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Identifying MUS81 interactors during interphase and mitosis using mass-spectrometry

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ABSTRACT

DNA replication can be perturbed by various factors, which are together referred to as ‘replication stress’. When replication is not completed in time, persistent DNA replication intermediates may remain and may be transmitted into mitosis. To faithfully segregate chromosomes during mitosis, these DNA intermediates need to be resolved. In this context, the MUS81-EME1 endonuclease complex is crucial for preserving genome stability through various mechanisms. Firstly, MUS81 promotes POLD3 recruitment to common fragile sites (CFSs) to facilitate mitotic DNA synthesis (MiDAS). MiDAS is considered a form of ‘back-up’ DNA replication to minimize chromosomal missegregation, although the exact molecular mechanism and the importance of MiDAS remain unclear. Secondly, Mus81, in conjunction with other endonucleases, can resolve branched DNA intermediates in mitosis. Specifically, MUS81 exhibits resolution activity towards 3’ flaps, replication forks, and Holliday junctions. In line with these findings, MUS81 was recently shown to be recruited to breaks and gaps at CFSs, which are induced by replication stress.

We here aim to identify novel mitotic interaction partners of MUS81. To this end, replication stress was induced in synchronized cells and mitotic cells were collected. We identified proteins directly associated with MUS81 using immune-purification of GFP-tagged MUS81. In addition to EME1 and other established MUS81 binding partners, we found MUS81 to associate with MAGEA1 both during interphase and mitosis.

INTRODUCTION

To divide, a cell must duplicate its genomic content during S-phase of the cell cycle. The process of DNA duplication is continuously challenged by endogenous or exogenous factors. For example, oncogene overexpression can interfere with the tightly regulated process of DNA replication. Alternatively, cross-linking of DNA strand due to aldehydes prevent DNA unwinding and prevent ongoing replication. Combined, all processes that slow or stall ongoing replication forks are collectively called ‘replication stress’.

Prolonged stalling of a replication fork can result in DNA under-replication or its collapse, leading to DNA double-strand breaks (DSBs).

These toxic DNA lesions are repaired in an error-free manner by the homologous recombination (HR) DNA repair pathway. HR-mediated DNA repair gives rise to the formation of a four-way DNA structure, also known as Holliday junctions (HJs). HJs can be processed by the BLM-TopoIIIα-RMI1-RMI2 (BTR) complex that promotes dissolution of the HJs. In addition, HJs can be resolved via endonucleases, for example by the endonuclease MUS81-EME1 complex. The MUS81-EME1 complex also functions during normal replication, in which MUS81 regulates the DNA replication rate by promoting replication fork progression and reducing the frequency of replication initiation.

To prevent loss or gain of genomic content, it is crucial that DNA lesions are resolved during interphase and are not transferred into mitosis. However, not all DNA lesions are resolved before mitotic entry and end up in mitosis. To ensure the faithful segregation of the two copies of each chromosome, cancer cells require mechanisms that cope with DNA lesions during mitosis. Although during mitosis most canonical DNA damage repair pathways are inactivated, some dedicated DNA repair pathways are active.

Beyond its roles during interphase, MUS81 also functions during mitosis. When DNA replication is perturbed, areas of under-replicated DNA are processed during mitosis. Under-replication occurs preferentially at common-fragile sites (CFSs). MUS81 localizes to common CFSs during mitosis, where it promotes formation of gaps and breaks. To process joint DNA molecules, such as those that result from incomplete DNA replication, MUS81 is associated with other nucleases during mitosis to form the SLX complex. Specifically, MUS81-EME1 in conjunction with SLX1-SLX4 and
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XPF are able to sever various joint DNA molecules during mitosis\textsuperscript{11}. The importance of MUS81 was underscored by the observation that depletion of MUS81 enhances mitotic aberrations and micronuclei formation\textsuperscript{10,12}.

