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RESEARCH ARTICLE

Condensation properties of stress granules and processing bodies are compromised in myotonic dystrophy type 1

Selma Gulyurtlu¹, Monika S. Magon¹, Patrick Guest¹, Panagiotis P. Papavasiliou¹, Kim D. Morrison¹, Alan R. Prescott² and Judith E. Sleeman¹,*

ABSTRACT

RNA regulation in mammalian cells requires complex physical compartmentalisation, using structures thought to be formed by liquid-liquid phase separation. Disruption of these structures is implicated in numerous degenerative diseases. Myotonic dystrophy type 1 (DM1) is a multi-systemic trinucleotide repeat disorder resulting from an expansion of nucleotides CTG (CTGexp) in the DNA encoding DM1 protein kinase (DMPK). The cellular hallmark of DM1 is the formation of nuclear foci that contain expanded DMPK RNA (CUGexp) (with thymine instead of uracil). We report here the deregulation of stress granules (SGs) and processing bodies (P-bodies), two cytoplasmic structures key for mRNA regulation, in cell culture models of DM1. Alterations to the rates of formation and dispersal of SGs suggest an altered ability of cells to respond to stress associated with DM1, while changes to the structure and dynamics of SGs and P-bodies suggest that a widespread alteration to the biophysical properties of cellular structures is a consequence of the presence of CUGexp RNA.

KEY WORDS: LLPS, Myotonic dystrophy type 1, P-bodies, Stress granules, Trinucleotide repeats

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is an autosomal dominant, multi-systemic disease (reviewed by Pettersson et al., 2015) with an estimated global prevalence of 1:20,000 (Theadom et al., 2014). Its symptoms are variable and can include myotonia, muscular atrophy, cardiac defects, cataracts and insulin resistance (Pettersson et al., 2015). DM1 is caused by an expansion of nucleotides cytosine, thymine and guanine (CTGexp) in the DM1 protein kinase (DMPK) gene. Transcription of mutant DMPK results in RNA transcripts containing a (CUG)exp repeat expansion (CUGexp) – with uracil (U) instead of thymine (T) – in their 3′ untranslated region (3′UTR), which then form an elongated metastable stem-loop structure (Pettersson et al., 2015). These RNA transcripts accumulate to form nuclear foci (CUGexp foci) and recruit RNA-binding proteins (RBPs), notably muscleblind-like protein 1 (MBNL1), which interacts dynamically with the CUGexp foci, showing rapid exchange with MBNL1 (Coleman et al., 2014; Ho et al., 2005). Similarities between DM1 and DM2 – the latter resulting from a similar repeat expansion within an unrelated gene (Liquori et al., 2001) – suggest that the nuclear CUGexp foci are the source of DM1 pathology. The mechanisms by which CUGexp foci result in cellular damage and the broad range of DM1 symptoms are not understood but are proposed to include disturbance of the normal cellular functions of MBNL1.

MBNL1 is the most abundant RNA metabolism regulator within the muscleblind-like protein family and expressed in most tissues (Konieczny et al., 2014). The protein has a key role as an alternative splicing regulator, acting antagonistically with a second splicing regulator, i.e. CUGBP Elav-like family member 1 (CELF1, also known as and hereafter referred to as CUGBP1) (Junghans, 2009; Mahadevan et al., 2006). The splicing activity of CUGBP1 is increased in DM1 and thought to be linked to accumulation of MBNL1 in CUGexp foci. Splicing alterations attributed to disturbance of the balance between active MBNL1 and CUGBP1 have been documented in DM1. For example, whereas MBNL1 inhibits exon-5 inclusion in mRNA of cardiac troponin T (TNNT2), CUGBP1 promotes it (Wang et al., 2007), and inclusion of exon-11 in the insulin receptor (INSR) is promoted by MBNL1 and inhibited by CUGBP1 (Savkur et al., 2001). However, both MBNL1 and CUGBP1 have additional diverse functions in gene regulation. MBNL1 is also implicated in RNA transport and stability, and in miRNA processing (Konieczny et al., 2014; Osborne et al., 2009; Rau et al., 2011; Wang et al., 2012). CUGBP1 is involved in translation, stability and decay of mRNA (Masuda et al., 2012; Teplova et al., 2010).

Cellular RNA metabolism is complex, and its physical organisation within the cell involves the formation of RNA-rich bodies within the nucleus and cytoplasm. In the nucleus, these include Cajal bodies, nuclear speckles and paraspeckles, whereas – in the cytoplasm – structures including processing bodies (P-bodies) and stress granules (SGs) play roles in RNA stability. These are all non-membranous, highly dynamic structures and there is evidence that at least some of them are formed by liquid-liquid phase separation (LLPS) (Banani et al., 2016; Jain et al., 2016; Kroschwald et al., 2015; Protter and Parker, 2016; Stane k and Fox, 2017). LLPS requires a high concentration of a molecule or a mixture of molecules, forming a network of weak multivalent interactions resulting in a separate phase. This allows structures to exhibit liquid-like properties, such as having spherical, droplet-like morphology resulting from reduced surface tension, and demonstrating fusion, fission and reversible disruption (Couchaine et al., 2016; Sawyer et al., 2019). Phase-separated structures normally allow rapid exchange of components, which distinguishes them from the more-solid protein aggregates (Markmiller et al., 2018; Staněk and Fox, 2017) that are implicated in pathologies, such as...
amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (Jain et al., 2016; Li et al., 2013; Markmiller et al., 2018; Ramaswami et al., 2013).

In mammalian cells, as in cells of other species, P-bodies and SGs (Jain et al., 2016; Protter and Parker, 2016) are cytoplasmic structures proposed to form by phase separation dependant on mRNA-protein interactions, with key roles in cellular signalling and the stress response (Anderson et al., 2015; Protter and Parker, 2016). They interact with each other both physically and functionally (Anderson et al., 2015; Protter and Parker, 2016; Shah et al., 2016), and share a number of protein components, although the precise relationship between the two structures is unclear. P-bodies contain the RNA decay machinery, including the decapping enzymes DCP1A, and DCP2, and decapping activators, such as EDC4 and DDX6 (Anderson et al., 2015). P-bodies appear to be built on a scaffold of mRNA (Banani et al., 2016; Ditlev et al., 2018) and have been specifically implicated in mRNA degradation, surveillance, translation repression and RNA-mediated gene silencing (Anderson et al., 2015; Protter and Parker, 2016). SGs derive from mRNAs stalled during translation initiation, typically resulting from forms of cellular stress, such as oxidative stress, heat shock, hypoxia, starvation and viral infection (Anderson et al., 2015; Protter and Parker, 2016; Roy and Rajaguru, 2018). In contrast to the smaller P-bodies, mammalian SGs appear to contain a combination of liquid and more-solid phases, where different areas within the SG exhibit varying levels of dynamic exchange of their components (Jain et al., 2016; Protter and Parker, 2016). In yeast, SGs have a more-solid structure (Ditlev et al., 2018; Jain et al., 2016; Kroschwald et al., 2015). Upon removal of stress, SGs disassemble (Hardy et al., 2017; Mateju et al., 2017; Protter and Parker, 2016; Turakhiya et al., 2018) and messenger ribonucleoproteins (mRNPs) that, during the stress response, had been sequestered within them return to active translation (Lee and Seydoux, 2019). Aberrant persistent SGs, which are predicted to be less dynamic than canonical SGs, have been implicated in neurodegenerative diseases, such as ALS and FTD (Jain et al., 2016; Markmiller et al., 2018; Wolozin and Ivanov, 2019; Zhang et al., 2018), and have been proposed to result from increased liquid-to-solid phase transitions within the SGs, with autophagy implicated in their clearance (Mateju et al., 2017).

