCR) with specific primers was performed.

During the steps of purification, Benzonase was added to degrade and remove input DNAs not protected by protein. Nevertheless, as it can be seen in the 1.5% agarose gel, all the PEG dialyzed MSBI1-transfected samples contain the desired DNA.

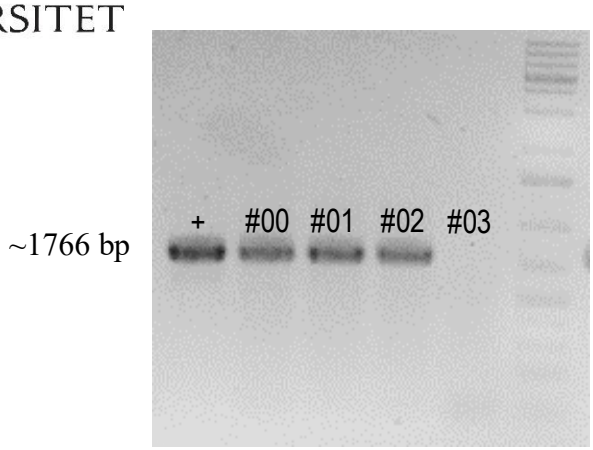


Fig 7: Agarose gel image of the MSBI1 Rep specific products obtained by LPCR using the primers indicated in Table 3. The LPCR of the dialyzed samples was conducted for determining the presence of DNA. The 1.5% agarose gel image where isolated DNAs from the second gradient was loaded. The desired band size for MSBI1 specific product is approximately 1766bp. PCR product from the samples of the second gradient detected DNA in all the transfected controls. Here, #00, #01, #02 are the transfected control with MSBI1 plasmid and #03 is the non-transfected control. The positive (+) sample is the pcDNA3.1 (-) MSBI1 Cod Opt HIS6 plasmid. A DNA ladder was used as the reference at the farthest right well.

In brief, DNA and low amount of proteins were detectable after density gradient centrifugation but more evidence is needed to confirm whether it forms infectious particle or extracellular vesicle.
The next strategy was to look for the possible interaction of MSBI1 Rep either with other BMMF Rep protein or with the full genome. Then, depending on the interaction, the combined interacting macromolecules will be subjected to viral particle preparation following the same CsCl density gradient centrifugation protocol.

3.2 Preparation and Characterization of C-Flag pcDNA3.1 (-) MSBI1 Rep Codon Optimized and N-Flag pcDNA3.1 (-) MSBI1 Rep Cod Optimized plasmids

Immunoprecipitation (IP) and Co-Immunoprecipitation (Co-IP) are powerful techniques to explore the interaction partners among the other BMMF isolates. Overexpressed plasmids which were Flag-tagged in C- or N- terminus were prepared and produced to proceed with IP and Co-IP experiments.

3.2.1. Plasmid preparation

The prepared plasmids were C-Flag pcDNA3.1 (-) MSBI1 Rep Codon Optimized and N-Flag pcDNA3.1 (-) MSBI1 Rep Cod Optimized plasmids. Overexpression plasmid allowing expression of Rep protein fused to an N- or C-terminal 3xFlag tag were generated by PCR production of the Rep gene insert with terminal primers including the 3xFlag encoding sequence and specific restriction sites for interstation of the linear PCR product into the target vector. After PCR, the products were digested, combined with the restricted target expression vector and ligated to embed the desired amplicon into the expression vector, pcDNA3.1 (-). The transformation was performed using highly efficient DH5α bacterial cells. The transformed solution was drop-plated on LB Amp agar plates. Thereafter, transformed bacterial plasmids exhibiting as the white colonies were observed on the next day of plating.

Single colonies were picked and incubated overnight in the LB Amp solution for DNA preparation (Mini-Prep), control restriction and sequencing to verify the successful insertion.

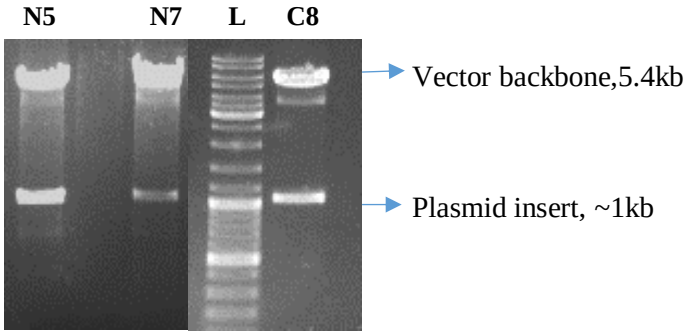


Fig 8: DNA characterized by agarose gel separation. Image of gel after mini-prep and control digestion with KpnI & BamHI. Three positive clones were identified where two clones N5, N7 for N- terminal Flag MSBI1 plasmid and one clone C8 for C-terminal Flag MSBI1 plasmid.

The DNAs were loaded on 1% Agarose gel and the gel electrophoresis showed three positive clones where two were for N-Flag pcDNA3.1 (-) MSBI1and the rest one for C-Flag pcDNA3.1 (-) MSBI1. On the gel, two distinct bands were seen for the same sample. The upper band is the vector backbone with the base pairs of 5.4 kb and the lower band is the predicted insert with the approximate band size of 1000 bp. Using the website of Eurofins company, the chosen primers were pcDNA3.1-FP and pcDNA3.1-RP1. These primers reside nearby the cloning cutting site which covers the desired insert.

3.2.2. Sequence verification

Positive cloned samples were further confirmed through sequencing.
The alignment was performed taking the original MSBI1 Rep sequence. The alignments of the two sequences were verified through Clustal Omega.

```
Desired sequence   GTGGATCCGCCATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATT
Fwd sequencing     GTGGATCCgccATGGACTACAAAGACCAtgACGgtgATTATAAAGATCATGACATCGATT
Rev sequencing     GTGGATCCGCCATgGACTACAAAGACCATGACgGtGAtTaTAAAGATCATGACATCGATT
                   ************************************************************

Desired sequence   ACAAGGATGACGATGACAAGGGAGCAAGCGACCTGATCGTGAAAGACAATGCCCTGATGA
Fwd sequencing     ACAAGGATGACGATGACAAGggagcaAGCGACCTGATCGTGAAAGACAATGCCCTGATGA
Rev sequencing     ACAAGGATGACGATGACAAGGGAGCAAGCGACCTGATCGTGAAAGACAATGCCCTGATGA
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Fig 9: Sequence data obtained using specific forward and reverse primers. The fragment of alignment (clustal omega O-1.2.4) showed the restriction sites for BamHI and KpnI (yellow) consisting of the desired plasmid insert (gray) with Flag sequence in C terminus (green).

Sequencing results indicate no unwanted point mutations or insertions/deletions when compared to the template sequence. This defines the verification of successful insertion of the MSBI1 Rep sequence and Flag tag sequence of the newly cloned plasmids.

3.2.3. Measurement of protein production over time

3.2.3.1. Measurement of protein production over time by Western Blot analysis

After verification of successful insertion of Rep and Flag sequence, a time-course Rep overexpression experiment was conducted with the newly produced plasmids. The overexpressed plasmids were transfected by Polyethyleneimine in HEK293TT cells and protein expression was performed for 24 h, 48 h, and 72 h. In this time-course experiment, overexpression of HIS6 tagged pcDNA3.1 (-) MSBI1 Rep Cod Opt was used as a reference. Protein expression levels were observed by immunodetection based on Western Blot and immunofluorescence microscopy.

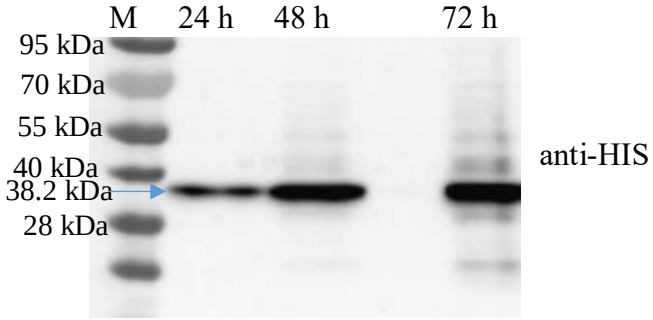

Fig 10: Western blot analysis of time-course pcDNA3.1 (-) MSBI1 Rep Cod Opt HIS6 preparations. Western blot analysis with anti-HIS antibody performed for samples. HEK293TT transfected with pcDNA3.1 (-) MSBI1 Rep Cod opt C-HIS was harvested at 24, 48 and 72 h after transfection. The harvested samples showed distinct bands and an increase protein production within time. The expected band size for the HIS tagged fusion protein is around 38.2kDa which showed up for the pcDNA3.1 (-) MSBI1 Rep Cod Opt HIS6 plasmid protein.

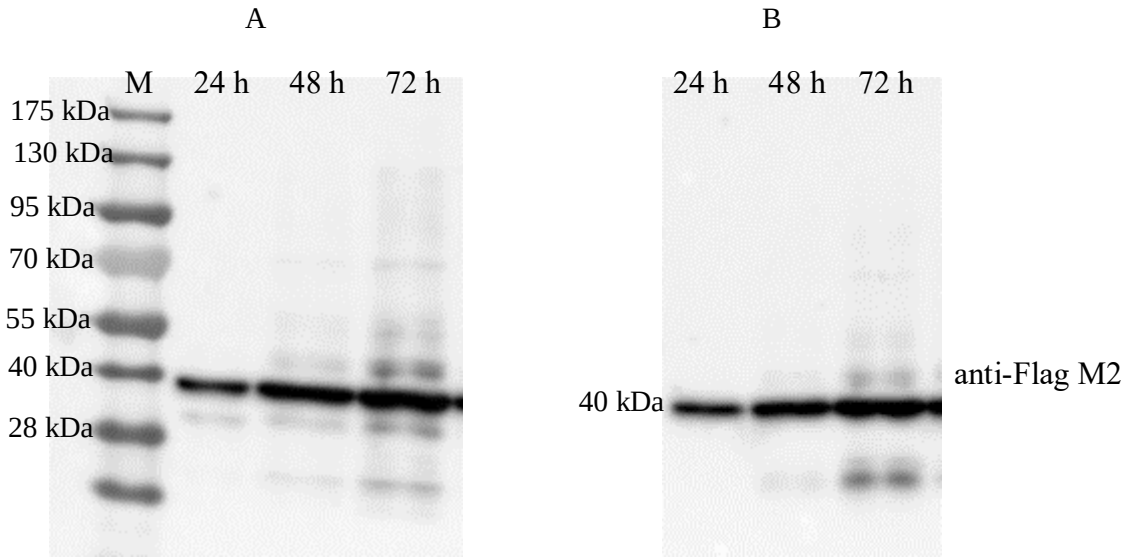

Fig 11: Western blot analysis of time-course (A) C-Flag and (B) N-Flag pcDNA3.1 (-) MSBI1 Rep Cod Opt preparations. Western blot analysis with anti-Flag M2 antibody for samples. HEK293TT transfected with C-Flag and N-Flag pcDNA3.1 (-) MSBI1 Rep Cod Opt was harvested at 24, 48 and 72 h. Immunodetection results showed clear bands of the MSBI1 Rep protein for the overexpressed plasmids.

Western blot analysis of overexpressed protein showed a high level of expression already after 24 h expression which still increases after 48 h and 72 h expression time for all three analyzed plasmids. The size of the band matched with the expected size of the Rep protein which is approximately 41kDa. The additional band might be the truncated variants of the modified Rep.