Recently, several additional pathways of mitotic resolution of joint DNA molecules have been described: Firstly, it has been described that the mitotic DNA synthesis (MiDAS) pathway aids in the process of faithful chromosome segregation by synthesizing DNA at under-replicated regions, in the early stages of mitosis\textsuperscript{13}. Upon replication stress, sites of DNA under-replication end up in mitosis. MUS81 localizes to these sites and promotes the recruitment of POLD3, which is the polymerase that facilitates DNA synthesis during MiDAS\textsuperscript{13}. Furthermore, several additional proteins are implicated to be involved in MiDAS, including SLX4, RAD52, FANCD2, and RECQL5\textsuperscript{13-15}. Despite the seemingly important role of MiDAS, the underlying molecular mechanism remains unclear. Secondly, DNA molecules that are still attached at the onset of anaphase can form ultrafine DNA bridges (UFBs)\textsuperscript{16}. These UFBs are resolved by BLM-Toposiomerase-RMI1/2 and Rif1\textsuperscript{18,19}, which facilitate UFB breakage to allow chromosome segregation\textsuperscript{20}. Although the DNA repair pathways that are available during mitosis allow chromosome segregation, they result in loss of genomic content\textsuperscript{20}.

Taken together, cancer cells experience an increased load of DNA lesions as a result of perturbed DNA replication. These lesions are not always repaired before mitotic onset, which potentially fuels genomic instability and may negatively impact cellular viability. Cancer cells have evolved mechanisms to cope with mitotic DNA lesions, although the mechanistic wiring of mitotic DNA repair is currently largely unclear. Since MUS81 appears to play an important role in mitotic DNA damage resolution, we here aimed to identify proteins involved in mitotic DNA repair by immuno-purification of MUS81 as an established mitotic repair factor.

RESULTS

Optimizing synchronization protocol and GFP-MUS81 overexpression

To study proteins involved in mitotic DNA lesion processing, we engineered U2OS cells to express GFP-tagged MUS81. GFP-MUS81 was confirmed by Western blot (Fig. 1A) as well as by elevated GFP signal in the whole cell population as measured by flow cytometry (Fig. 1B), and immunofluorescence microscopy (Fig. 1C).

We aimed to identify mitotic MUS81 interaction partners as MUS81 was shown to play an important role in the resolution of DNA damage during mitosis. In order to do so, we tested different synchronization protocols to identify the most effective protocol to enrich mitotic U2OS cells with replication stress. To this end, cells were first treated with 2mM thymidine for 24 hours to allow accumulation at G1/S-phase of the cell cycle. Next, cells were released for 8 hours to allow progression into S/G2, with or without a low dose (0.2) of the DNA polymerase inhibitor aphidicolin to induce replicative stress. We tested two different protocols to enrich for mitotic cells. Firstly, cells were released from the thymidine block for 8 hours to allow accumulation in S/G2-phase. Next, cells were treated with the microtubule poison nocodazole (100ng/ml) to enrich for mitotic cells. Alternatively, following the 8 hours release from a thymidine block, the CDK1 inhibitor RO-3306 (5) was added for 4 hours to allow accumulation at the G2/M border. Next, cells were released into mitosis for 1 hour through wash-out of RO-3306 (Fig. 1D-F). As synchronization of cells can induce DNA lesions, we tested to what extent the synchronization protocol affected DNA lesions ending up in mitosis. To this end, we quantified the amount of FANCD2 foci in mitotic cells following synchronization. As expected, cells treated with aphidicolin showed an increase in FANCD2 foci as compared to untreated cells (Fig. 1D, 1E). An increase was observed in FANCD2 foci in cells synchronized using RO-3306 as compared to nocodazole-synchronized cells (median: 0 in nocodazole treated cells vs 1.0 in RO-3306 synchronized cells) (Fig. 1E). Furthermore, the number of mitotic cells was assessed using flow cytometry (Fig. 1F). Nocodazole synchronization resulted in more mitotic cells when compared to RO-3306 synchronization, both in untreated (10.3% in nocodazole-treated cells vs 4.4% in RO-3306 treated cells) and aphidicolin treated (5.5% nocodazole vs 1.3% RO-3306) (Fig. 1F). Taken together, these data indicate that synchronization using nocodazole leads to
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Figure 1. Optimizing synchronization protocol and GFP-MUS81 overexpression