Increased cellular stress, resulting from the presence of expanded DMPK RNA (CUGexp) RNA foci, has been suggested to be a pathological effector in DM1 (Huichalaf et al., 2010; Isara et al., 1995; Kumar et al., 2014; Toscano et al., 2005; Usuki and Ishiura, 1998; Usuki et al., 2000). Both MBNL1 and CUGBP1 have been identified in SGs of mammalian cells (Fujimura et al., 2008; Onishi et al., 2008), and increased numbers of SGs have been reported in DM1 myoblasts as compared to SG numbers in control myoblasts grown under normal conditions (Huichalaf et al., 2010). Furthermore, a defect in SG formation in response to treatment grown under normal conditions (Huichalaf et al., 2010).}

**RESULTS**

The hallmark cellular defect in DM1 is the presence of CUGexp RNA foci formed by mutant DMPK transcripts in the cell nucleus, within which the multi-functional RBP MBNL1 accumulates. While some DMPK mRNA is clearly exported to the cytoplasm and translated (Gudde et al., 2017), the global effect of the presence of CUGexp RNA and the formation of nuclear CUGexp foci on cellular RNA metabolism is not understood. Lens cataract is the most prevalent symptom in DM1 (Romeo, 2012; Smith and Gutmann, 2016), and we have previously determined that the CUGexp foci in HLECs derived from DM1 patients contain only 0.2-0.5% of cellular MBNL1 (Coleman et al., 2014), making sequestration of MBNL1 within the foci an unlikely explanation for DM1-associated cataract. Transparency of the lens is achieved by the differentiation of a stem cell pool within the lens epithelium, during which transcription is shut down, and all cellular organelles and inclusions are lost (Dahm and Prescott, 2002; Gribben et al., 2002). Lens formation relies on forms of autophagy, i.e. the same process that is responsible for clearance of SGs under certain conditions (Hardy et al., 2017; Mateju et al., 2017; Protter and Parker, 2016; Turakhiya et al., 2018). Together, evidence for alteration of SGs in DM1 and our previous observation that MBNL1 localises to SGs following transcriptional arrest in HLECs (Coleman et al., 2014) led us to investigate the presence of the DM1-associated proteins MBNL1 and CUGBP1 in cytoplasmic structures involved in RNA metabolism.

MBNL1 and CUGBP1 colocalise within P-bodies in HLECs

Our initial examination of the subcellular distribution of endogenous CUGBP1 and MBNL1 in HLECs (four patient-derived cell lines and two age-matched control cell lines) revealed a striking contrast between their distributions in the nucleus, where MBNL1 localises to CUGexp foci and CUGBP1 does not, and the cytoplasm, where the two proteins colocalise in discrete punctate structures (Fig. 1A and Fig. S1). Further analysis demonstrated these structures to be P-bodies, identified by using the P-body marker GE1 (Fig. 1A). Whereas accumulation of CUGBP1 has been reported in P-bodies (Yu et al., 2013), MBNL1 has not previously been reported as a P-body component. Our observation of both these proteins in P-bodies points to close links between the cytoplasmic functions of MBNL1 and CUGBP1.

MBNL1 and CUGBP1 both accumulate within SGs of HLECs following treatment with NaAsO₂

MBNL1 and CUGBP1 have both been reported to accumulate within SGs (Fujimura et al., 2008; Kedersha and Anderson, 2009) in different cell types, using varied strategies to induce cellular stress. Furthermore, myoblasts from DM1 patients have been reported to display SGs under normal growth conditions, suggesting a higher basal level of stress compared to controls (Huichalaf et al., 2010). Although we saw no evidence of SGs in the DM1 HLEC lines under basal level of stress compared to controls (Huichalaf et al., 2010).
SGs show no delay in formation but disperse more quickly in DM1 HLECs compared to SGs in control cells

Having shown that SGs are not present in DM1 HLECs under normal growth conditions – suggesting that any increase in basal stress levels is insufficient to drive SG formation – and that SGs containing MBNL1 and CUGBP1 are readily formed in response to NaAsO2, we next sought to investigate the kinetics of SG formation and SG loss in DM1 HLECs compared to control cells. When fixed after differing lengths of treatment with NaAsO2, neither DM1 HLECs nor control HLECs showed any delay in the formation of SGs detected using endogenous TIA1 (Fig. 2A). In contrast, DM1 HLECs that were pre-treated with NaAsO2 to induce SGs and then allowed to recover showed a more rapid loss of SGs detected using endogenous TIA1 and MBNL1, compared to control HLECs (Fig. 2B). This suggests that DM1 cells differ from control cells in their ability to recover from stress.

MBNL1 dynamics are unaffected within SGs in DM1 HLECs but altered in P-bodies

We next used fluorescence recovery after photobleaching (FRAP) to examine the dynamics of MBNL1 in SGs and P-bodies, by using transiently expressed GFP-tagged MBNL1 (hereafter referred to as GFPMBNL1), previously validated to behave similarly to endogenous MBNL1 in these cell lines (Coleman et al., 2014). Previous studies have shown that, in mammalian cells, these two related structures are both highly dynamic, displaying properties of liquid droplets (Kroschwald et al., 2015). Persistent SGs have long been associated with degenerative conditions, notably ALS (Li et al., 2013; Wolozin and Ivanov, 2019), but the dynamics of MBNL1 within SGs and P-bodies in the context of DM1 has not been examined.

To analyse the dynamics of SGs, HLECs transiently expressing GFPMBNL1 were treated for 45 min with 0.5 mM NaAsO2 to induce SGs. To analyse the dynamics of P-bodies, HLECs were transiently transfected to express both GFPMBNL1 and mCherry-Dcp1a, a marker for P-bodies, to enable their unambiguous identification. A diffraction-limited spot of laser excitation was used to bleach GFPMBNL1 in SGs or P-bodies and the subsequent recovery fitted to a one-phase exponential recovery model (Fig. 2C-E and Fig. S3). This modelling (Fig. 2C) showed no statistically significant changes in the mobile fraction or half-time of recovery of GFPMBNL1 in SGs in DM1 cells compared to controls, suggesting that SGs, once formed, are not defective in DM1 cells in terms of MBNL1 dynamics. The mobile fraction of GFPMBNL1 within P-bodies in both DM1 and control cells was significantly larger than that for SGs in DM1 cells (Fig. 2C), suggesting that GFPMBNL1 has a more-dynamic relationship with P-bodies than with SGs, although the small number of data points for SGs in control cells prevents a strong conclusion from being drawn (69.6±17.2% for P-bodies in DM1, 70.1±25.2% for P-bodies in control, 43.9±15.2% for SGs in DM1, 50.6±20.2% for SGs in control). A significant increase in the half-time of recovery for GFPMBNL1 in P-bodies was seen for DM1 cells compared to controls, indicating an altered relationship of MBNL1 with P-bodies associated with DM1 (3.4±2.3 s for DM1, 2.0±1.2 s for control).