3.2.3.2. Visualization of protein production over time by Immunofluorescence

The production and analysis of overexpressed Flag-tagged Rep fusion protein were also analyzed by immunofluorescence microscopy. Therefore, HEK293TT cells were transiently transfected with the two overexpression plasmids followed by overexpression for 24 h, 48 h, and 72 h. Then immunofluorescence microscopy was performed based on two fixation protocols (methanol or Paraformaldehyde, shortly PFA) and following preparation. Specific anti-Flag M2 primary mouse antibodies were used in combination with anti-Rep GP primary antibody produced in the guinea pig. GP anti-Rep as a polyclonal antibody detects both cytoplasmic and aggregates Rep. Immunodetection acquired with the mouse primary detection antibodies was coupled with goat-anti mouse Alexa Fluor 488 secondary antibodies and the signaling from guinea pig species with goat anti-guinea pig Alexa Fluor 546 secondary antibody.

Protein production was observed at comparable levels for all fusion proteins analyzed. Also, the cell distribution of the detection signals was comparable for all applied antibodies. The increase of signal from 24 h to 72 h was detected in all transfected conditions which are depicted in Figure 13. Mild differences in signal detection were observed in some samples dependent on the type of fixative used. These differences could occur due to various compatibility of antibodies and cell structure fixing reagents as well as due to technical reasons.

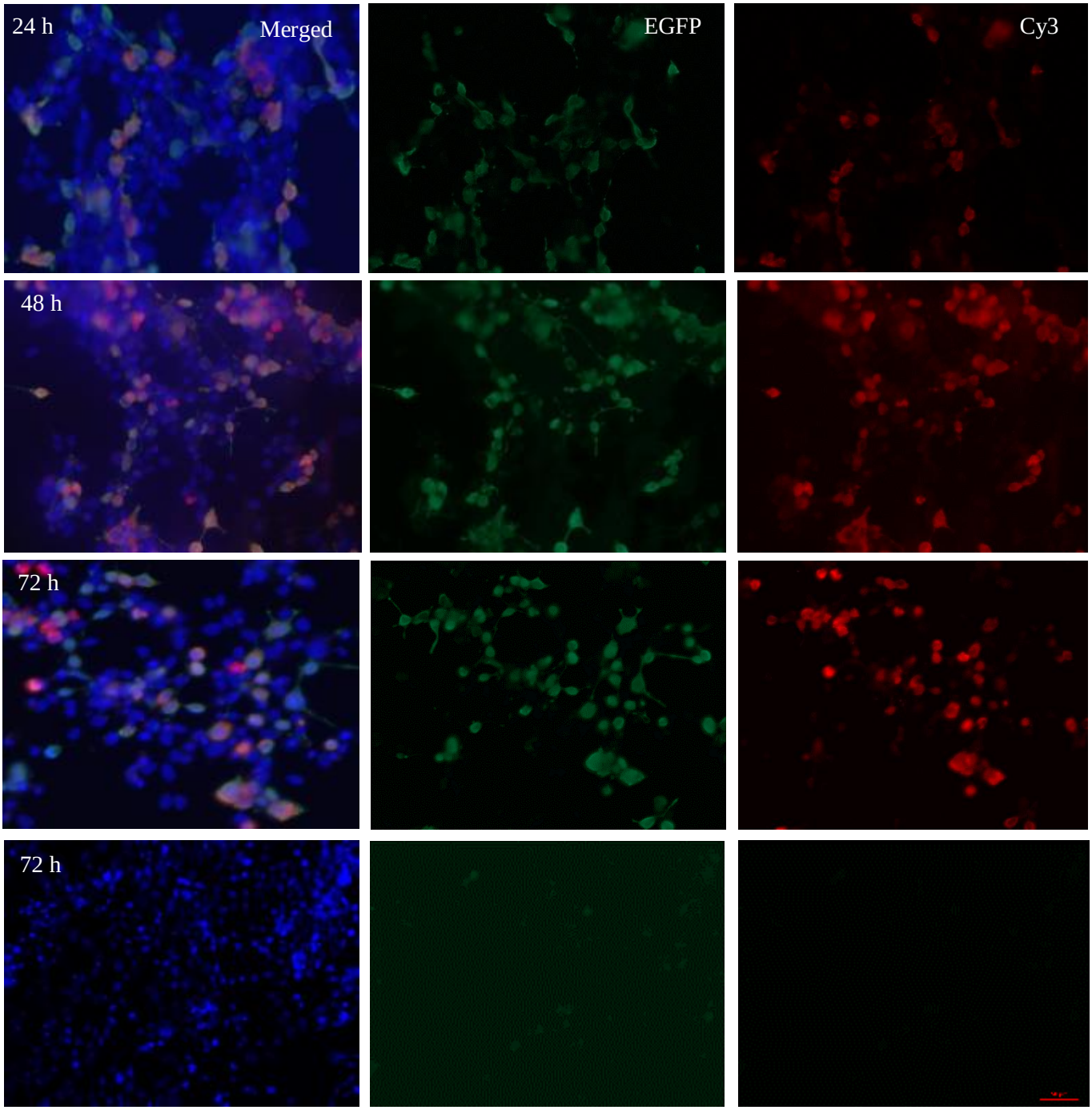

Fig 12: Immunofluorescence staining of HEK293TT cells with C-Flag pcDNA3.1 (-) MSBI1 Rep Cod Opt protein production incubated with anti-Flag M2 and anti-Rep-GP primary antibodies for 24 h, 48 h, and 72 h and negative control after fixation with methanol. Blue stain represents the staining of cell nuclei under the DAPI channel. For 24 h protein observation, the parallel three pictures correspond to the exposure upon merged (blue+green+red), mouse (green) and GP (red) detection. Bottom pictures represent a "secondary" negative control (plasmid transfected cells exposed to secondary antibodies) after 72h of incubation.

Very discrete red and green signals were seen along with the clear cytoplasmic distribution when fixed with PFA chemical. The Flag M2 specific epitope tag defining antibody was used to identify the aggregates which are frequently observed for MSBI1.176 Rep overexpression. Due to protein translation and accumulation in the cell, it increases with longer incubation times. Immunofluorescence assays showed clear signal distribution for both cases of N-Flag and C-Flag plasmids. Herein, anti-tag based detection was followed through using Flag M2 antibody since both plasmids were Flag-tagged. Rep protein was detectable already after 24 hours after transfection and its production increased within time.

3.3 Immunoprecipitation and Co-Immunoprecipitation: Rep-Rep Interaction

To elucidate the interaction between BMMF proteins through immunoprecipitation, co-Immunoprecipitation was also followed for detecting the potential interaction partner. Before initiating the IP/Co-IP experiments, the desired proteins were overexpressed based on transiently-transfected HEK293TT cells in 10 cm culture plates as mentioned above. 72 h after transfection, cells were washed and harvested in ice-cold 1xPBS. To prevent protein degradation, all the steps of harvesting were done on ice in the presence of 1% protease inhibitor cocktail (Sigma, p8340). Cells were centrifuged again and frozen at -80 °C.

Combinations of different BMMF proteins have been analyzed to check interaction with each other which might be important for complementing assembly of infectious particles or vesicles. To establish a robust protocol for immunoprecipitation, codon-optimized expression plasmids were used to allow maximum expression levels of Flag- and HIS-tagged MSBI1 Rep.

C-terminal and N-terminal Flag-tagged MSBI1 Rep codon-optimized proteins have been prepared to observe the effects of tags and protein overexpression itself, as an N-terminal Flag tag might have different consequences for protein interaction compared with a C-terminal Flag tag. Also, protein stability might be modified in different ways. Agarose A/G beads were used to bind the tag-specific Flag-antibody which is coupled to the Flag-tagged target protein. In co-immunoprecipitation, the tagged beads will capture the similar tag protein (bait) which may drag down the interacting proteins (prey) therefore, the interacted protein complex can be precipitated and detected through western blot analysis when exposing to prey protein-specific antibodies. In co-immunoprecipitation, immunodetection with other tag-based antibodies will identify the presence of prey protein if the suspected interacting molecule exists. In IP and Co-IP experiment, double plasmid transfection with two different tags of overexpressed plasmid was used to observe the transfection. IP and Co-IP experiments were being performed through changing different lysis, washing buffer conditions and attempting various steps so that clear detection of these proteins from the BMMF1 group can be perceived.

To identify the desired MSBI1 Rep proteins as well as protein networks, the specific HIS or Flag-tagged Rep antibodies were used. Yet, a set of major specifications is needed for optimization and full characterization of the generated assemblies of proteins and nucleic acids.

3.3.1. Immunoprecipitation and Co-Immunoprecipitation using Flag M2 antibody

Mechanism: Immunoprecipitation of Flag-tagged protein performed based on agarose beads coupling with Flag M2 antibody which leads to precipitation only the Flag-tagged proteins. In the case of co-immunoprecipitation, one tag biased protein will pull down the whole complex if there exists any interacting macromolecule. After loading the precipitated protein samples into the gel, the transferred blot paper was incubated overnight with each of the tag-based antibodies. Since Flag-tag-based immunoprecipitation was performed, the incubation with mouse anti-Flag M2 antibody will detect only the Flag-tagged proteins. On the other hand, the

incubation with mouse anti-HIS antibody will detect only the HIS tagged protein, which can only be detected if co-immunoprecipitation occurred.

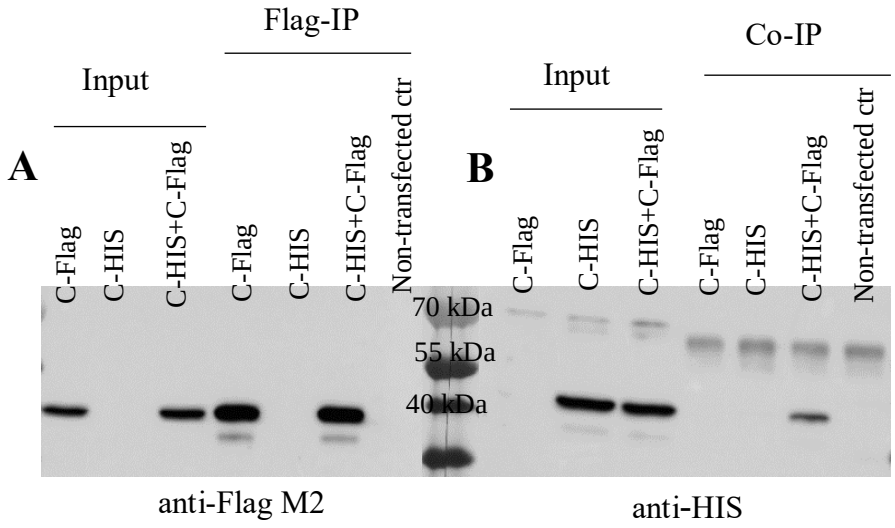

Fig 13: Immunoblot analysis of IP and co-IP assay to demonstrate the interaction of Flag- and HIS-tagged MSBI1 proteins. Immunoprecipitation performed with Flag antibody and agarose beads (A, B). Input defines sample before incubation with Flag antibody and agarose beads; IP-immunoprecipitations with Flag antibody and agarose beads with anti-Flag M2 immunodetection; Co-IP- coimmunoprecipitation when the interacting HIS tag protein detected in the whole protein complex when immunodetection with anti-HIS antibodies.

To establish the experiment, single plasmid transfection (pcDNA 3.1 (-) C-Flag MSBI1 Rep Cod Opt or pcDNA 3.1 (-) C-HIS MSBI1 Rep Cod Opt), the combination of two plasmids (pcDNA 3.1 (-) C-Flag MSBI1 and pcDNA 3.1 (-) C-HIS MSBI1 Rep Cod Opt) were used.

The determined molecular weight for the MSBI1 HIS fusion protein is 38.2 kDa and 3xFlag tag fusion protein is 40.1 kDa. HEK293TT cells were single and double transfected with C-Flag and C-HIS pcDNA3.1 (-) MSBI1 Rep Cod Optimized plasmids. After 72 hours, cells were harvested and subjected to Flag tag- and antibody-based immunoprecipitation. The results showed the need to optimize the lysis and washing conditions so several steps of method adjustments were performed. In order to get the optimum protein detection Lysis Buffer 1 (50 mM Tris, 150 mM NaCl, 1% Triton X-100+1% protease Inhibitor) and Washing buffer X (50 mM Tris, 150mM NaCl, 0.5% Triton X-100, 0.05% SDS +1% protease Inhibitor) were optimal for immunoprecipitation with Flag M2 antibodies and agarose beads.