A. U2OS cells parental or engineered to overexpress GFP or GFP-MUS81 constructs. Immunoblot shows MUS81, GFP and β-actin protein levels. B. GFP intensity (A.U.) of U2OS parental or GFP-MUS81 were analyzed by flow cytometry on a Becton Dickinson FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA). A minimum of 10,000 events was analyzed per sample. C. GFP expression of U2OS parental or GFP-MUS81 were analyzed using fluorescent microscopy. D, E. U2OS parental cells were treated with thymidine (2mM) for 24 hours. Next cells were treated with aphidicolin (0.2 µM) for 8 hours and synchronized with nocodazole for 5 hours or with RO-3306 (5 µM) for 4 hours followed by 1 hour release. Cells were fixed and stained FANCD2 (green) and counter-stained with DAPI (blue). Representative immunofluorescence images are shown. Scale bar indicates 7.5 µm nucleus per condition analyzed n>30. F. Cells are treated as described in D cells were harvested for flow cytometry analysis DNA content (propidium iodine) and MPM2-Alexa-488 were assessed by flow cytometry on a Becton Dickinson FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA). A minimum of 10,000 events was analyzed per sample. G. A schematic representation of treatment is shown.

better enrichment of mitotic cells and induces fewer FANCD2 foci than RO-3306 based synchronization. Hence, we decided to proceed with a further experiment using the nocodazole synchronization protocol (Fig. 1G).

Immune-purification of GFP-MUS81 and mass spectrometry (MS) analysis
To identify proteins that are potentially involved in mitotic processing of DNA lesions, we aimed to analyze MUS81 interactors upon replication stress during mitosis. To that end, we treated GFP-MUS81-expressing U2OS cells according to the optimized synchronization protocol (Fig. 1G). Mitotic cells were harvested by mitotic shake-off, followed by GFP-MUS81 immunopurification (IP). Westernblot analysis showed that cells overexpressing GFP-MUS81 showed enrichment for MUS81 in the pulldowns (Fig. 2A). Additionally, enrichment of the established MUS81 interactor EME1 was observed. As a control, GFP pull-downs were performed, which did not pull down EME1 nor MUS81 (Fig. 2A). To confirm that the pull-downs were performed in mitotic cells, flow cytometry analysis was performed in parallel (Fig. 2B). As expected, the majority of the cells used in the pull-down experiment were found to be mitotic as judged by MPM2 positivity (Fig. 2B). To conclude, we successfully pulled down MUS81 from lysates of mitotic cells.
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GFP-MUS81 IPs were analyzed by mass spectrometry (MS) to identify interactors of MUS81. Firstly, as a control, we analyzed MUS81 protein enrichment in the GFP-MUS81 IP samples compared to the GFP control IP. As expected, MUS81 was found to be most highly enriched both in GFP IPs from interphase and mitotic lysates (Fig. 2C, D). MUS81 was previously described to have different binding partners, depending on the phase of the cell cycle. The established MUS81 interactor EME1 was found to be enriched in both interphase and mitotic pulldowns (Fig 2C, D). SLX4 - another known MUS81 interactor - was only observed in the interphase IP and not in the mitotic pull-down (Fig. 2C). Surprisingly, we did not observe specific enrichment for proteins upon treatment with aphidicolin. Of note, MAGEA1 was found to be enriched in both interphase and mitotic IPs. MAGEA1 has not previously been described to interact with MUS81. Gene set analysis of MAGEA1 and MUS81 predicted similar expression patterns, reflecting a cell cycle dependent expression profile (Suppl. Fig. 1A-B). Taken together, our proteomic analysis identifies MAGEA1 as a potential novel interactor of MUS81 during both interphase and mitosis.