Simultaneous expression of a DMPK minigene and GFPMBNL1 models DM1 cells with CUGexp foci detected in living cells

Patient-derived HLECs are a highly relevant cell model in which to study the cellular pathology of DM1. However, the use of patient cell lines comes with the key drawback that the lines are of diverse...
genetic origins, making disease onset and severity as well as length of CUG repeats different between them. To assess the effect of CUGexp RNA on SG structure and dynamics directly, we generated an inducible HeLa cell model for DM1. The stable HeLa cell lines comprising this model contain a bi-directional tetracycline-responsive promoter that drives the simultaneous expression of GFPMBNL1 and a DMPK minigene. To mimic the DM1 phenotype, this DMPK minigene contains an interrupted 960-nucleotide-long CTG repeat (CTG960), yielding model cell line HeLa_CTG960_GFPMBNL1. However, as the disease only manifests in the presence of >50 repeats (Lee et al., 2012), the DMPK minigene in the matched control cell line, i.e. HeLa_CTG12_GFPMBNL1, contains only12 CTG repeats (CTG12).

Fig. 2. Kinetics analysis of SGs and P-bodies reveals increased rates of SG loss during stress recovery, and slightly altered MBNL1 dynamics in DM1 compared to control cells. (A) SG formation. Control and DM1 HLECs treated with 0.25 mM NaAsO2 were followed over 1.5 h during which the proportion of cells containing SGs was detected with antibody against endogenous TIA1. No significant differences were observed between DM1 and control cells. (B) SG recovery after pre-treatment with NaAsO2. Cells were pre-treated with NaAsO2 (0.5 mM for 90 minutes), washed thoroughly, and the proportion of cells containing SGs was detected over 2 h of recovery with antibodies against endogenous TIA1 and MBNL1. Compared with control cells, significantly fewer DM1 cells retain SGs at the 1 h time point. P<0.05 one-way ANOVA (n=50 cells per cell line per time-point); data shown are pooled from two independent experiments, obtained from three DM1 cell lines and two control cell lines (see Fig. S2 for individual data from each cell line). (C) Mobile fraction (left) and half-time of fluorescence recovery (right) of GFPMBNL1 within P-bodies (PB) and SGs (SG) in control and DM1 HLECs. *P<0.05; ***P<0.001; ****P<0.0001 Kruskal–Wallis. (D,E) Plotted is the relative fluorescence recovery of GFP-tagged MBNL1 (GFPMBNL1) in P-bodies (PB; panel D) and SGs (SG; panel E) over 15 s, following photobleaching, using a 488 nm laser pulse for 2.5 s. Closed circles indicate P-bodies or SGs in control cells [HLECs from CCat1 and CCat3 (see ‘Cell lines and cell culture’ in Materials and Methods), n=39 total for P-bodies and n=6 for SGs]; open circles indicate P-bodies or SGs in DM1 cells [HLECs from DMCat1, DMCat2 and DMCat4 (see ‘Cell lines and cell culture’ in Materials and Methods), n=17 total for P-bodies and n=17 for SGs]. All data are displayed as the mean±s.d. Representative images before photobleaching are shown below, magnifications of the boxed areas are shown on the right of each image, before and immediately after photobleaching, and following recovery. Scale bars: 7 µm.
Fluorescence microscopy and immunoblotting confirmed that no detectable GFP-MBNL1 was expressed in the absence of the tetracycline equivalent doxycycline (Dox), whereas addition of Dox resulted in expression of GFP-MBNL1 in both cell lines (GFP-MBNL1 in Fig. 3A and 67 kDa band in Fig. 3B). In the control cell line HeLa_CTG12-GFP-MBNL1, P-bodies were less numerous compared to the control line, i.e. 40% of P-bodies contained CUGBP1 but to the usual distribution in >95% of expressing cells (Fig. 3A and Fig. S4). These were confirmed as CUGexp foci by using RNA fluorescence in situ hybridisation (FISH) with a Cy3-CAG10 probe against the trinucleotide repeats (CUGexp in Fig. 3A), allowing nuclear GFP-MBNL1 accumulation to be used as a marker for CUGexp foci in subsequent experiments. There are typically several CUGexp foci per nucleus, also seen in HLECs derived from DM1 patients (Fig. 3D see also Figs 5, 8, Figs S1, S4, Movies 2 and 4 and Coleman et al., 2014). The partition of GFP-MBNL1 between cytoplasm and nucleus was not affected by the presence of CUGexp foci (Fig. 3F). Comparison of the signals for endogenous MBNL1 and GFP-MBNL1, detected with antibody against MBNL1 by immunoblotting, revealed moderate overexpression of GFP-MBNL1 in both cell lines (Fig. 3B,G), with <1% of GFP-MBNL1 accumulating in CUGexp foci (Fig. 3C). The number of foci did not change significantly between 24 h and 72 h of induction (Fig. 3D) but the foci were significantly larger than those previously characterised in DM1 HLECs (Fig. 3E; Coleman et al., 2014).

In DM1 HeLa cell models, MBNL1 and CUGBP1 show subtle changes in colocalisation within P-bodies and sub-regions of SGs, associated with the presence of CUGexp foci

In unstrressed Dox-induced HeLa_CTG12-GFP-MBNL1 and HeLa_CTG960-GFP-MBNL1 cells, P-bodies were less numerous and less distinct than in HLECs (Fig. 4A, Fig. S5). Many of the P-bodies present in the control HeLa_CTG12-GFP-MBNL1 cell line accumulated small amounts of both GFP-MBNL1 and CUGBP1, with 86% of P-bodies containing CUGBP1 and 56% of P-bodies both proteins (n=57 cells). In the DM1 model line HeLa_CTG960-GFP-MBNL1, very few P-bodies contained detectable levels of GFP-MBNL1, while fewer P-bodies contained CUGBP1 compared to the control line, i.e. 40% of P-bodies contained CUGBP1 but only 3% contained both proteins (n=53 cells) (Fig. 4G). This suggests that P-body components, particularly MBNL1, were altered by the presence of the CTG960 expansion. Following treatment with NaAsO2 to induce cellular stress, staining for the P-body marker GE1 showed increased numbers of prominent P-bodies in both cell lines as well as SGs containing GFP-MBNL1, CUGBP1 and the canonical SG protein TIA1 (Figs 4B,C and 7D,E). In NaAsO2-treated cells of either cell line most P-bodies contained GFP-MBNL1 but GFP-MBNL1 levels in P-bodies as a percentage of total cellular GFP-MBNL1 was smaller in HeLa_CTG960-GFP-MBNL1 cells compared with HeLa_CTG12-GFP-MBNL1 control cells (0.08±0.1% compared with 0.1±0.1%, respectively) (Fig. 4F). As can be seen in Figs 4B and 8A, MBNL1 and CUGBP1 were better markers for SGs in these cells than TIA1, the latter also showing some staining around the plasma membrane. This was not seen with MBNL1, CUGBP1 or polyadenylated [poly(A)] RNA. Using superresolution airsycan microscopy, localisation of both GFP-MBNL1 and CUGBP1 was revealed to be non-uniform within each SG and largely co-incident (Fig. 4D). In both cell lines colocalisation of GFP-MBNL1 and CUGBP1, measured using the Pearson correlation coefficient, was very high. However, a statistically significant increase was seen in the HeLa_CTG960-GFP-MBNL1 cell line compared to the HeLa_CTG12-GFP-MBNL1 control line (Fig. 4E) (0.90±0.05 compared to 0.87±0.07, respectively), suggesting a subtle change in SG architecture with respect to the distribution of MBNL1 and CUGBP1, associated with the presence of CUGexp RNA.