As seen from Figure 14, after immunoprecipitation with Flag M2 antibody two bands, as expected, were detected after membrane incubation with anti-Flag antibody – in single and co-transfection samples. Incubation of the same samples with anti-HIS antibody allowed to detect a HIS-tagged protein in the co-transfected sample indicating the interaction of Flag-tagged and His-tagged Rep. But, dimers were not observed for the double plasmid transfection positive control.

3.3.2. Pulldown using HIS beads

HIS-tagged proteins were pulldown with Ni-NTA agarose which is most commonly used for His-tag purification since it gives higher yield. Low concentrations of imidazole were applied to provide higher specificity and purity of procedure. Imidazole competes with the His-tag for binding to the metal-charged resin. A low concentration of imidazole is added to both binding and wash buffers to interfere with the weak binding of other proteins and to elute any proteins that weakly bind.

To get the optimum protein detection and to avoid the non-specificities, different composition of lysis and washing buffer was used from where the optimum results were obtained. Finally for HIS-pulldown experiment Lysis Buffer 3 (50mMTris, 150mM NaCl, 1% Triton X, 10mM imidazole +1% protease Inhibitor) and Washing Buffer N (50mMTris, 150mM NaCl, 0.5% Triton X, 0.05% SDS, 30mM imidazole +1% protease Inhibitor) showed the desired or expected results.

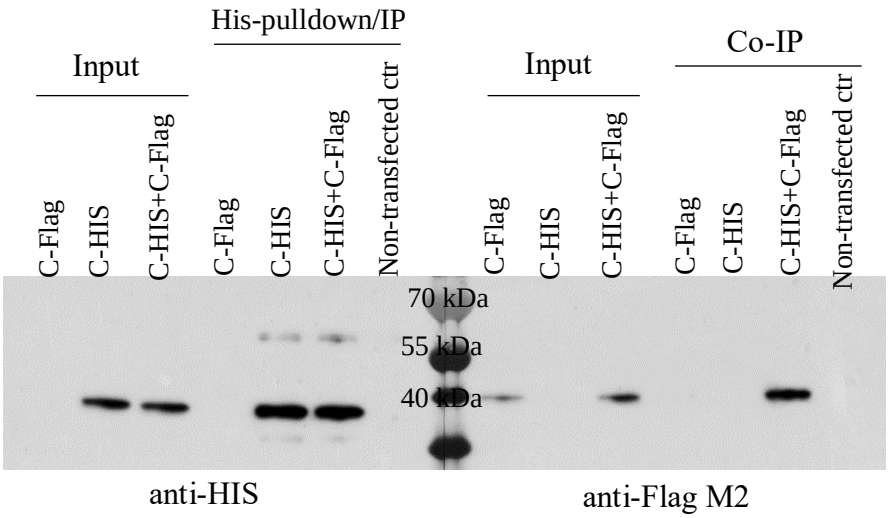

Fig 14: Immunoblot analysis of IP and co-IP assay to demonstrate the interaction of Flag- and HIS-tagged MSBI1 proteins. Pulldown performed with HIS beads (C, D). Input defines sample before incubation with HIS beads; IP- immunoprecipitations with HIS beads with anti-HIS immunodetection; Co-IP- coimmunoprecipitation when the interacting Flag tag protein detected in the whole protein complex when immunodetection with anti-Flag antibodies.

HIS-pulldown experiment verified data obtained with Flag-based IP presented above. The interaction of Flag and HIS tagged Rep proteins observed from the representative images (Figure 14) since the desired MSBI1 Rep protein band of the Co-IP showed up for the double transfected plasmids upon exposure with anti-Flag antibody.

Upon exposure to anti-HIS and anti-Flag antibodies, distinct bands were seen as expected for pcDNA3.1 (-) MSBI1 Rep cod opt and combination plasmids. In HIS beads based IP & Co-IP, dimers were observed only in a single (C-HIS MSBI1 Rep) and double(C-HIS+C-Flag MSBI1 Rep) transfected plasmids when exposed to anti-HIS antibodies. Unfortunately, no dimers were observed for the Flag beads and antibody-based IP and Co-IP experiments.

So, HIS pull-down and Flag Immunoprecipitation and Co-immunoprecipitation worked perfectly well. Based on these optimized conditions next steps of IP and Co-IP with transfections of BMMFs isolates from groups 1 and 2 can be conducted.

3.4. Identification of cellular Rep-interaction proteins

A pull-down assay based on His-tagged MSBI1.176 Rep was performed using HIS-resin beads. This pull-down assay was used to screen cell lysate preparation of four different humans immortalized cancer cell lines (Colo 678, RKO, MCF-7, HEK293TT) and a macrophage lysate preparation for the presence of Rep-interaction proteins. In this experiment, HIS affinity beads were used to immobilize the desired tagged protein, MSBI1 Rep HIS, and also putative Rep interaction partners. Rep HIS containing samples were produced by Rep overexpression insoluBL21 bacterial cells. Here, pulled down assay was performed where the tag-based beads attach with the bait protein (MSBI1 Rep) and later, the immobilized protein was incubated with the cell lysates of different cell lines assuming a putative interaction partner could specifically bind the immobilized Rep. The amount and purity of the bead-coupled pull-down protein was assessed by protein quantification by Bradford Assay and Coomassie protein staining. In the colorimetric assay, serial dilutions of bovine serum albumin (BSA) was used as the standard protein control. Also, Bradford reagent was used to measure the concentration of the protein based on the absorbance level of the staining dye. Additionally, Coomassie staining was conducted to confirm the presence of adequate amounts for Rep proteins for incubation with the five different cell lysates. In Coomassie staining, dilutions of Bovine Serum Albumin (BSA) with known concentration were loaded onto the gel as controls allowing densitometric estimation of protein amounts.

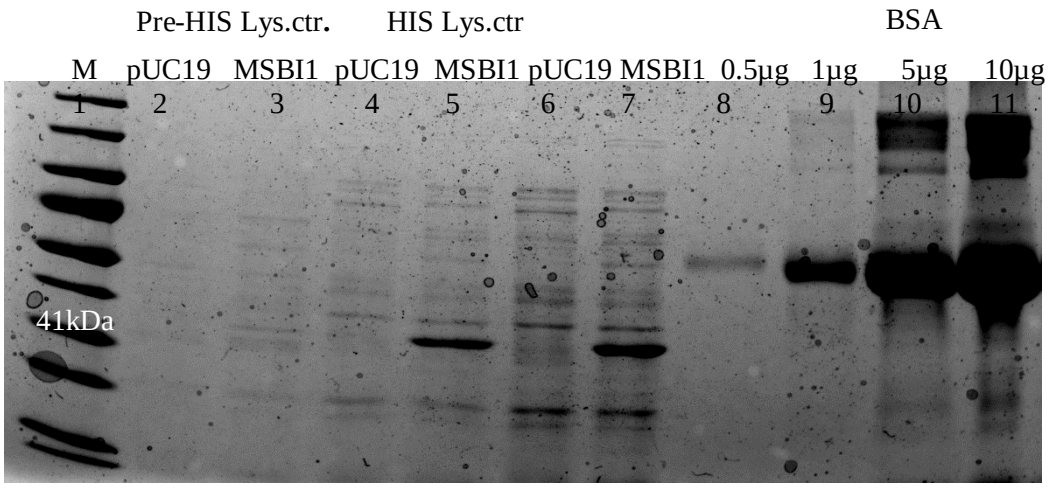

Fig 15: Coomassie-stained gel representation which demonstrates the confirmation of the presence of MSBI1 Rep protein after protein preparation based on soluBL21 cell system. Coomassie staining with BSA as the control. pUC19 is the control vector and MSBI1 is the sample of interest. Pre-HIS lys.ctr- the lysate controls before adding the beads and after HIS lys. ctr is the lysates after pulling down with HIS beads. Two different concentrations 5 µl and 10 µl of MSBI1 Rep lysates were loaded on the gel (lanes 5 and 7, respectively). Four different concentration of BSA was loaded which include 0.5 µg, 1 µg, 5 µg, 10 µg (lanes 8-11).

The pattern of protein distribution was similar between positive and negative control samples. The positive MSBI1 band showed dominant at the expected lane of 41kDa and was absent in the pUC19 sample.

From the above coomassie staining, there were the positive bands for pExp5 MSBI1 Rep-6HIS detected for the loaded concentration with 5 µl and 10 µl. The pull-down assay proceeded and the 4 µg of pull-down protein was incubated with the cell lysates to allow protein interaction. Five different cell lysate preparations were chosen to observe whether interaction takes place with any of the cellular proteins: HEK293TT, rectal carcinoma cell line RKO, colon carcinoma cell line colo 678, breast cancer tissue MCF-7 and peripheral blood. Then a washing procedure was performed based on two individual protocols including either milder washing with 15 mM imidazole or a harsher washing condition with 30 mM imidazole to remove non-interacting background proteins of the cell lysates. After that, the immobilized interaction complexes were pulled down by complete denaturation in the Lämmli loading buffer. As a negative control, the empty vector pUC19 was also taken as a reference for the Rep-containing pull-down preparations.

Hence, protein detection through western blot was employed to confirm the presence of the desired protein in pulling down with different cell lysates. All the blots were exposed to an anti-Rep mix antibody for immunodetection of the Rep antigen of BMMF.