Interaction of MUS81 and MAGEA1

To validate the interaction between MUS81 and MAGEA1 (Fig. 2C, D), we repeated the pull-down experiment in asynchronous U2OS GFP-MUS81 cells. Immunoblot analysis of the GFP-MUS81 pull-down confirmed the interaction between GFP-MUS81 and MAGEA1 (Fig. 3A). We next set out to investigate if the interaction between MUS81 and MAGEA1 also occurs at endogenous levels. To test this, MAGEA1 immuno-precipitation (IP) was performed (Fig 3B). Immunoblot analysis did not confirm co-IP of MUS81 in these pulldowns (Fig. 3B). Conversely, we performed IPs of endogenous MUS81, MUS81 enrichment in both anti-MUS81 and IgG control IPs was
observed, indicating that the MUS81 IP was not specific (Fig. 3C). Combined, the interaction between MUS81 and MAGEA1 was only observed using GFP-MUS81, but could not be confirmed using IPs of the endogenous proteins.

Depletion of MUS81 has been reported to sensitize cells towards cisplatin and aphidicolin 12,21. We investigated if depletion of MAGEA1 would yield a similar phenotype as described for MUS81 depletion. To this end, U2OS cells were transfected with siRNAs targeting MAGEA1 for 24 hours (Fig. 3D). Immunoblot analysis showed a knockdown of MAGEA1 with siRNA #1 but not with siRNA #2. Next, siMAGEA1 cells were plated for colony survival and treated with the indicated concentration of cisplatin (Fig. 3E, F) or aphidicolin (Fig. 3G). Upon increasing concentration of cisplatin number of colonies observed was reduced in all cells (Fig. 3E). Quantification of colonies showed that depletion of MAGEA1 by siRNA #1 effectively sensitized cells to cisplatin treatment (Fig. 3F). Aphidicolin treatment did effectively reduced the formation of colonies in both control and siMAGEA1 treated cells. However, there was no sensitization effect observed upon MAGEA1 depletion (Fig. 3G). To conclude, the cisplatin sensitization observed upon MAGEA1 depletion is in line with the known phenotype for MUS81 depletion. This could indicate that MAGEA1 and MUS81 act in the same pathway.
DISCUSSION

In this study, we used GFP-MUS81 pull downs combined with MS analyses to identify specific mitotic and interphase MUS81 interactors. We found established MUS81 interactors, such as EME1, ERCC1 and SLX4. Additionally, we found MAGEA1 to specifically interact with GFP-MUS81 in the osteosarcoma cell line U2OS. By performing co-IP experiments using the endogenous MAGEA1, we were not able to confirm the reciprocal interaction of MAGEA1 with endogenous MUS81. Further experiments should focus on validating the interaction of MUS81 and MAGEA1. MUS81 co-IPs did not work with the experimental setup that we used. Additional experiments with antibodies targeting other epitopes of MUS81 could be performed and may allow IP of MUS81. Additionally, it would be informative to see if MAGEA1 and MUS81 co-localize, this can be studied by for example proximity ligation experiments.

We find siRNA-mediated knockdown of MAGEA1 to increase sensitivity towards cisplatin. Surprisingly, in a genome-wide genetic screen for drug sensitivity, MAGEA1 inactivation was not observed to result in sensitization towards cisplatin\(^22\). These different results can be explained by a difference in cell lines; whereas the genetic screen was performed in the untransformed RPE-1 cell line, our experiments were performed in the U2OS osteosarcoma cell line. Furthermore, it may be that the observed difference is a result of knockout versus knockdown. Additionally, there may be a difference in the sensitivity of the two assays. The detection limit of a genome-wide screen is lower when compared to doing experiments with individual genes. For example, MUS81 knock-out is known to enhance sensitivity towards PARP inhibitor\(^23,24\); however, MUS81 knock out was not found to enhance olaparib sensitivity in the genetic screen\(^25\).