**SG formation and dispersal are altered by CUGexp RNA expression in our inducible HeLa cell model**

To investigate further the effects the presence of CUGexp RNA has on GFP-MBNL1-containing SGs, we used time-lapse imaging of CTG960- and CTG12-expressing cells during the response to and the recovery from stress induction following treatment with NaAsO2. First, HeLa_CTG960-GFP-MBNL1 and HeLa_CTG12-GFP-MBNL1 cells were induced for 24 h with Dox. Following addition of NaAsO2 (0.25 mM), time-lapse microscopy was carried out immediately and z-stacks of images were taken every 4 min until the formation of SGs was clearly observed (Fig. 5A). This revealed a pronounced delay in the formation of SGs in cells expressing CUGexp RNA compared with those that did not (HeLa_CTG960-GFP-MBNL1, 36±12 min compared with HeLa_CTG12-GFP-MBNL1, 15±2 min, respectively) (Fig. 5B,C). This change was not seen when using endogenous MBNL1 and TIA1 to detect SGs in cells not induced with Dox (Fig. S7). As cellular stress induced by NaAsO2 is reversible and dispersal of SGs at least as important for their regulation as is their formation, we next investigated the dispersal time of GFP-MBNL1-containing SGs in HeLa_CTG960-GFP-MBNL1 compared to control cells. Following induction with Dox for 24 h or 72 h cells were treated for 45 min with 0.5 mM NaAsO2 to ensure full induction of SGs in both lines. Upon thorough removal of NaAsO2, cells were imaged during recovery, with cells marked as recovered when no SGs were observed anymore (Fig. 5A). In contrast to the delay in formation of SGs associated with CUGexp RNA, GFP-MBNL1-containing SG dispersal occurred more quickly in cells containing CUGexp RNA compared to control cells (HeLa_CTG960-GFP-MBNL1, 133±38 min at 24 h and 79±11 min at 72 h; HeLa_CTG12-GFP-MBNL1, 158±30 min at 24 h and 159±28 min at 72 h). The increased speed of dispersal was more pronounced after 72 h than after 24 h (Fig. 5B,C). The disassembly seen when using MBNL1 as a marker for SGs closely resembled that reported previously when using GFP-G3BP1 as a marker in human U-2 OS osteosarcoma cells (Wheeler et al., 2016), with breakdown of large SGs into much smaller fragments (Movies 3 and 4). This suggested that we were visualising the disassembly of SGs, rather than simply the loss of MBNL1 from SGs, as the latter would be visualised as a gradually weakening signal in an SG of unchanged size. Taken together, these data show that the presence of the CUGexp RNA affects both SG formation in response to NaAsO2 treatment and SG dispersal upon stress removal, suggesting an overall defect in SG regulation associated with the presence of the CUGexp RNA characteristic of DM1.