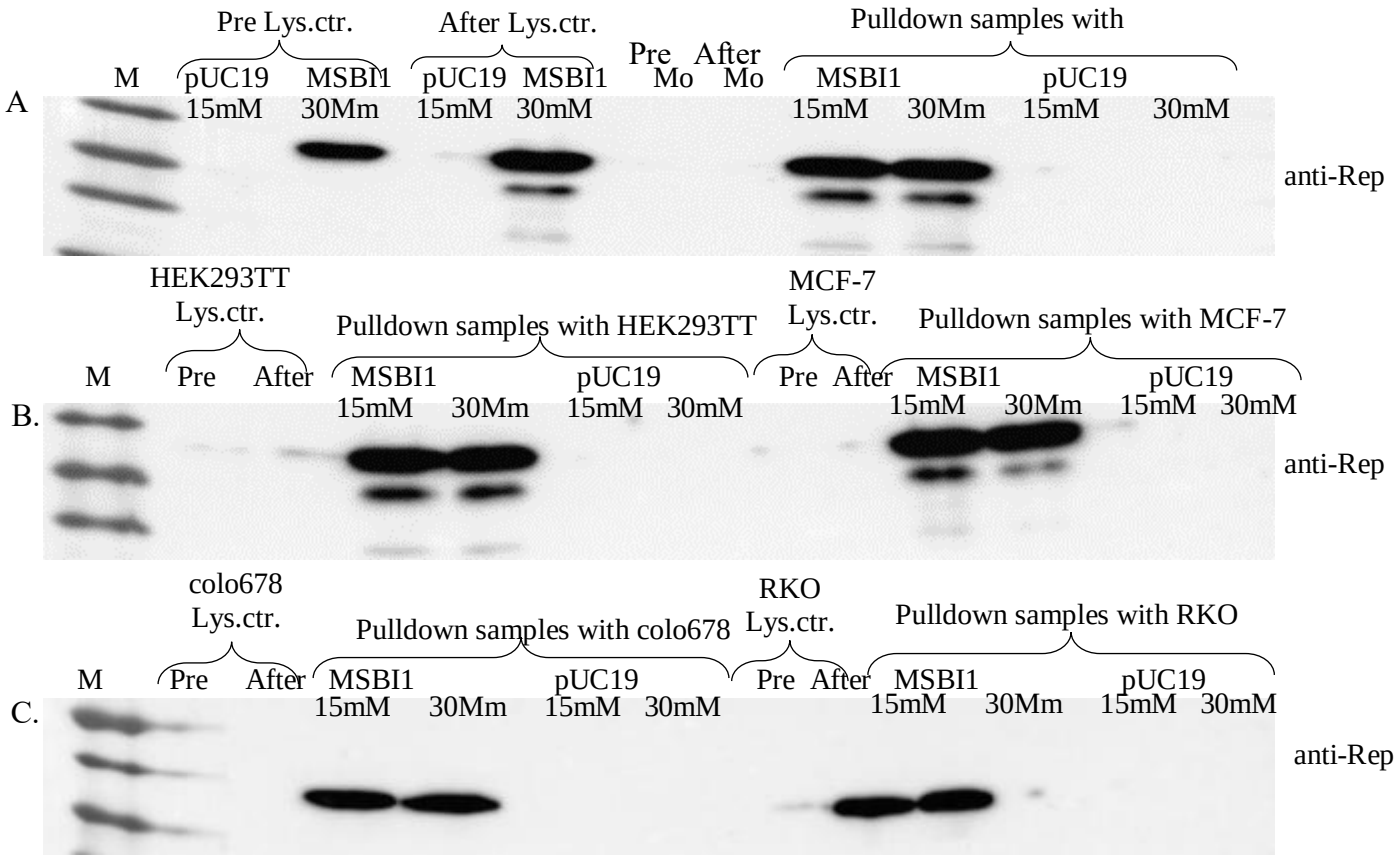

Fig 16: Western blot analysis of MSBI1 protein complexes with (A) Monocytes (B) HEK293TT & MCF-7 (C) Colo678 & RKO cell lines. Immunodetection with anti-HIS antibodies performed for cell lysates after a pull-down

experiment. Western blot analysis where pUC19 is the protein of expression vector and MSBI1 is the positive BMMF1 Rep protein. Protein bands at the lane of 41kDa for the positive control MSBI1 showed up. Two different concentrations of Imidazole 15mM and 30mM with the same pulled down samples of five different cell lines with pUC19 and MSBI1 were observed. No bands for negative control pUC19 and protein bands for positive control MSBI1 were seen as expected. Pre Lys.ctr-a lysate control of cells/MSBI1/pUC19 before adding HIS beads and After Lys.ctr-a lysate control of cells/MSBI1/pUC19 after adding HIS beads.

From the blots, bands were observed at the size of 38 kDa where all the positive MSBI1 samples were pulled down with the lysates meaning that the MSBI1 protein is present in the positive protein complexes with five different cell lysates. No protein product was observed in the negative control of pUC19 indicating no contamination by the positive MSBI1 Rep control. After data verification presented all samples were sent to the DKFZ Core Facility for mass spectrometry analysis where assay, as well as statistical analysis, were performed.

Resulting peptides have been loaded on a cartridge trap column, packed with Acclaim PepMap300 C18, 5 µm, 300 Å wide pore (Thermo Scientific) and separated in a 120 min gradient from 3% to 40% ACN on a nanoEase MZ Peptide analytical column (300 Å, 1.7 µm, 75µm x 200 mm, Waters). Eluting peptides have been analyzed by an online coupled Q-Exactive-HF-X mass spectrometer.

Data analysis was carried out by MaxQuant (version 1.6.3.3). In total 32387 peptides and 3312 proteins could have been identified by MSMS based on an FDR cutoff of 0.01 on the peptide level and 0.01 on the protein level. Data significance calculated with Student's T-test and q-value (FDR-adjusted p-value). Quantification was done using a label-free quantification approach based on the MaxLFQ algorithm (Cox J.et al., 2014). In total 3307 proteins were quantified.

A general identification and quantification numbers on peptide and protein levels among all analyzed cells showed two overrepresented proteins. These proteins, RFWD2 and CEP83, were identified with a high fidelity level (q-value of 0.000 and 0.012, respectively) as putative interaction partners for MSBI1 Rep protein in all tested cells. The data is graphically represented in a heatmap representation allowing global visualization of the data to identify clusters of proteins and/or samples with consistent behavior (Figure 18).

Data of cell lines separately showed a list of potential interaction partners which not necessarily overlap between tested cells. For HEK293TT cells, 218 proteins with q value <0.05 were identified. Among the top five are angio-associated migratory cell protein (AAMP), E3 ubiquitin-protein ligase (RFWD2), rabankyrin-5 (ANKFY1), cyclin-dependent kinase 2/3 (CDK2; CDK3), transcription factor BTF3 homolog 4 (BTF3L4). Here, AAMP is known to be involved in angiogenesis and cell migration (Beckner, M. E et al., 1999). Rabankyrin-5 (ANKFY1) protein is needed for endocytic trafficking and retromer function (Zhang, J et al., 2012).

In monocytes, 23 target proteins were detected. Among them, the top 5 are TATA-binding protein-associated factor 2N, Erlin-2, Myosin-IIIb, E3 ubiquitin-protein ligase RFWD2, and Protein Wiz. The TATA-binding protein-associated factor 2N protein is required in transcription initiation in distinct promoters (Jobert, L et al., 2009). And, Erlin-2 protein is involved in the regulation of cellular cholesterol homeostasis (Huber, M. D et al., 2013). Interestingly erlin-2, E3 ubiquitin-protein ligase, and parafibromin were found in both HEK293TT and monocyte cells as a potential interaction partner of MSBI1 Rep protein. The tumor suppressor protein Parafibromin involved in hematopoiesis and inhibits cancer cell

growth through arresting the G1 phase (Zhang, C et al., 2006). Also, Tctex1 domain-containing protein 1 was identified in two cell types – monocytes and RKO cell lines; this common protein is associated with intraflagellar transport.In Colo678, RKO and MCF-7 cells, RFWD2 also showed a distinguishably higher abundance compared to the other hits in these samples.

MSBI1 Rep protein pulled down with five different cell lines before Mass Spec (HEK293TT, RKO, Colo678, MCF-7 & monocytes) Mass Spectrometry data was analyzed based on q-values.

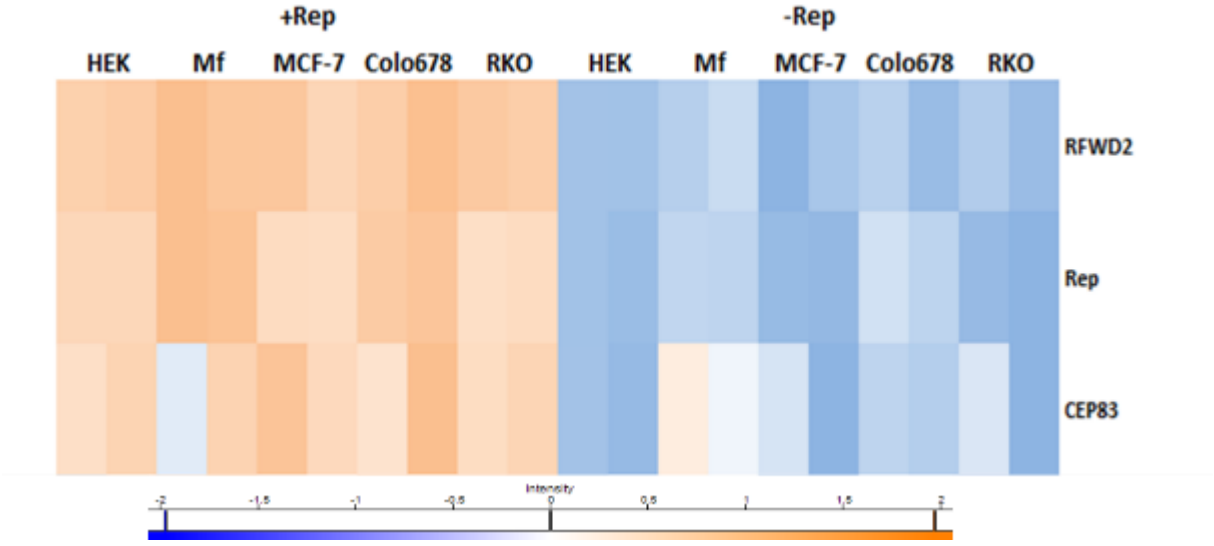

Figure 17: Color mapping. Log-2 transformed LFQ-values have been normalized via z-scoring. Proteins are ordered according to their statistical significance top to bottom (qvalue-based). Two-sample t-tests with a permutation-based false discovery rate (FDR) of 0.05 were used. Orange refers to an enrichment, blue color refers to a depletion.

4. DISCUSSION

Bovine milk and meat factors are defined as the putative pathogenic agent in a certain colon, breast cancer, and neurodegenerative diseases. The physiological appearance of these BMMF agents needs to be addressed to know about the mode of action. Relatively, episomal BMMF DNA is more likely to exist with associated protein rather than the naked DNA. Therefore, the virus particle or vesicle formation was followed by overexpressing a plasmid construct harboring the MSBI1 DNA to analyze the association of BMMF DNA and Rep proteins by CsCl density gradient centrifugation. CsCl density gradient centrifugation based experiments allowed the isolation of high molecular weight fraction containing MSBI1.176 Rep protein and DNA.

In previous studies, the interaction between different BMMFs was identified which suggested that the formation of an infectious entity may depend on the interactions to construct larger assemblies. Hence, the overexpressed plasmids were designed and prepared with Flag-tags at both termini to look for the rep-rep interaction by IP and Co-IP experiment. After multiple optimization steps of Flag IP and HIS pull down, the C-Flag and C-HIS overexpressed MSBI1 plasmid were immunodetected with single and double plasmid transfection. Optimization steps were successful for optimum precipitation of single and double plasmid proteins. Dimers were expected at the double plasmid transfected controls where both C termini Flag and HIS MSBI1 overexpressed plasmids (Rep proteins) are supposed to interact. But, dimers were only observed for the HIS pulled down samples but not in Flag antibody or agarose beads based IP and Co-IP.

Apart from the Rep-Rep interaction within the BMMF groups, Rep interaction with the host proteins was also observed. Interactome studies of BMMF Rep will give an overview of the Rep protein- host protein interactions for exploring the signaling pathways of the disease manifestation. The interaction with five different human cell lines was analyzed by following the pull-down assay and Mass Spectrometry based characterization. The identified putative Rep interaction partners were RFWD2 and CEP83 within all cell lines.

4.1 BMMF particle/vesicle preparation using the Cesium Chloride density gradient centrifugation

BMMF particle preparation was performed to analyze whether the Rep possess any function in capsids assembly or vesicle formation in addition to replication. Previous results showed that BMMF1 DNA, as well as protein, can be detected in human tissues and in serum. In order to analyze the particular function, characterization of a putative infectious agent either within the physical state of a particle or vesicle was essential. Since free DNA cannot efficiently enter and circulate in the body, it is expected that a protective surrounding like protein coat might stabilize the BMMF DNA. The characterization of BMMF particle preparation might help to understand the physical appearance of bioactive BMMFs. In density gradient centrifugation, such potential agents are being purified by different strategies enriching putative BMMF target structures and depleting contaminating and soluble proteins at the same time.

Starting from the isolating source (milk); how can the agent enter into the human body and how does it persist and circulate as (active) infectious agent eventually provoking diseased condition? All these inquiries might be better accessible once it is known whether BMMFs might persist as particles or vesicles which is a central part of this study.

After transfection with pcDNA3.1 (-) MSBI1 Rep plasmid in HEK293TTcells, high amounts of target protein (Rep) were produced. Sample preparation for CsCl density gradient centrifugation was based on using specific detergents, salts and protein inhibitors to prevent protein degradation and allow particle formation. Then, after ultracentrifugation, the gradient was differentially analyzed by splitting the sample into fractions allowing the search for putative BMMF DNA Rep-containing target structures.