In this study, we aimed to identify proteins involved mitotic DNA repair by immune-purification of MUS81. MUS81 is described to play an important role in resolving mitotic DNA damage upon replication stress. In the early stages of mitosis, or even in late G2, MUS81 is involved in MiDAS\(^13,23\). Additionally, MUS81 assembles in a nuclease complex together with SLX1-SLX4 and XPF to cleave persistently joint DNA molecules\(^11\).

In our synchronization protocol we used nocodazole to allow mitotic accumulation. Nocodazole is a microtubule poison that prevents the transition from metaphase to anaphase of mitosis\(^25\). Thus, cells treated with nocodazole accumulate in prometaphase. We detected SLX4, a member of the SLX nuclease complex, in the interphase pull downs but not in our mitotic pull downs. The interphase cells that we analyzed are mainly G2 cells, and the assembly of the SLX1-SLX4, XPF complex together with MUS81 is favored in G2/M stage of the cell cycle\(^11\). It is not known exactly when the interaction between MUS81 and the nuclease complex is disassembled.

MiDAS is described to occur during prophase, the earliest phase of mitosis\(^13\). It is not known when exactly during mitosis MUS81 localizes to the sites of MiDAS and whether MUS81 remains at the site of MiDAS. Since we are analyzing cells that have progressed from prophase to prometaphase it is possible that the MUS81 species that we pull down are not interacting with MiDAS factors anymore.

The synchronization protocol that we used results in cells accumulating in the early phases of mitosis. This stage of mitosis is described to be the cell cycle phase when MUS81 functions to mitigate replication stress. Despite our synchronization protocol we did not detect previously identified MiDAS factors. This could be explained by the fact that MUS81 does not physically interact with MiDAS factors or that these interactions are transient and during prometaphase these factors do not interact anymore. Furthermore, in literature, there is no consensus on which proteins are essential for MiDAS and which are dispensable\(^13-15\). MiDAS was first described to require MUS81 and POLD3\(^13\), later RAD52 was shown to facilitate MiDAS\(^15\). However, data has been presented that disputes the role of RAD52 in facilitating MiDAS\(^26\). Specifically, Minocherhomj and colleagues describe that MUS81 facilitates MiDAS\(^15\), whereas Calzetta and colleagues dispute the role of RAD52 and argue that MiDAS is solely dependent on nucleotide availability\(^26\). Moreover, a recent report challenges the model in which MiDAS as a mitotic pathway but rather a late-G2 phase phenomenon\(^27\). Additionally, it was shown in this report that MiDAS is solely depending on RAD52 and that both POLD3 and MUS81 are dispensable\(^27\). These contradictory data indicate that MiDAS is a process that may be highly context dependent. Further investigation into MiDAS should take into account when MiDAS is happening during the cell cycle.
MATERIAL & METHODS

Cell culture
The U2OS osteosarcoma cell line was obtained from ATCC (#HTB-96). U2OS cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS), 50 units/mL penicillin, 50 µg/mL streptomycin, in a humidified incubator supplied with 5% CO₂ at 37°C. Cell lines were verified by STR profiling (Baseclear, the Netherlands).

Immunofluorescence microscopy
U2OS cells were seeded on glass coverslips in 6-well plates. When indicated, U2OS cells were treated with aphidicolin (0.2 µM) for 18 hours. For FANCD2 (Novusbio, NB100-182), cells were treated for 60 seconds with PEM (100 mM PIPES pH 6.9, 1 mM MgCl₂ and 10 mM EGTA). Next, cells were simultaneously fixed and permeabilized (20 mM PIPES pH 6.8, 0.2% Triton X-100, 1mM MgCl₂, 10 mM EGTA, 4% paraformaldehyde) for 10 minutes at room temperature. Cells were then incubated with corresponding Alexa-488 conjugated secondary antibodies, and counterstained with DAPI (Sigma). For analysis of DNA damage response components, prophase and prometaphase cells were included for scoring. Specifically, mitotic cells with condensed chromosome, but prior to metaphase alignment Images were acquired on a Leica DM6000B microscope using a 63x immersion objective (PL S-APo, numerical aperture: 1.30) with LAS-AF software (Leica).