**MBNL1 dynamics within SGs show subtle alterations in HeLa cells expressing CUGexp RNA**

To investigate the effect of CUGexp RNA on the dynamics of MBNL1 within SGs, FRAP analysis was used on HeLa_CTG12-GFP-MBNL1 and HeLa_CTG960-GFP-MBNL1 cells that had been induced with Dox for 24 h prior to treatment with NaAsO2 for 45 min (Fig. 5C-E). This analysis revealed a smaller mobile fraction
Fig. 3. Simultaneous expression of a DMPK minigene and GFPMBNL1 models DM1. (A) HeLa cells containing a DMPK minigene with 12 (left) or 960 (right) CTG trinucleotide repeats and GFP-tagged MBNL1 (GFPMBNL1), under a bi-directional tetracycline responsive promoter. Cells grown in the absence of doxycycline (−Dox) (top row) show no expression of GFPMBNL1 in the cytoplasm or nucleus (counterstained with DAPI, blue on overlay). Cells induced with 1 µg/ml Dox (+Dox) for 48 h show clear expression of GFPMBNL1 (middle row, green on overlay), forming distinct nuclear foci (arrows) in the CTG960 line and the characteristic localisation of GFPMBNL1 to the nucleus and cytoplasm in the CTG12 line. RNA FISH using a probe against the CUGexp RNA (bottom row, magenta on overlay) confirms that the GFPMBNL1 nuclear foci are genuine CUGexp foci in the CTG960 line (right) and that no CUGexp RNA is detected in the CTG12 line (left). Scale bar: 10 µm. (B) Immunoblotting of PAGE-separated whole-cell lysates using antibodies against GFP (AlexaFluor790; top) and MBNL1 (AlexaFluor680; centre) confirmed successful expression of GFPMBNL1 (67 KDa) in both cell lines, i.e. CTG12 (left) and CTG960 (right), following treatment with Dox for 48 h – but not in uninduced cells (−Dox). All samples show the characteristic double-band just above the 40 kDa marker for endogenous MBNL1 (centre). Ponceau S staining of total protein (bottom) was used as loading control. (C) The percentage of total cellular GFPMBNL1 localised to foci is small (<1%) and does not change between 24 h and 72 h of induction (n=48 cells at 24 h and n=88 cells at 72 h, pooled from two independent experiments; unpaired t-test). (D) The number of CUGexp foci per nucleus is small (mean 1.7 and 2.4 after 24 h and 72 h, respectively; n=84 cells at 24 h and n=117 cells at 72 h, pooled from two independent experiments; Mann–Whitney U test). (E) CUGexp foci are larger in HeLa cells than in DM1 HLECs (n=419 cells for HeLa, n=86 cells for HLEC; ****P<0.0001 Mann–Whitney U test). (F) The proportion of total cellular GFPMBNL1 localised to the cytoplasm is similar in CTG12 and CTG960 cell lines after 72 h of induction (n=10 cells per line). (G) Expression levels of endogenous MBNL1 are not significantly different in CTG12 and CTG960 cells, whether induced with Dox or not (ANOVA test, mean of two biological repeats adjusted for gel loading by using tubulin. Expression levels of GFPMBNL1 and the ratio of GFPMBNL1 to endogenous MBNL1 as shown in panel B are similar in CTG12 and CTG960 cells, adjusted for the number of cells expressing GFPMBNL1 in each cell line. All data are displayed as the mean±s.d.
Fig. 4. MBNL1 and CUGBP1 colocalise in P-bodies and sub-regions of SGs in HeLa models of DM1. (A) Unstressed HeLa_CTG12_GFPMBNL1 (CTG12, left) and HeLa_CTG960_GFPMBNL1 (CTG960, right) stained for CUGBP1 (red), GFP-tagged MBNL1 (GFPMBNL1; green) and GE1 (blue). For CTG12 cells, the merged image (top) shows colocalization of GE1 with CUGBP1 and GFPMBNL1 in cytoplasmic P-bodies. For CTG960 cells, the merged image (top) shows colocalization of GE1 with CUGBP1 in P-bodies, but GFPMBNL1 (green) is not detectable. Magnified images of the boxed P-bodies are shown below. Scale bars: 10 µm. Red and green signals in these images have been adjusted to visualise the cytoplasmic P-bodies, resulting in saturation of the nuclear signal. (B) CTG12 (top) and CTG960 (bottom) cells after treatment with NaAsO$_2$, showing SGs (arrows) containing TIA1 (red in merged image), GFPMBNL1 (green in merged image) and CUGBP1 (blue in merged image). Scale bars: 10 µm. (C) CTG12 (left) and CTG960 (right) cells treated with NaAsO$_2$. SGs (yellow arrows) show colocalisation of CUGBP1 (red in merged images) and GFPMBNL1 (green in merged images). Blue arrows highlight large number of P-bodies (detected with GE1, blue in merged images) also present in both cell lines. Nuclear foci of GFPMBNL1 (green in merged image) are only present in CTG960 cells (green arrow). Dotted lines show the approximate outlines of nuclei by using GFPMBNL1 signal. Scale bars: 10 µm. (D) Super-resolution airyscan images of SGs showing non-uniform distribution of CUGBP1 (magenta in merged images) and GFPMBNL1 (green in merged images) in CTG12 (left) and CTG960 (right) cells. Scale bars: 1 µm. (E) Graph of Pearson correlation coefficient of colocalisation between CUGBP1 and GFPMBNL1 in SGs of CTG12 and CTG960 cells (****P<0.00001; n=204 SGs and n=229 SGs in CTG960 and CTG12 cells, respectively, from three independent experiments; unpaired t-test). (F) Analysis of the amount of GFPMBNL1 in the P-bodies of CTG12 and CTG960 cells that had been treated with NaAsO$_2$. The percentage of GFPMBNL1 in P-bodies of CTG960 cells (n=670 P-bodies from 53 cells) was reduced compared with that in P-bodies of CTG12 cells (n=924 P-bodies from 34 cells); ****P<0.0001 unpaired t-test. (G) Unstressed CTG960 and CTG12 cells were induced with doxycycline for 72 h. P-bodies (in %) that contain CUGBP1 or CUGBP1 plus MBNL1 are reduced in CTG960 cells compared with levels in CTG12 cells (n=100 cells per cell line per experiment). All data are displayed as the mean±s.d. (see also Fig. S5).
Fig. 5. Kinetics analysis of SGs reveals altered formation and dispersal of SGs in an inducible cell model of DM1, and increased variance in MBNL1 dynamics. (A) Selected time-points from representative time-lapse images showing the formation (top) and dispersal during recovery (bottom) of SGs (arrows) in doxycycline (Dox)-induced CTG12 (left) and CTG960 (right) HeLa cell lines treated with NaAsO2 to induce SGs. The ‘formation time’ of SGs was recorded as the time-point at which at least five SGs were first detected. The ‘dispersal time’ of SGs was recorded as the first time-point at which SGs could no longer be clearly detected. Scale bars: 10 µm. See also Movies 1-4. (B) CTG12 control and CTG960 DM1 model cells were induced with Dox (+DOX), and time of SG formation (left) and SG loss (right) were analysed after treatment with NaAsO2 (analysed immediately for SG formation, and after 90 minutes of pre-treatment and drug removal for SG dispersal). Bar graphs show the time of SG formation after induction for 24 h (left) and time of SG loss after induction for 24 h or 72 h (right) in CTG960 cells normalised to the mean values measured in control (CTG12) cells (set as 100%). CTG12, n=19 cells; CTG960, n=27 cells for SG formation; ****P<0.0001. CTG12, n=35 cells; CTG960, n=25 cells for SG loss at 24 h; *P<0.05. CTG12, n=33; CTG960, n=18 cells for SG loss at 72 h; ****P<0.0001 unpaired t-tests. Each graph is representative of two independent experiments. (C) The proportion of cells containing SGs was plotted against time for the full time-course of treatment with or recovery from treatment with NaAsO2. This emphasises the difference in dynamics of SG formation (top) between cells expressing CTGexp RNA (DM1) and control RNA, but also the differences regarding SG loss when cells were Dox-induced for 24 h (centre) or 72 h (bottom). The increased difference seen with RNA induction for 24 h or 72 h confirms that these changes are due to CUGexp expression, rather than random variation between the cell lines. (D) The mobile fraction (left) and half-time of fluorescence recovery (right) of GFP-tagged MBNL1 (GFPMBNL1) in SGs is similar in CTG960 and CTG12 HeLa cells (Mann–Whitney U test; ns, not significant). (E) Plotted is the relative fluorescence recovery of GFPMBNL1 in SGs over time in response to photobleaching (488 nm laser, 2.5 s pulse), see also Fig. S7. Closed circles, SGs in HeLa_CTG12_GFPMBNL1 cells (n=41); open circles, SGs in HeLa_CTG960_GFPMBNL1 cells (n=25). All data are displayed as the mean±s.d.
and shorter half-time of recovery for GFPMBNL1 in HeLa cells compared to HLECs (Fig. 2), suggesting GFPMBNL1 is less able to exchange within SGs in HeLa cells compared to HLECs (mobile fraction 28.7±9.5% for HeLa_CTG12, GFPMBNL1 and 34.8±16.2% for HeLa_CTG960, GFPMBNL1; half-time of recovery 1.8±1.1 s for HeLa_CTG12, GFPMBNL1 and 2.2±1.7 s for HeLa_CTG960, GFPMBNL1). No statistically significant differences in the mobile fraction of GFPMBNL1 or half-time of recovery between the two HeLa cell lines were detected (Fig. 5D). Visualisation of individual data sets, however, suggested increased recovery between the two HeLa cell lines were detected (Fig. 5D).

Further investigation of the characteristics of P-bodies in stressed cells (Fig. 7B,D). Subsequent treatment with 1,6-hexanediol resulted in a marked reduction in the number of P-bodies per cell back to a level comparable to that seen in unstimulated cells in both DM1 and control cells (Fig. 7B,D). This suggests that the majority of NaAsO2 stress-induced P-bodies in HLECs are formed by LLPS, in common with those seen in unstimulated cells; however, a minority of P-bodies might represent more-stable structures showing insensitivity to 1,6-hexanediol treatment.

**NaAsO2 stress-induced P-bodies in HeLa cells that express CTGexp are resistant to treatment with 1,6-hexanediol**

Further investigation of the characteristics of P-bodies in stressed cells was made using the inducible HeLa cell model, allowing a more direct assessment of any effect the presence of CTGexp RNA has in a genetically homogeneous background. Exposure of cells from lines HeLa_CTG12, GFPMBNL1 and HeLa_CTG960, GFPMBNL1 to 1,6-hexanediol without pre-treatment with NaAsO2 resulted in complete P-body dispersal in both lines, consistent with the liquid-like properties for P-bodies in unstimulated cells (Fig. 7E). Treatment with NaAsO2 resulted in an increased number of P-bodies, similar to that seen in HLECs (Fig. 7C,E). Subsequent
treatment with 1,6-hexanediol significantly reduced P-body numbers in both cell lines but, in contrast to the results from HLECs, the number of P-bodies per cell did not return to pre-stressed levels (Fig. 7C,E). Notably, the average number of P-bodies per cell remained higher in cells containing CUGexp RNA than in those without. Taken together, these data suggest that the P-bodies present in NaAsO₂-stressed cells represent a diverse population with differing degrees of sensitivity to 1,6-hexanediol. Furthermore, the increase in 1,6-hexanediol-resistant P-bodies in HeLa_CTG960_GFPMBNL1 suggests that the presence of CUGexp RNA pre-
disposes cells to the formation of more-stable P-bodies when subjected to stress.