After CsCl density gradient centrifugation, fractions were collected and immunodetected with anti-HIS antibodies after each gradient cycle to identify the high molecular weight fractions with the presence of MSBI1 Rep protein. From the previous experiences of the same DKFZ group, the fraction of interest was fraction 8-9. In general, it is expected, that particles or vesicles might be present within a high molecular weight fraction. Mostly, the fractions from 10 to 20 (lower molecular weight) contain impurities, soluble protein or other oligomeric proteins. Also, the modification step was followed using the synthetic Polyethyleneglycol polymer (PEG8000) which has a role in up concentrating the viral vesicle or particle suspension. The fractions were treated with PEG8000 and compared with the samples not treated with PEG8000. By using the PEG8000 polymer within a preceding PEG particle precipitation, a better resolution of the distribution of proteins from high to lower density fractions was observed within the gradient fractions displaying the presence of Rep protein at a lower section (higher molecular weight) of the gradient.

To get the cleaner and purer protein fraction before visualizing the formation of capsids from the protein of interest, two gradient centrifugation cycles were performed before dialysis. From the fraction of interests of the first gradient cycle, all desired proteins were identified when exposed upon the anti-HIS monoclonal antibody. The difference between the first and second gradient is that the pattern of the protein bands gets shifted to lower molecular weight. Because of the longer centrifugation gradient cycle, it is assumed that the higher molecular weight complexes might dissociate into smaller structures.

The fractions were collected after the second gradient and were dialyzed to remove CsCl which might dissociate complexes over time. Then, the dialyzed fractions were combined and analyzed by western blot and immunodetection. Here, the western blot was exposed to anti-Rep and anti-His antibodies. The result showed the presence of MSBI1 Rep protein for all the transfected samples. Cellular background impurities were accessed by the detection with the anti-γ-tubulin antibody. Very low amounts of cellular impurities were observed.
Notably, non-transfected control showed lower detection levels of cellular background in the high molecular weight fractions compared to transfected cells. Therefore, it is speculated that MSBI1 Rep might co-precipitate cellular proteins towards the higher molecular weight fractions.

It is known that upon viral protein accumulation in the cell viral DNA as well as host cell DNA fragments can be encapsidated during viral particle/ virus-like particle formation (Buck, C. B., & Thompson, C. D., 2007). During sample preparation, non-specific DNA binding may occur. Upon capsomere formation plasmid DNAs up to 10 kb, as well as human DNA fragments of appropriate size, can be incorporated into particle.

In addition to protein detection, the presence of the MSBI1.176 Rep expression plasmid was characterized by Long Polymerase Chain Reaction. The PCR products were separated on 1.5% agarose gels. MSBI1.176 genomic DNA was detected represented by bands at around 1800 bp. MSBI1.176 DNA was detected within all the combined fractions which were

transfected with MSBI1.176. This defines the presence of input DNA in the transfected controls even though Benzonase was used to remove unprotected, naked DNA like chromosomal and naked plasmid DNAs.

In brief, it is confirmed that the BMMF DNA seems to be protected by a protein shield. Though MSBI1 proteins and DNA were observed, the presence of particle or vesicle needs to be verified. Indeed, future investigations are required to confirm by visualizing through electron microscopy or Rep-specific immunogold electron microscopy and infection assays. In this context, one BMMF gene alone might not be sufficient for the formation of particles/vesicles. It might be possible that BMMF agents need interaction with other viruses, cellular or milk proteins or other BMMF group members for the establishment of functional bioactive structures.

Milk contains tiny, vesicle-like structures which are known as milk vesicles or milk exosomes. Apart from the controversies, the extensive study of knowing about the biological activity of milk vesicles is still ongoing. Until now, the milk exosomes containing a large number of varieties of proteins have been described acting as the bioactive cargo for cell-to-cell communication (Samuel, M et al., 2017). Also, the study of Arntz et al (2015) observed that milk vesicles contain microRNAs which has a role in the modification of immune pathways for delaying the onset of arthritis. Milk vesicles could contain original BMMF DNAs or protein and may have any unrecognized function in disease manifestation when exposed to humans. Hence, there might be a possibility that bovine milk vesicles deliver the BMMF DNA or proteins which possibly migrate to the site of infection (colon, liver and breast tissue) in humans. Also, cellular components within milk, like bovine T- or B-cells, other blood cells or prokaryotic cells might conduct the transfer of BMMF into the human host.

Vesicles are considered as a plausible carrier of disease factors because of their potential characteristics of intercellular communication through trafficking or macromolecule transmission. Other studies confirmed the hypoxic sections of tumor cells secreting exosomes (extracellular vesicle) which have a potential role in enhancing angiogenesis or metastasis (Park, J.E et al., 2010). Consistently, the finding of Thakur et al (2014) proved the existence of double-stranded DNA in tumor-induced exosomes. All these collective information supports the prediction of the BMMF entity towards vesicle more.

4.2 Preparation and Characterization of C-Flag pcDNA3.1 (-) MSBI1 Rep Codon Optimized and N-Flag pcDNA3.1 (-) MSBI1 Rep Cod Optimized plasmids

Mainly, to characterize protein-protein interaction with Flag tags at both Rep termini, overexpression plasmid allowing codon-optimized overexpression of Flag-tagged Rep were produced. The epitope tags alone do not possess any characterized biological function, but the tag, attached to the terminus within a fusion protein, might have an effect on protein folding, localization, interaction, stability or translation. To explore the possible effect of Flag tag on expressed Rep fusion proteins, cloning of expression plasmids with both C- and N-terminal Flag was performed based on a codon-optimized Rep gene, where specific codons of the original gene were changed into codons more frequently used in the human host (mentioned at 3.1.1 section).

The sequencing of the produced plasmids showed the correct localization of the Flag tags and no mutations. Thus, the expression plasmids were produced in higher quantities for expression experiments. Particularly, the construct of interest showed higher level of protein translation upon transfection compared to non-codon optimized constructs.

Time-course experiments were conducted to characterize the level of Rep protein expression. Western blot assays, as well as immunofluorescence-based microscopic examination with several Rep-specific antibodies, showed similar protein expression profiles both for N- and C-terminally Flag-tagged Rep fusion proteins. Expression profiles were also identical to expressions observed for a control plasmid transfection allowing expression of a Rep fused with a C-terminal 6xHis tag used as a control in previous studies. Although N- and C-terminally Flag-tagged Rep fusion proteins both have a predicted molecular weight of about 40 kDa, the C-terminally tagged Rep was running a bit higher, even though both of the samples were run on the same gel. This might be explained by slightly differently retained secondary structures during SDS-PAGE. The difference in spatial differences might be explained by conformational differences of Rep protein. No dimers or further oligomeric structures were detected on WB membranes after exposure to the anti-Flag M2 antibody. It can be deduce that the tag (N or C) has no effect on expression and there is no difference in protein stability wherever the tag is (N or C).

The same time course experiment was performed with the purpose to visualize the Rep expression products by immunofluorescence applying a combination of specific anti-Rep antibodies in mouse and guinea pig as well as with mouse anti-Flag antibodies. The result showed detectable N-Flag/C-Flag and C-HIS MSBI1 Rep protein after 24 h and a systematic increase with time in all transfected conditions. Slight differences in fluorescence detection were observed in samples. For example, better detection was seen when Rep GP and FLAG M2 antibodies applied after methanol cell fixation. In the case of immunostaining with Flag M2 antibody, clearer signals were observed for C-Flag MSBI1 Rep protein with PFA fixation. From the results of immunodetection, the translation level of newly designed proteins was compatible with the reference C-HIS tagged MSBI1 protein, which was previously characterized by the F200 research group.

In summary, the two newly generated plasmids showed a high level of protein expression of N- or C-terminally 3xFlag-tag fusion proteins which can be used for future applications like immunoprecipitation and Co-immunoprecipitation.

4.3 Immunoprecipitation and Co-Immunoprecipitation: Rep-Rep interaction

Characterization of Rep-Rep interaction is important because for Rep dimerization or multimerization an interaction of Rep proteins is mandatory. Rep interaction from monomers into dimers or multimers could lead to a conformational switch and allow formation of pre-amyloid oligomers, responsible for neurodegenerative disease. For observing the Rep-Rep interaction two overexpressed fusion protein with free N-terminal tags were used in IP & Co-IP experiment.

IP is commonly used to immobilize one (mostly known) protein as the ‘bait’ protein to trap a second defined interaction partner (prey) or a whole mix of putative unknown interaction partners. In Co-IP, interacting molecules are being identified together with the immunoprecipitated bait within an immunoprecipitated complex. From the analysis of IP and Co-IP, the interaction can be estimated through immunodetection. An optimization experiment was initiated using different lysis and washing buffer composition for optimum protein detection. The chosen proteins were Flag- and HIS-tagged Rep proteins. For Flag-tag-based immunoprecipitation, Flag M2 antibody and agarose A/G beads were used to immunoprecipitate the Flag-Rep bait. In the case of HIS based immunoprecipitation, only His-reactive Ni-NTA resin beads were used to pull down the HIS-tagged MSBI1 protein. Because,

in this case, no antibody is used to immobilize the target protein, it is not called immunoprecipitation but pulldown.

During cellular processes or stress conditions, protein-protein interaction is fundamental for pathway signaling. Sometimes, protein only interacts in the form of a dimer with additional interaction partners. Our suspect is that MSBI1.176 Rep might exhibit pathogenicity upon forming dimers or multimeric MSBI1.176 Rep arrangements or even filamentous aggregates. Very recently, the group of Fernandez (2016) found the presence of H6-RepA-WH1 oligomers in immunodetection confirming the amyloidogenic nature of the Rep protein, which is highly homolog to the MSBI1.176 Rep protein structure. Therefore, it was expected that Rep protein might also form dimers and that N- or C-terminal Flag tags might differentially affect dimer formation (Fernandez et al, 2016).

In neurodegeneration, the formation of pre-amyloid oligomers and prionoid aggregates are common phenomena. It is interesting, that the protein of interest, MSBI1.176 Rep, is encoded on a BMMF isolated from Multiple sclerosis patients, for which there might be the possible formation of aggregates due to amyloidosis. The monomeric conformation of Rep protein initiate transcription and the dimeric form act as the auto-repressor. The conformation of Rep protein is crucial because it determines the plasmid copy number. Even though the dimeric form of Rep is described replication-inert, the persisting dimer may play the role of domain swapping through interaction. From a previous observation, it was found that the free N- or C- terminal proteins have the potential to form dimers by domain swapping (Liu Y et al., 2002). Consistently, similar BMMFs have the identical structural element which can be exchanged by forming a domain-swapped dimer. This domain swapping contributes to amyloid fibrillation, causing neurodegeneration (Nilsson, M. *et al., 2004*). On that account, observing rep-rep interacting dimer was one of the main goals using double transfections allowing to check the interaction of MSBI1.176 Rep fused to a Flag tag and MSBI1.176 Rep fused to a HIS tag at C-terminal.