RNA interference
For siRNA (small interference RNA) transfection, siRNAs (Ambion Stealth RNAi, Thermofisher) targeting MAGEA1 (sequence 1: GCAAAGCCUCUGAGUCCUU and sequence 2: GUUCUGUGUAUAUAGUUUA), or a siSCR (scrambled) control sequence (sequence #12935300) were used at a final concentration of 40 nM. Transfections were performed with oligofectamine (Invitrogen) by manufacturer’s guidelines.

Western blotting
Cell lysis was performed using Mammalian Protein Extraction Reagent (MPER, Thermo Scientific), supplemented with protease inhibitor and phosphatase inhibitor (Thermo Scientific). Protein concentrations were measured using a Bradford assay. Cells were harvested and MPER was added in a 1ml per 1mg pellet weight. 1 µl of benzonase (EMD, CA80601-766, 250 U) was added and cell pellets were incubated on a nutator at 4 °C for 20 min. Lysate supernatants were added to pre-washed GFP-trap beads (chromotek), or g-protein dynabeads (Thermo Scientific). Proteins were affinity-purified at 4°C on a nutator for 3 hours.

Mass-spectrometry
Each sample was isolated from SDS-page gel from a single slice. Online chromatography of the extracted tryptic peptides was performed using an Ultimate 3000 HPLC system (Thermo Fisher Scientific) coupled online to a Q-Exactive-Plus mass spectrometer with a NanoFlex source (Thermo Fisher Scientific), equipped with a stainless-steel emitter. Tryptic digests were loaded onto a 5 mm x 300 µm internal diameter (i.d.) trapping micro column packed with PepMAP100, 5 µm particles (Dionex) in 0.1% formic acid at
the flow rate of 20 µl/minute. After loading and washing for 3 min, trapped peptides were back-flush eluted onto a 50 cm × 75 µm i.d. nanocolumn, packed with Acclaim C18 PepMAP RSLC, 2 µm particles (Dionex). Eluents used were 100:0 H2O/acetonitrile (volume/volume (V/V)) with 0.1% formic acid (Eluent A) and 0:100 H2O/acetonitrile (v/v) with 0.1% formic acid (Eluent B). The following mobile phase gradient was delivered at the flow rate of 250 nl/min: 1–50% of solvent B in 90 min; 50–80% B in 1 min; 80% B during 9 min, and back to 1% B in 1 minutes and held at 1% A for 19 which results in a total run time of 120 minutes. MS data were acquired using a data-dependent acquisition (DDA) top-10 method dynamically choosing the most abundant not-yet-sequenced precursor ions from the survey scans (300–1650 Th) with a dynamic exclusion of 20 seconds. Survey scans were acquired at a resolution of 70,000 at mass-to-charge (m/z) 200 with a maximum inject time of 50 milliseconds or AGC 3e6. DDA was performed via higher energy collisional dissociation fragmentation with a target value of 1x10E5 ions determined with predictive automatic gain control in centroid mode. Isolation of precursors was performed with a window of 1.6 m/z. Resolution for HCD spectra was set to 17,500 at m/z 200 with a maximum ion injection time of 50 milliseconds. Normalized collision energy was set at 28. The S-lens RF level was set at 60 and the capillary temperature was set at 250°C. Precursor ions with single, unassigned, or six and higher charge states were excluded from fragmentation selection. The MS raw data were analyzed with MaxQuant (version 1.3.0.5) containing the integrated Andromeda search engine and searched against the UniProt human proteome build 20132802 with a false discovery rate of 0.01. Two biological replicates were performed each sample was measured once.

REFERENCES


**Supplement Figure 1**
Predicted geneset membership of MUS81 and MAGEA1 based on co-expression using gentICA-network^23^.