SGs in HeLa cells expressing CTGexp contain less poly(A) RNA and show altered docking events with P-bodies, suggesting defects in SG function

The main function of SGs is the temporary sequestration of untranslated mRNA; so, we next sought to address the consequences of CUGexp repeat RNA for the recruitment of poly(A) RNA into SGs. Dox-induced cells from lines HeLa_CTG12_GFPMBNL1 and HeLa_CTG960_GFPMBNL1 were treated with NaAsO₂, and FISH to detect poly(A) RNA was carried out using a PolydT probe alongside a probe for CUGexp RNA (Fig. 8A). The percentage of total cellular poly(A) RNA in SGs (identified by using GFPMBNL1) was decreased in cells expressing CUGexp RNA compared to control cells (Fig. 8B) (3.5±1.1% for CTG960 versus 4.2±1.4% for CTG12). This occurred despite the absence of CUGexp RNA from SGs themselves. Close physical interactions or docking events between SGs and P-bodies are often seen. The reason for these is unclear, but it has been proposed that, through these events, mRNPs can be exchanged between SGs and P-bodies (Protter and Fig. 7. See next page for legend.
P-bodies and SGs both show characteristics of LLPS structures, with PB stability increased by induction of CTGexp repeats. (A) Graph showing the predicted PrLDs for MBNL1 (left) and CUGBP1 (right), generated using PLAAC software (Lancaster et al., 2014). Parameters used were a core length of 30 amino acids and a background frequency for homo sapiens (see Materials and Methods). PrLDs are depicted where the probability is >0.5 on the y-axis. Red lines, PrLD probability score; black line, background probability score. (B) DM1 patient (DMCat3, top row) and control (CCat1, bottom row) HLECs (see: ‘Cell lines and cell culture’ under Materials and Methods) treated with NaAsO2 (left) or with NaAsO2 followed by 1,6-hexanediol (right). SGs (green arrows) containing GFP-tagged MBNL1 (GFPMBNL1; green merged images) are clearly seen cells treated NaAsO2 alone but absent in cells treated with NaAsO2 followed by 1,6-hexanediol. P-bodies (magenta arrows) detected with antibodies against endogenous GE1 (magenta in merged images) are numerous in cells treated with NaAsO2 alone but less numerous in cells treated with NaAsO2 followed by 1,6-hexanediol. Scale bars: 10 µm. (C) HeLa_CTG960_GFPMBNL1 cells treated with NaAsO2 (left) or with NaAsO2 followed by 1,6-hexanediol (right). SGs (green arrows) containing GFPMBNL1 (green in merged images) are clearly seen in NaAsO2-treated cells but absent in cells treated with NaAsO2 followed by 1,6-hexanediol. P-bodies (magenta arrows) detected with antibodies against endogenous GE1 (magenta in merged images) are numerous in cells treated with NaAsO2 alone but less numerous in cells treated with NaAsO2 followed by 1,6-hexanediol. Scale bars: 10 µm. (D) Shown are the average number of P-bodies per cell in DM1 (DMCat3) and control (CCat1) HLECs treated with 0.5 mM NaAsO2 or treated with 0.5 mM NaAsO2 followed by 5% 1,6-hexanediol compared to untreated cells. n = 53 (DMCat3), n = 53 (CCat1); *P < 0.05, ****P < 0.0001; one-way ANOVA, pooled from three independent experiments in each case. All data are displayed as the mean ± s.d. (E) P-bodies per cell in HeLa cells with (HeLa_CTG960, GFPMBNL1) and without (HeLa_CTG12, GFPMBNL1) CTGexp foci treated with 0.5 mM NaAsO2 or treated with 0.5 mM NaAsO2 followed by 5% 1,6-hexanediol compared to untreated cells. *P < 0.05, ****P < 0.0001. n = 108 (HeLa_CTG960, GFPMBNL1); n = 69 (HeLa_CTG12, GFPMBNL1); Kruskal–Wallis, pooled from three independent experiments in each case. All data are displayed as the mean ± s.d.

DISCUSSION

DM1-associated proteins MBNL1 and CUGBP1 are both components of P-bodies and SGs

In DM1 the activities of two multi-functional RNA-binding proteins, MBNL1 and CUGBP1, are altered. MBNL1 is recruited to aberrant nuclear CUGexp foci that are the hallmark of DM1, while the activity of CUGBP1 is upregulated. There is evidence that this leads to changed alternative splicing patterns in DM1, with MBNL1 and CUGBP1 acting antagonistically as splicing regulators (de Haro et al., 2006; Konieczny et al., 2014; Pettersson et al., 2015; Wang et al., 2015). The full extent of the effects of DM1 on RNA production and turnover within the cell, however, are not understood.

Following our observation that MBNL1 and CUGBP1 colocalise in small punctate cytoplasmic structures, despite showing no obvious colocalisation within the nucleus, we investigated the localisation and dynamic behaviour of MBNL1 and CUGBP1 in the cytoplasm in two independent cell culture models of DM1. In HLECs from DM1 patients and controls (Fig. 1) MBNL1 and CUGBP1 colocalise in both P-bodies in cells grown under normal conditions and in and SGs following NaAsO2 treatment. P-bodies and SGs are closely related structures involved in cytoplasmic mRNA regulation, and there is some evidence that components of P-bodies relocate to SGs in stressed cells (Anderson et al., 2015; Proctor and Parker, 2016; Shah et al., 2016). In our novel inducible HeLa cell model of DM1 (Figs 3 and 4), the situation is slightly more complex. CUGBP1 and MBNL1 colocalise in SGs following NaAsO2 treatment but, in unstressed cells – in which CUGBP1 accumulates within P-bodies regardless of the presence of CUGexp RNA – MBNL1 is largely absent from P-bodies of cells that contain CUGexp foci within their nuclei. This observation suggests that the role of MBNL1 in P-bodies is disrupted by the presence of CUGexp RNA.

SGs are not detected in HLECs from DM1 patients or in an inducible DM1 HeLa model cell line grown under normal conditions

In contrast to previous reports of SGs in DM1 myoblasts under normal growth conditions (Huichalaf et al., 2010; Ravel-Chapuis et al., 2016), suggesting that DM1 leads to a higher basal level of stress, we did not see evidence of SGs in either of our cell models grown under standard culture conditions. The reason for this is unclear but the difference is unlikely to be a simple correlation with the size of the CUGexp foci that, although small in HLECs from DM1 patients (Coleman et al., 2014), are large in our inducible HeLa cell model. The difference might reflect the use of different cell types and highlights the complexity of the cellular pathology of DM1, a multi-systemic condition with highly variable symptoms. DM1 patients are highly vulnerable to cataract development. Lens epithelial cells act as stem cells for lens development throughout life (Smith and Gutmann, 2016); so, increased basal stress in HLECs could be involved in DM1-associated cataract formation. Our results, however, suggest that a basal stress level sufficient to induce SGs is not present in DM1 HLEC cell lines. It is possible, though, that an underlying increased basal stress response is present in cells of the lens at later stages of differentiation, in vivo only or at levels insufficient to induce SGs.