The representative image of overall Rep-Rep interaction study is mentioned below:

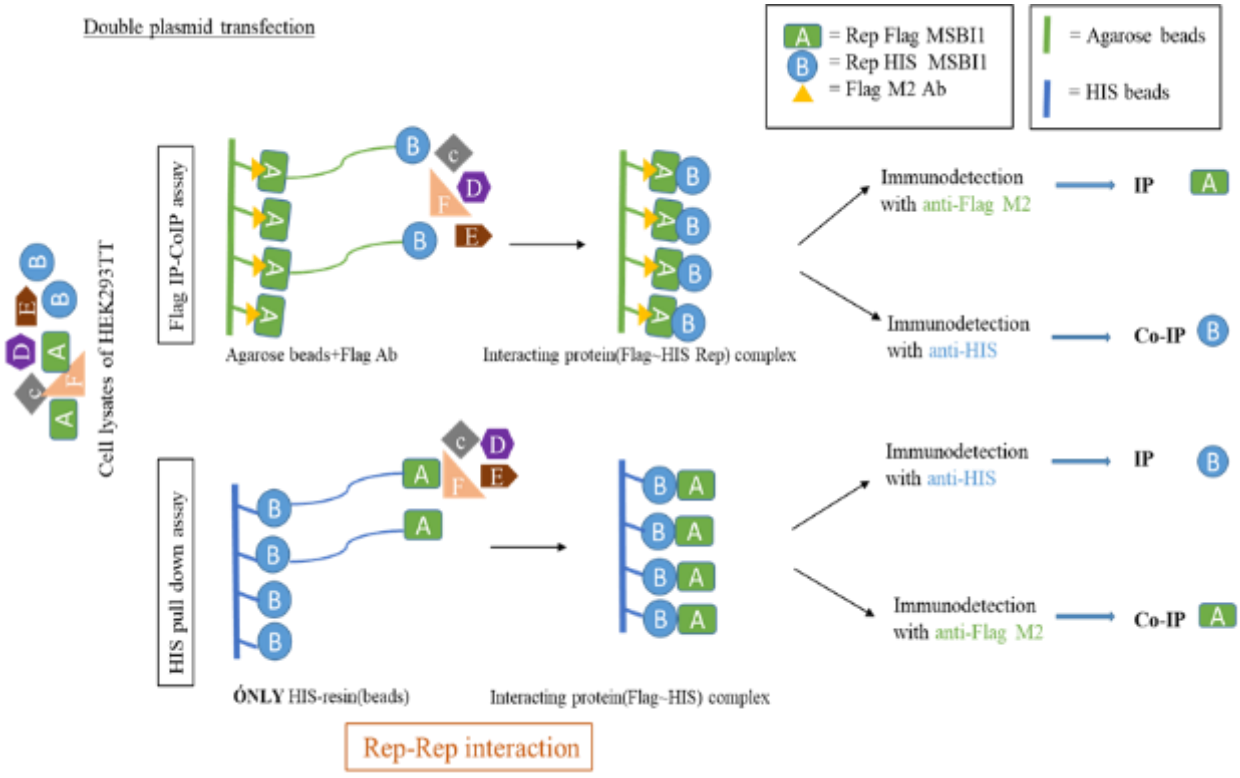

Fig 18: Representative image of Immunoprecipitation and Co-immunoprecipitation assay based on immunodetection for observing Rep-Rep interaction.

After optimization, the main purpose of the IP & Co-IP experiment was to observe the rep-rep interacting dimers. In that case, double plasmid transfected C-Flag and C-HIS encoded fusion MSBI1 Rep protein were the positive controls for dimer observation. Immunoblot analysis of Flag antibody and agarose based IP and Co-IP assay detected the distinct protein band at the expected size of the Rep protein which is approximately 41kDa. But, no dimers were observed in any of the IP and Co-IP assay of Flag antibody and agarose beads based experiment.

Immunoblot analysis of HIS beads based IP and Co-IP assay also detected the distinct protein band at the expected size of the Rep protein which is approximately 41kDa. Interestingly, dimers were observed only in HIS pull-down/IP assay for the single plasmid transfected (C-HIS MSBI1 Rep) and double transfected plasmid(C-HIS+C-Flag MSBI1 Rep) transfected proteins. Unfortunately, no dimers were observed for the Co-IP assay of HIS beads based pull down. The dimeric structure might get fully denatured by SDS-PAGE. Ultimately, dimerization of the misfolded or domain-swapped proteins coincides with the phenomena of amyloidogenesis which is responsible for neurodegenerative disorder. The exact characterization of the BMMF specific MSBI1 sample, also taken from multiple sclerosis patients will further help us to define that BMMFs is the putative pathogenic agent for neurodegeneration.

To optimize the optimum protein precipitation and to remove the non-specific cellular background proteins, multiple attempts were applied. Different compositions of lysis and washing buffer were used for optimization. In Flag antibody and agarose beads based IP and Co-IP optimization, 0.05% SDS detergent was added in the washing buffer to purify the bait protein. For HIS pull-down experiments, lysis buffer with 10 mM Imidazole and washing buffer with 35 mM imidazole showed optimum results with high specificity. Imidazole was used to remove non-specifically interacting background proteins. Eventually, optimized conditions for Flag antibody and agarose beads based on Immunoprecipitation and HIS pull-down protocols were successfully determined. As a next step, combinatorial transfections of C-Flag/N-Flag MSBI1 Rep and Reps within the BMMF2 group (C-HIS Mi-1/ C-HIS Mi-8/ C-HIS Mi-15) are scheduled for future experiments to characterize putative interaction of BMMF1 Rep proteins with Reps and additional proteins of BMMF group 2.

The very important purpose of IP, Co-IP experiment is that it will help to identify possible interaction partners among chosen isolates. Besides that, the identification of the interaction partner will help to identify vesicle packed or particle structured entity by CsCl density gradient ultra-centrifugation. Potentially, the physiology of BMMFs can be acquired which allows knowing more about their mode of action.

4.4 Identification of cellular Rep-interaction proteins

Four different cell lines and a monocyte preparation were used to analyze which proteins might be interacting with the specific MSBI1 Rep protein by pull-down experiments and mass spectrometry. In this experiment, Human Embryonic Kidney 293TT cells served as the control cell line, as bioactivity of the MSBI1.176 was already shown by replication, transcription and translation analysis (Eilebrecht S et al., 2018). To date, the presence of BMMFs proteins is found in breast and prostate cancer tissues, the brain of healthy donors and diseased patients while both BMMF DNA and proteins were observed in colon tissues (Timo B., et al., unpublished data). Considering these relevant examples, colon carcinoma cell line colo678, rectal carcinoma cell line RKO and breast carcinoma MCF-7 were chosen for observing the interactions of the proteins within the cancer context. Also, a positive Rep signal was obtained in colocalizations of Rep and macrophages in the lamina propria of colorectal cancer patients after immunohistochemistry, which is why peripheral blood monocytes were also chosen to characterize possible interactions (Bund T et al., unpublished).

The interaction of BMMF proteins with host proteins within cancer cell lines has not been examined so far. The present study is the first study to use an established pull-down protocol to identify specific BMMF protein interactions.

In order to confirm that the presence of MSBI1 Rep protein and also to check whether the pull-down protocol worked or not, Coomassie staining and western blot was conducted. As expected, the samples containing the purified input material, MSBI1 Rep were tested positive on Coomassie staining and western blot whereas the control input with the empty vector pUC19 was negative. To produce protein complexes of pUC19 and MSBI1.176 Rep HIS 6 *E.coli* system was used. After coupling with the HIS beads, the purified pulled down protein was prepared. Then, the purified MSBI1 Rep protein was incubated with the cell line lysates and after that pulled down with the cell line interaction proteins.

The data from pulled down samples were clustered in terms of the identified interactors and presented as a heatmap reflecting their stoichiometry. All twenty affinity purifications were measured using Mass Spectrometry and data files were simultaneously analyzed. The difference in data analysis between pUC19 and MSBI1 Rep suggests that MSBI1 might interact with different cellular proteins. To investigate the interaction partner, comprehensive Mass Spectrometry analysis was performed on HIS affinity beads based purified samples incubation with both pUC-X and MSBI1-X where X defines the five different cell lines HEK293TT, RKO, Colo678, MCF-7, and monocytes. All HIS tag affinity purifications were performed in duplicates where two washing buffer condition was observed. It includes milder washing buffer composition with 15mM Imidazole and harsher washing buffer composition with30mM Imidazole.

Two major interaction partners of MSBI1.176 Rep protein were identified within all the tested cell lysates: RFWD2 and CEP83. The E3 Ubiquitin –protein ligase, RFWD2. It is also known as constitutively photomorphogenic 1 protein, shortly COP1, (Holm et al., 2002). It contains the recognizing motif through which its substrate specifically binds to the E3 ligase for ubiquitination and proceeding to degradation via the 26S proteasome complex (Schnell, J. D., & Hicke, L., 2003).

RFWD2:

Ubiquitination or ubiquitin-proteasome proteolytic pathway degrades the unnecessary proteins during the cellular process through forming proteasomes. This post-translational modification is required to maintain the overall cell activity. The maintenance of cell activity includes proper cell division, differentiation, transduction and protein trafficking for controlling cell cycle as well as dynamic protein networks. However, halting or aberrations in any of the crucial steps of ubiquitination maybe lead to pathogenesis, malignancies, neurodegeneration and even lead to cancers (Mukhopadhyay, D., & Riezman, H. 2007). Due to the dysfunction of the ubiquitylation system, the aberrant or misfolded proteins are formed and get deposited as heavy aggregates resulting in resistance to proteolysis. Eventually, this may give rise to prion diseases like Creutzfeldt-Jakob disease, Alzheimer's diseases (Ciechanover A, Brundin P.; 2003).

Moreover, protein-protein interaction may results in aberrant interactomics for the human species which causes protein clump or aggregate deposition. This further leads to neurological disorders like Creutzfeldt-Jakob disease, Alzheimer's diseases or specific kinds of cancers. The full name of the pulled-down treated BMMF isolate is Multiple Sclerosis Bovine Isolate 1, where the isolate is derived from the brain of Multiple Sclerosis patients. One of the causes of inherent and acquired neurodegeneration is the accumulation of proteolysis resistant aggregates of certain proteins in tissues. One may speculate that MSBI1 Rep together with cellular protein might be involved in the pathogenesis of multiple sclerosis.

An earlier report resolving expression analysis showed the ubiquitous expression of the COP1 protein-coding gene in testis and colon (Fagerberg, L et al., 2013). COP1 is found to be upregulated in Binet C-phase of Chronic Lymphocytic Leukemia patients which were marked through a higher level of the prognostic marker, ZAP-70. Further, COP1 downregulates the p53 activation by disrupting the formation of the p53-Brn-3a complex which is needed for apoptosis. The earlier studies reported the downregulation of FOX01 and p21 (FU.C et al., 2015). The RNA binding protein FOX01 is an important transcription factor when, in response

to oxidative stress, it regulates metabolic processes and insulin signaling. Also, the FOX01 gene has a role in inhibiting metastasis and in provoking apoptosis, acting as the tumor suppressor (Wang, Y., Zhou, Y., & Graves, D. T., 2014). The p21 protein is essential for p53 activity through DNA repair and cell cycle arrest (Bunz, F., 1998).

The identified substrates of mammalian COP1 are C-Jun, ETV1, p53, acetyl co-A, carboxylase, and FOX01. The ligase activity through binding with the substrates triggers proteasome-dependent degradation in many biological processes. Increased expression of COP1 was found specifically in breast and ovarian carcinomas. Interaction of non-phosphorylated COP1 with the substrate p53 showed inhibition of p-53dependent transcription and apoptosis (Dornan.D et al, 2004). Also, COP1 directly interacts with the VPA motif of tumor suppressor protein, p27 which is needed for repair or prompting apoptosis (Choi et al., 2015).

CEP83:

Another identified putative interaction partner of MSBI1 Rep protein is the Centrosomal protein of 83kDa (CEP83), which is involved in ciliogenesis. Centrioles are the cylindrical organelle composed of tubulin protein and surrounded by nine distal appendages (DAP). The centriole coming at an earlier stage of the cell cycle is named as the mother or mature centriole whereas the later one is termed daughter centriole. These two centrioles in the centrosome are tied with one another but they are different in terms of age or activity. The mature centriole has a polar structure with two ends; one proximal and the other distal end. The proximal end is microtubule minus attaching with daughter centriole and the distal end is microtubule plus with radiating appendages. These two ends, together, have a role in spindle fiber contact or duplication before cilia formation at the interphase stage. Cilia is the protrusions from the eukaryotic cells which separated into two groups; motile and non-motile. The motile group of cilia features the rhythmic vibrating motions and can be found in the lungs, respiratory tract, and middle ear. The motile cilia have a role in clearing away the dirt at the airways and in propelling sperm. The other non-motile cilia are the sensory organelles, often called cellular antenna or primary cilia. The latter cilia grow from the distal end of the mature centriole and are enlarged within cell cycle progression (Azimzadeh, J., & Bornens, M., 2007).

Ciliogenesis is mediated by the distal appendage structure at the mature centriole. In ciliogenesis, the distal appendages (DAP) of the centrioles assist in the anchoring of the cilia to the plasma membrane for cilia initiation and proper assembly. One of the DAP proteins is Centrosomal protein of 83kDa, shortly CEP83 containing 701 residues. Because of its coiled-coil domain, this protein is also called as Coiled-coil domain-containing protein 41, shortly CCDC41. Together with Intraflagellar transport IFT20 protein, CEP83 delivers the ciliary membrane proteins from the Golgi to assemble cilia. Association of appendage proteins in mother centrioles is essential because only mother centrioles can carry the appendages for centriole-to-membrane-docking before ciliogenesis. Previous RNAi study revealed that CEP164 is essential for ciliogenesis but the loss of one of the DAP protein (CEP83) delocalizes the CEP164 which eventually halts the complete cilia formation (Tanos, B. E., 2013). Overall, the CEP83 has a key role in cilium assembly, protein localization to centrosome and vesicle docking.

Cilia dysfunction or defective cilia due to mutations of the CEP83 gene cause human ciliopathies, namely nephronophthisis and orofaciodigital syndrome. Nephronophthisis is autosomal recessive kidney disorder which can lead in the end-stage to renal failure. Because of the disease manifestation and identified mutation in the CEP83 gene, it is also symbolized as Nephronophthisis 18. Other clinical features include retinal degeneration and some neurological disorder (Failler.M et al., 2014). Additionally, the CEP83 is also called renal carcinoma antigen NY-REN-58. In the SEREX diagnostic report, NY-REN-58 showed increased positive renal cancer serum reactivity when compared to the activity of normal sera (Scanlan, M. J., 1999).

Between two interaction protein partners, E3 Ubiquitin ligase is involved in p53 dependent pathways whereas the other CEP83 is associated with ciliogenesis. There is a set of additional putative interaction partners identified for cell-lysate specific analysis. Most of them seem to be involved in angiogenesis (AAMP), endocytic trafficking (ANKFY1), cholesterol homeostasis (Erlin-2), inhibiting cancer cell growth (parafibromin) and intraflagellar transport (Tctex1). More or less, most of the putative interacting proteins are related to signaling pathways relevant to cancer induction and in-migration. Thus, it might be speculated that the interaction of these cellular proteins with BMMF protein interaction might be important for the transportation of the infectious agent or might be involved in altering the cellular system.

This interactome experiment certainly will help to better understand the role of the BMMF in cancer. Based on this extensive study, additional functions of the Rep protein have been identified. Future experiments have to ascertain whether these interactions might also be involved in capsomers/vesicle formation and/or transport.

Conclusion

This study covers the characterization of BMMF particles/vesicles, Rep-Rep protein interaction, and identification of BMMF1 Rep-interacting proteins within the human host. As for the characterization of BMMF particles/vesicles, there is no specific formation of structured particles/vesicles based on MSBI1.176 Rep overexpression in HEK293TT cells, although protected DNA, as well as BMMF Rep, was specifically identified within heavy molecular weight fractions after gradient ultracentrifugation. Several optimization steps of CsCl gradient centrifugation allowed to achieve higher Rep-protein yield and better separation within fractioning, still, based on electron microscopy analysis, only very heterogeneous and unstructured protein assemblies were observed. BMMFs might be represented within vesicles, cell contaminations of the bovine host within the milk or bacterial cells, instead. Here, no assembly with the Rep protein would be expected. In conclusion, the generation of a high amount of high molecular weight Rep was optimized.

Still, only the MSBI1 Rep-overexpression plasmid itself was transfected in HEK293TT cells for packing by the overexpressed Rep protein as the basis to form particles or vesicles. Hence, particle or vesicle preparation by transfection with two plasmids, the original MSBI1.176 genome, and the overexpression plasmid, should be characterized, in the future. This may give an insight into whether the formulation needs the iterons which are only present within the full genome.

For observing Rep-Rep protein interaction, Immunoprecipitation and Co-immunoprecipitation was performed. Biomolecular protocols were successfully optimized for two different Rep fusion proteins (3xFlag-Rep or Rep-HIS) with both Flag Ab+agarose- based immunoprecipitation and HIS beads-based pull-down and following analysis of co-immunoprecipitation. The results showed that the dimerization of Rep-Rep interaction is possible. This gives a hint that oligomerization and possibly, larger assemblies or filaments may form which have cytotoxic and pathogenic activity.

Putative cellular interaction partners of the BMMF1 Rep were identified within an interactome study based on four related carcinoma cell lines and macrophage cell preparations. From the mass spectrometry-based data analysis, two interaction partners were identified: RFWD2 and CEP83. The RFWD2 protein is responsible for p-53 pathway downregulation and the protein CEP83 has a crucial role in ciliogenesis. Such systemic interactions might hint for a specific function of the BMMF protein and might play a crucial role in cancer induction.

Current results are significantly expanding our understanding of BMMF physiology and possible mechanisms in disease manifestation. Further research will clarify the ways of entry and/or BMMF circulation as well as BMMF functions allowing setting up new strategies for prevention and modulation of BMMF infection.

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REFERENCES:

1. Whitley C., Gunst, K., Müller, H., Funk, M., zur Hausen, H., de Villiers, EM. Novel replicationcompetent circular DNA mole-cules from healthy cattle serum and milk and multiple sclerosis-affected human brain tissue. Genome Announc 2014; 2(4):e00849-14.
2. Eilebrecht, S, Hotz-Wagenblatt, A, Sarachaga, V, et al. Expression and replication of virus-like circular DNA in human cells. Sci Rep 2018; **8**: 2851.
3. Alisson-Silva, F, Kawanishi, K, Varki, A. Human risk of diseases associated with red meat intake: Analysis of current theories and proposed role for metabolic incorporation of a non-human sialic acid. Mol Aspects Med 2016; **51**: 16– 30.
4. Zur Hausen, H, Bund, T, Villiers, E-M. Infectious agents in bovine red meat and milk and their potential role in cancer and other chronic diseases. Curr Topics Microbiol Immunol 2017; **407**: 83– 116.
5. Zur Hausen, H, Villiers, E-M. Dairy cattle serum and milk factors contributing to the risk of colon and breast cancers. Int J Cancer 2015; **137**: 959– 67.
6. Funk, M., Gunst, K., Lucansky, V., Muller, H., zur Hausen, H., & de Villiers, E.-M. (2014). Isolation of Protein-Associated Circular DNA from Healthy Cattle Serum. Genome Announcements, 2(4).
7. Gunst, K., zur Hausen, H., & de Villiers, E.-M. (2014). Isolation of Bacterial Plasmid-Related Replication-Associated Circular DNA from a Serum Sample of a Multiple Sclerosis Patient. Genome Announcements, 2(4).
8. Lamberto, I., Gunst, K., Muller, H., zur Hausen, H., & de Villiers, E.-M. (2014). Mycovirus-Like DNA virus Sequences from Cattle Serum and Human Brain and Serum Samples from Multiple Sclerosis Patients. Genome Announcements, 2(4).
9. Falida, K., Eilebrecht, S., Gunst, K., zur Hausen, H., & de Villiers, E.-M. (2017). Isolation of Two Virus-Like Circular DNAs from Commercially Available Milk Samples. Genome Announcements, 5(17).
10. De Villiers, E.-M., Gunst, K., Chakraborty, D., Ernst, C., Bund, T., & zur Hausen, H. (2019). A specific class of infectious agents isolated from bovine serum and dairy products and peritumoral colon cancer tissue. Emerging Microbes & Infections, 8(1), 1205–1218.
11. Gerald Karp, Cell and molecular biology: Concepts and experiments, fourth edition, 2005, Von Hoffman press

12. Fisher, W.D., Cline, G.B. and Anderson, N.G., 1964. Density gradient centrifugation in angle-head rotors. Analytical biochemistry, 9(4), pp.477-482.
13. Hull, R.2014. Plant Virology: Assay, Detection, and Diagnosis of Plant Viruses; fifth edition. Academic press, John Innes Centre, UK.1118pp
14. Kaboord, B., & Perr, M. (2008). Isolation of Proteins and Protein Complexes by Immunoprecipitation. 2D PAGE: Sample Preparation and Fractionation, 349–364.
15. Chang, I.-F. (2006). Mass spectrometry-based proteomic analysis of the epitope-tag affinity purified protein complexes in eukaryotes. PROTEOMICS, 6(23), 6158–6166.
16. Buck, C. B., & Thompson, C. D. (2007). Production of Papillomavirus-Based Gene Transfer Vectors. Current Protocols in Cell Biology, 37(1), 26.1.1–26.1.19.
17. Samuel, M., Chisanga, D., Liem, M., Keerthikumar, S., Anand, S., Ang, C.-S., … Mathivanan, S. (2017). Bovine milk-derived exosomes from colostrum are enriched with proteins implicated in immune response and growth. Scientific Reports, 7(1).
18. Arntz, O. J., Pieters, B. C. H., Oliveira, M. C., Broeren, M. G. A., Bennink, M. B., de Vries, M., … van de Loo, F. A. J. (2015). Oral administration of bovine milk derived extracellular vesicles attenuates arthritis in two mouse models. Molecular Nutrition & Food Research, 59(9), 1701–1712.
19. Park, J. E., Tan, H. S., Datta, A., Lai, R. C., Zhang, H., Meng, W.,Lim,S.K., Sze, S. K. (2010). Hypoxic Tumor Cell Modulates Its Microenvironment to Enhance Angiogenic and Metastatic Potential by Secretion of Proteins and Exosomes. Molecular & Cellular Proteomics, 9(6), 1085–1099.
20. Thakur, B. K., Zhang, H., Becker, A., Matei, I., Huang, Y., Costa-Silva, B., … Lyden, D. (2014). Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Research, 24(6), 766–769.
21. Marianayagam, N. J., Sunde, M., & Matthews, J. M. (2004). The power of two: protein dimerization in biology. Trends in Biochemical Sciences, 29(11), 618–625.
22. Khalifa, N. B., Hees, J. V., Tasiaux, B., Huysseune, S., Smith, S. O., Constantinescu, S. N., Kienlen-Campard, P. (2010). What is the role of amyloid precursor protein dimerization? Cell Adhesion & Migration, 4(2), 268–272.
23. Giraldo R, Fernández-Tornero C, Evans PR, Díaz-Orejas R, Romero A (2003) A conformational switch between transcriptional repression and replication initiation in the RepA dimerization domain. Nature Struct. Biol. 10: 565-571.
24. Fernández, C., González-Rubio, G., Langer, J., Tardajos, G., Liz-Marzán, L. M., Giraldo, R., & Guerrero-Martínez, A. (2016). Nucleation of Amyloid Oligomers by RepA-WH1-Prionoid-Functionalized Gold Nanorods. Angewandte Chemie, 128(37), 11403–11407
25. Shimozuma, Y., Ito, T., Inokuchi, M., Uchikoshi, M., Miyashita, M., Nozawa, H., Imawari, M. (2010). Reactivation of epstein-barr virus in B cells of patients with chronic hepatitis C. Journal of Medical Virology, 82(12), 2064–2072.
26. Y Sugawara, et al.(2010). Enhancement of hepatitis C virus replication by Epstein-Barr virus-encoded nuclear antigen 1 Retrieved November 1, 2010, The EMBO Journal, October 1999.