SG turnover is altered in cell culture models of DM1

DM1 HLECs treated with NaAsO2 to induce oxidative stress showed no increase in the rate of SG formation compared to age-matched controls (Fig. 2). In our novel HeLa cell model that contains larger CUGexp foci than DM1 patient-derived HLECs, a pronounced delay in SG formation was seen (Fig. 5). Impaired SG formation, in the absence of a detectable underlying stress response, has been reported previously in fibroblasts from DM1 patients, induced to adopt a myoblast phenotype by exogenous expression of MyoD (Ravel-Chapuis et al., 2016), but not in the unmanipulated fibroblasts. This was interpreted as a cell type difference as myoblasts – but not fibroblasts – are affected in DM1. However, nuclear foci of the myoblast cells also were larger compared to those of the parental fibroblasts. Our new results are in broad agreement with these observations, and further suggest a complex interplay between cell type and size of CUGexp foci affecting cellular responses to stress.

In contrast to the delay seen in SGs formation, both of our cell models showed increased rates of SG dispersal in cells containing the CUGexp RNA when stress was removed. In the inducible HeLa cell model, this effect was more pronounced when CUGexp foci were induced for longer periods of time (72 h versus 24 h) (Fig. 5B), suggesting that the changes are directly linked to the duration of expression of CUGexp RNA. SGs are transient structures, with the mRNAs sequestered within them destined to return to active

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Dispersal of SGs is a tightly regulated process, sometimes involving a form of autophagy (Hardy et al., 2017; Mateju et al., 2017; Protter and Parker, 2016; Turakhiya et al., 2018). The rapid loss of SGs from cells containing CUGexp foci may indicate alterations or impairment in the mechanisms required for regulated SG dispersal associated with the presence of CUGexp RNA. This would require considerable additional analyses but may be of importance for understanding cataract development associated with DM1. Defects in autophagy are associated with cataract development, although the mechanisms and types of autophagy involved are, as yet, unclear (Costello et al., 2013; McWilliams et al., 2019; Morishita et al., 2013; Morishita and Mizushima, 2016).

Alterations to SG turnover cannot solely be attributed to the sequestration of MBNL1 in CUGexp foci

The sequestration of MBNL1 in CUGexp foci has long been cited as a main cause of cellular defects in DM1. However, we have previously reported that the amount of total cellular MBNL1 present within nuclear foci in HLECs from DM1 patients is extremely small, i.e. <0.2% (Coleman et al., 2014). Even in our HeLa cell model, which appears to contain large and very bright nuclear foci when viewed using GFPMBNL1, the amount of cellular GFPMBNL1 contained in the foci is <1% (Fig. 3C). Delayed formation of SGs was also seen in HeLa cells without CUGexp foci but with shRNA-mediated MBNL1 depletion (Fig. 6). While this does implicate loss of MBNL1 as a direct cause of disruption to SG formation, the delay seen following MBNL1 reduction by shRNA was much less pronounced than that resulting from CUGexp foci induction, despite the almost complete loss of MBNL1 seen in shRNA-treated cells. The small effect on SG turnover of almost complete MBNL1 depletion compared to that seen in cells containing CUGexp foci, in which only very small amounts of MBNL1 are mislocalised to foci, strongly suggests that sequestration of MBNL1 in CUGexp foci is not the primary cause of the alterations to SG turnover. It is likely, though, that MBNL1 is also bound to CUG repeats in DMPK mRNA outside nuclear foci.

Fig. 8. Reduced amounts of poly(A) RNA in SGs, and altered docking events between P-bodies and SGs suggests defects in their function in cells expressing CUGexp RNA

(A) RNA FISH of HeLa_CTG12_GFPMBNL1 (top) and HeLa_CTG960_GFPMBNL1 (bottom) cells treated for 90 min with NaAsO2. Poly(A) RNA (first images; red in merged images) accumulates in the nucleus and in cytoplasmic SGs of both cell lines. CUGexp RNA (third images; blue in merged images) is detected only in the nuclear foci (arrows) of HeLa_CTG960_GFPMBNL1 cells. GFP-tagged MBNL1 (GFPMBNL1) accumulates in SGs of both cell lines and in the CUGexp foci when they are present (second images; green in merged images). (B) The total amount of cellular poly(A) RNA (in %) in SGs is reduced in cells that express CUGexp RNA (CTG960) than in control cells (CTG12). Data shown are pooled from two independent experiments. n=71 (CTG12), n=84 (CTG960); **P<0.01, unpaired t-test. (C) The number of docking events between SGs and P-bodies is lower in cells that express CUGexp RNA (CTG960) than in control cells (CTG12). Data shown are pooled from two independent experiments (CTG12, n=57 cells; CTG960, n=60 cells); ***P<0.001, Mann–Whitney U test. (D) HeLa_CTG12_GFPMBNL1 (top) and HeLa_CTG960_GFPMBNL1 (bottom) cells, treated with NaAsO2 for 90 min. P-bodies detected with antibodies against GE1 (indicated by white arrows in first and third images; shown in red in merged and magnified images), show docking events (indicated by yellow arrows in merged images and shown magnified in panels on the right). SGs are detected by staining for GFPMBNL1 (indicated by arrowheads and shown in green in merged images). All nuclei are counterstained with DAPI (white in merged image in A; blue in merged image in B). Scale bars: 10 µm (A,D); 2 µm (magnified images in D).
MBNL1 and CUGBP1 are likely to be core components of SGs and might be recruited from P-bodies

MBNL1 and CUGBP1 have different dynamics within SGs, with GFP-tagged CUGBP1 showing both a larger mobile fraction and faster recovery in FRAP experiments than GFPMBNL1 (Fig. 6). Previous studies of various SG proteins in different cell types have reported highly divergent dynamic behaviours (Bley et al., 2015), with proteins essential for SG formation exhibiting faster kinetics within SGs – i.e. with half-time of recovery between 2 s and 3 s – and larger mobile fractions than those dispensable for their formation. MBNL1 exhibits behaviour similar to that published for TIA1, a well-established SG marker whose depletion results in impaired SG formation (Bley et al., 2015; Gilks et al., 2004). Together with the delayed formation of SGs in cells with disrupted MBNL1 expression, this suggests that MBNL1 is important for SG integrity. CUGBP1 – although exhibiting a rapid exchange within SGs similar to that of MBNL1 – has a small mobile fraction previously reported to be characteristic of non-essential SG components (Bley et al., 2015). Reduction of CUGBP1 expression, however, also caused a delay in SG formation, which would not be expected for a non-essential component, emphasising further the complexity of these structures.