27. Borkosky, S. S., Whitley, C., Kopp-Schneider, A., zur Hausen, H., & deVilliers, E.-M. (2012). Epstein - Barr virus Stimulates Torque Teno Virus Replication: A Possible Relationship to Multiple Sclerosis. PLoS ONE, 7(2), e32160.

28. Liu, Y., & Eisenberg, D. (2002). 3D domain swapping: As domains continue to swap. Protein Science, 11(6), 1285–1299.
29. Nilsson, M. et al. (2004).Prevention of domain swapping inhibits dimerization and amyloid fibril formation of cystatin C. Use of engineered disulfide bridges, antibodies and carboxymethylpapain to stabilize the monomeric form of cystatin C. J. Biol. Chem.
30. Cox J.et al., Accurate Proteome-wide Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction, Termed MaxLFQ. Mol Cell Proteomics, 2014, 13, pp 2513-2526
31. Beckner, M. E., Peterson, V. A., & Moul, D. E. (1999). Angio-Associated Migratory Cell Protein Is Expressed as an Extracellular Protein by Blood-Vessel-Associated Mesenchymal Cells. Microvascular Research, 57(3), 347–352.
32. Zhang, J., Reiling, C., Reinecke, J. B., Prislan, I., Marky, L. A., Sorgen, P. L., … Caplan, S. (2012). Rabankyrin-5 Interacts with EHD1 and Vps26 to Regulate Endocytic Trafficking and Retromer Function. Traffic, 13(5), 745–757.
33. Jobert, L., Argentini, M., & Tora, L. (2009). PRMT1 mediated methylation of TAF15 is required for its positive gene regulatory function. Experimental Cell Research, 315(7), 1273–1286.
34. Huber, M. D., Vesely, P. W., Datta, K., & Gerace, L. (2013). Erlins restrict SREBP activation in the ER and regulate cellular cholesterol homeostasis. The Journal of Cell Biology, 203(3), 427–436.
35. Zhang, C., Kong, D., Tan, M.-H., Pappas, D. L., Wang, P.-F., Chen, J., … Teh, B. T. (2006). Parafibromin inhibits cancer cell growth and causes G1 phase arrest. Biochemical and Biophysical Research Communications, 350(1), 17–24.
36. Holm, M., Ma, L.G., Qu, L.J., and Deng, X.W. (2002). Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in Arabidopsis. Genes Dev. 16, 1247–1259.
37. Schnell, J. D., & Hicke, L. (2003). Non-traditional Functions of Ubiquitin and Ubiquitin-binding Proteins. Journal of Biological Chemistry, 278(38), 35857–35860.
38. Mukhopadhyay, D., & Riezman, H. (2007). Proteasome-Independent Functions of Ubiquitin in Endocytosis and Signaling. Science, 315(5809), 201–205.
39. Ciechanover A , Brundin P. (2003). the ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. Neuron,40(2):427-46.
40. Fagerberg, L., Hallström, B. M., Oksvold, P., Kampf, C., Djureinovic, D., Odeberg, J., Uhlén, M. (2013). Analysis of the Human Tissue-specific Expression by Genome-wide Integration of Transcriptomics and Antibody-based Proteomics. Molecular & Cellular Proteomics, 13(2), 397–406.
41. FU, C., GONG, Y., SHI, X., SHI, H., WAN, Y., WU, Q., & XU, K. (2015). Expression and regulation of COP1 in chronic lymphocytic leukemia cells for promotion of cell proliferation and tumorigenicity. Oncology Reports, 35(3), 1493–1500.
42. Wang, Y., Zhou, Y., & Graves, D. T. (2014). FOXO Transcription Factors: Their Clinical Significance and Regulation. BioMed Research International, 2014, 1–13.
43. Bunz, F. (1998). Requirement for p53 and p21 to Sustain G2 Arrest after DNA Damage. Science, 282(5393), 1497–1501.

44. Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G. D., Dowd, P., … Dixit, V. M. (2004). The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature, 429(6987), 86–92.
45. Choi, H.H., Phan, L., Chou, P.C., Su, C.H., Yeung, S.C., Chen, J.S., and Lee, M.H. (2015). COP1 enhances ubiquitin-mediated degradation of p27Kip1 to promote cancer cell growth. Oncotarget 6, 19721–19734.
46. Uljon, S., Xu, X., Durzynska, I., Stein, S., Adelmant, G., Marto, J. A., … Blacklow, S. C. (2016). Structural Basis for Substrate Selectivity of the E3 Ligase COP1. Structure, 24(5), 687–696.
47. Olofsson, L. E., Orho-Melander, M., William-Olsson, L., Sjöholm, K., Sjöström, L., Groop, L., Olsson, B. (2008). CCAAT/Enhancer Binding Protein α (C/EBPα) in Adipose Tissue Regulates Genes in Lipid and Glucose Metabolism and a Genetic Variation in C/EBPα Is Associated with Serum Levels of Triglycerides. The Journal of Clinical Endocrinology & Metabolism, 93(12), 4880–4886.
48. Newton, K., Dugger, D. L., Sengupta-Ghosh, A., Ferrando, R. E., Chu, F., Tao, J., Dixit, V. M. (2018). Ubiquitin ligase COP1 coordinates transcriptional programs that control cell type specification in the developing mouse brain. Proceedings of the National Academy of Sciences, 201805033.
49. Azimzadeh, J., & Bornens, M. (2007). Structure and duplication of the centrosome. Journal of Cell Science, 120(13), 2139–2142.
50. Tanos, B. E., Yang, H.-J., Soni, R., Wang, W.-J., Macaluso, F. P., Asara, J. M., & Tsou, M.-F. B. (2013). Centriole distal appendages promote membrane docking, leading to cilia initiation. Genes & Development, 27(2), 163–168.
51. Failler, M., Gee, H. Y., Krug, P., Joo, K., Halbritter, J., Belkacem, L., … Saunier, S. (2014). Mutations of CEP83 Cause Infantile Nephronophthisis and Intellectual Disability. The American Journal of Human Genetics, 94(6), 905–914.
52. Scanlan, M. J., Gordan, J. D., Williamson, B., Stockert, E., Bander, N. H., Jongeneel, V.; Old, L. J. (1999). Antigens recognized by autologous antibody in patients with renal-cell carcinoma. International Journal of Cancer, 83(4), 456–464.
53. Hurd, T. W., & Hildebrandt, F. (2011). Mechanisms of Nephronophthisis and Related Ciliopathies. Nephron Experimental Nephrology, 118(1), e9–e14.
54. Speedy, A. W. (2003). Global Production and Consumption of Animal Source Foods. The Journal of Nutrition, 133(11), 4048S–4053S.
55. Zur Hausen, H., Bund, T., & de Villiers, E.-M. (2018). Specific Nutritional Infections Early in Life as Risk Factors for Human Colon and Breast Cancers Several Decades Later. International Journal of Cancer.
56. De Villiers, E.-M., Gunst, K., Chakraborty, D., Ernst, C., Bund, T., & zur Hausen, H. (2019). A specific class of infectious agents isolated from bovine serum and dairy products and peritumoral colon cancer tissue. Emerging Microbes & Infections, 8(1), 1205–1218.
57. Zur Hausen, H., & de Villiers, E.-M. (2014). Cancer "Causation" by Infections—Individual Contributions and Synergistic Networks. Seminars in Oncology, 41(6), 860–875.
58. Lippi G, Mattiuzzi C, Cervellin G (2016) Meat consumption and cancer risk: a critical review of published meta-analyses. Crit Rev Oncol Hematol 97:1–14

59. Farvid MS, Cho E, Chen WY, Eliassen AH, Willett WC (2015) Adolescent meat intake and breast cancer risk. Int J Cancer 136:1909–1920
60. Zur Hausen, H., Bund, T., & de Villiers, E.-M. (2017). Infectious Agents in Bovine Red Meat and Milk and Their Potential Role in Cancer and Other Chronic Diseases. Viruses, Genes, and Cancer, 83–116.
61. Chait, B. T. (2011). Mass Spectrometry in the Postgenomic Era. Annual Review of Biochemistry, 80(1), 239–246.
62. Moreno-del Álamo, M., de la Espina, S. M.-D., Fernández-Tresguerres, M. E., & Giraldo, R. (2015). Pre-amyloid oligomers of the proteotoxic RepA-WH1 prionoid assemble at the bacterial nucleoid. Scientific Reports, 5(1).
63. Li D, Sinha KK, Hay MA, Rinaldi CR, Sauntharajah Y, Nucifora G. RUNX1-RUNX1 homodimerization modulates RUNX1 activity and function. J. Biol. Chem. 2007
64. Bowers, S. R., Calero-Nieto, F. J., Valeaux, S., Fernandez-Fuentes, N., & Cockerill, P. N. (2010). Runx1 binds as a dimeric complex to overlapping Runx1 sites within a palindromic element in the human GM-CSF enhancer. Nucleic Acids Research, 38(18), 6124–6134.
65. Chandra, A. (2015). Role of Amyloid from a Multiple Sclerosis Perspective: A Literature Review. Neuroimmunomodulation, 22(6), 343–346.
66. Guo, P. 1994. Principles, perspectives and potential applications in viral assembly. Semin. Virol. 5**:**27-37.
67. Bina M., Ng,S.C. and Blasquez,V. (1983) Simian virus 40 chromatin interaction with the capsid proteins. J. Biomol. Struct. Dyn., 1, 689–704.
68. Black L.W. (1989) DNA packaging in dsDNA bacteriophages. Annu. Rev. Microbiol., 43, 267–292.
69. Chejanovsky, N., and B. J. Carter. 1989. Mutagenesis of an AUG codon in the adeno-associated virus rep gene: effects on viral DNA replication. Virology 173**:**120-128.
70. Muzyczka, N., and K. I. Berns. 2001. Parvoviridae: the viruses and their replication, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
71. Fraenkel-Conrat H, Williams RC. Reconstitution of Active Tobacco Mosaic Virus from Its Inactive Protein and Nucleic Acid Components. Proc Natl Acad Sci U S A. 1955;41:690–698.
72. King, J. A. (2001). DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. The EMBO Journal, 20(12), 3282–3291.
73. Kilick, T., Papov, N, A.,Burk-Korner, A., Kromyslova, A., Zur Hausen, H., Bund, T., & Hansman, G, E.-M. (2019). Structural analysis of a replication protein encoded by a plasmid isolated from a multiple sclerosis patient.Acta Crystallogr D Struct Biol, 498-504.
74. Longkumer, T., Kamireddy, S., Muthyala, V. R., Akbarpasha, S., Pitchika, G. K., Kodetham, G., … Siddavattam, D. (2013). Acinetobacter phage genome is similar to Sphinx 2.36, the circular DNA copurified with TSE infected particles. Scientific Reports, 3(1).
75. Raab-Traub, N., & Dittmer, D. P. (2017). Viral effects on the content and function of extracellular vesicles. Nature Reviews Microbiology, 15(9), 559–572.
76. Altan-Bonnet, N. (2016). Extracellular vesicles are the Trojan horses of viral infection. Current Opinion in Microbiology, 32, 77–81.

77. Canli, Ö., Nicolas, A. M., Gupta, J., Finkelmeier, F., Goncharova, O., Pesic, M., … Greten, F. R. (2017). Myeloid Cell-Derived Reactive Oxygen Species Induce Epithelial Mutagenesis. Cancer Cell, 32(6), 869–883.e5.
78. Thuman-Commike, P. A., Greene, B., Malinski, J. A., Burbea, M., McGough, A., Chiu, W., & Prevelige, P. E. (1999). Mechanism of Scaffolding-Directed Virus Assembly Suggested by Comparison of Scaffolding-Containing and Scaffolding-Lacking P22 Procapsids. Biophysical Journal, 76(6), 3267–3277.