It has been suggested that SGs in mammalian cells are composed of a mixture of liquid and solid phases (Protter and Parker, 2016) with a solid core structure surrounded by a more dynamic shell (Jain et al., 2016; Markmiller et al., 2018; Protter and Parker, 2016). High-resolution airyscan microscopy reveals close colocalisation of MBNL1 and CUGBP1 in SGs, with both proteins showing punctate accumulations similar to those described as G3BP-containing SG core by Jain et al. (2016) using STORM microscopy. Moreover, CUGBP1 has previously been identified in the proteome of SG core structures (Jain et al., 2016; Kuechler et al., 2020; Youn et al., 2018), which strengthens the evidence that the two DM1-associated proteins MBNL1 and CUGBP1 are core SG proteins. This represents a slightly unusual situation, as proteins required for the formation of a particular RNP structure are usually confined to that structure (reviewed by Corbet and Parker, 2019), whereas MBNL1 and CUGBP1 are also found in P-bodies as well as in specific nuclear regions (Coleman et al., 2014; Fujimura et al., 2008).

In our inducible HeLa cell model of DM1, the presence of CUGexp RNA resulted in a slight decrease in the Pearson correlation coefficient regarding the colocalisation of GFPMBNL1 and endogenous CUGBP1 within SGs, suggesting an alteration in the interactions of these two proteins with SGs. In the same unstressed cells, both CUGBP1 and GFPMBNL1 were detected within P-bodies when CUGexp repeats were not expressed; however, GFPMBNL1 was absent from P-bodies in cells that did express CUGexp repeats. Upon NaAsO2-induced stress, GFPMBNL1 was seen in a subset of P-bodies in both our DM1 cell lines, but the amount of GFPMBNL1 in P-bodies was significantly reduced in cells with CUGexp foci. It is conceivable that this loss of MBNL1 from P-bodies – as a core SG component – contributes to the delayed formation of SGs as seen in these cells following treatment with NaAsO2.

Alterations to LLPS might be associated with DM1

PrLDs within low-complexity sequences promote the formation of LLPS structures, including mammalian SGs (Ditlev et al., 2018; Markmiller et al., 2018; Protter and Parker, 2016). Analysis of the sequences of MBNL1 and CUGBP1 (Fig. 7) reveals small predicted PrLDs in both MBNL1 and CUGBP1, which might account for their propensity to accumulate SGs. The domain in MBNL1 is longer (28 aa) than that in CUGBP1 (10 aa).

Induction of CUGexp RNA in our DM1 HeLa cell model changed the distribution of the GFPMBNL1 half-time of recovery values, showing a subset of SGs with a much slower rate of recovery. The explanation for this is unclear but this reduced rate might be due to increased tendency for the formation of more solid SGs in cells containing nuclear foci. However, in cells with MBNL1 reduced to very low levels by using shRNA (Fig. 6), we observed an increase in the rate of CUGBP1 exchange within SGs, suggesting an increased fluidity of the structures overall. This apparent contradiction, taken together with the differences in rates of exchange of MBNL1 and CUGBP1 in SGs despite their close colocalisation, suggest that the dynamics of LLPS structures are extremely complex (reviewed by A and Weber, 2019) and that FRAP of individual constituent proteins is not a perfect method to examine the biophysical nature of cellular structures. It also provides further evidence that alterations to SGs associated with DM1 are not entirely due to loss of MBNL1.

1,6-Hexanediol has been extensively used to disrupt structures predicted to form by LLPS, in vitro and in vivo (Kroschwald et al., 2015; Molliex et al., 2015; Patel et al., 2007; Ribbeck and Gorlich, 2002; Updike et al., 2011). As with FRAP, this is not a fail-safe way to assess the LLPS nature of cellular structures, as the solvent is toxic to mammalian cells at higher concentrations (Wheeler et al., 2015). Treatment of HLEC & inducible HeLa cell models of DM1 with 1,6-hexanediol resulted in complete loss of SGs, as would be expected for a phase-separated structure. The effect on P-bodies was more complex. In unstressed cells, virtually no P-bodies were visible after treatment with 1,6-hexanediol. However, in HeLa cells pre-treated with NaAsO2 large numbers of additional P-bodies were formed during the stress response and a proportion of these remained following 1,6-hexanediol treatment, suggesting that they had lost their liquid-like properties. Of particular interest for understanding DM1 is that the number of 1,6-hexanediol-resistant P-bodies was significantly higher in HeLa cells containing CUGexp nuclear foci. This suggests that the presence of the DM1-associated CUGexp RNA influences the biophysical characteristics of P-bodies in stressed cells, perhaps as a result of the loss of MBNL1 from them.

Functional defects might occur in SGs in cells containing CUGexp RNA

P-bodies and SGs show significant overlap in their components (reviewed by Youn et al., 2019), and it has been known for some time that SGs and P-bodies are closely linked structures that can dock to and fuse with each other, potentially facilitating the exchange of mRNPs between them (reviewed by Stoecklin and Kedersha, 2013). We now show that MBNL1 is a component of both SGs and P-bodies, and is partially lost from P-bodies in DM1 model HeLa cells that express CUGexp RNA (Fig. 4 and Fig. S6). Docking events between SGs and P-bodies are also reduced in these cells compared to controls, as is the proportion of total cellular poly(A) RNA present in SGs. This suggests that the function as well as dynamics of SGs is compromised in the presence of the CUGexp RNA that causes DM1. This occurs despite the absence of CUGexp RNA from SGs (Fig. 8) and might be mediated through altered biophysical interactions between P-bodies and SGs.

Implications of altered SG and P-body dynamics for DM1 pathology

Defects in the normal physiology of cytoplasmic LLPS structures, particularly SGs, have been widely implicated in human diseases. The spontaneous conversion of PrLDs into an aggregated state, and
subsequent transition from liquid to solid to form solid RNP aggregates has been proposed as a pathological mechanism in conditions including ALS and FTD (Li et al., 2013; Ramaswami et al., 2013). The presence of aberrant or persistent SGs is also associated with the cellular pathology of age-related diseases, neurodegeneration and some cancers (Mateju et al., 2017; Proctor and Parker, 2016; Turakiya et al., 2018). Furthermore, increased basal stress levels and defects in cellular stress responses have been suggested to occur in DM1 (Huichalaf et al., 2010; Ibara et al., 1995; Kumar et al., 2014; Toscano et al., 2005; Usuki and Ishiura, 1998; Usuki et al., 2000). Here, we report delayed SG formation and premature SG loss in cell culture models of DM1, together with altered biophysical characteristics of the closely related P-bodies. This strongly suggests that the presence of CUGexp RNA, perhaps partly as a consequence of the sequestration of MBNL1 by the CUG expansion within nuclear foci or elsewhere, results in defects in cellular responses to stress. The assembly and disassembly of SGs are complex, regulated processes (Wheeler et al., 2016). Failure to disassemble SGs in a controlled manner could impair the ability of cells to respond effectively to repeated stress events and, furthermore, might suggest a defect in autophagy associated with DM1. Defects in autophagy have recently been implicated in several degenerative conditions (Ranganathan et al., 2020) and autophagy is known to contribute to the generation of optically transparent lens fibre cells during differentiation of the lens epithelial stem cell population (Costello et al., 2013; Morishita et al., 2013; Morishita and Mizushima, 2016).

In summary, our data show alterations to the structure and regulation of SGs and P-bodies – two key LLPS structures involved in cytoplasmic mRNA metabolism and cellular stress responses in cell culture models of DM1. These changes are associated with the presence of CUGexp RNA and might be linked to deficiency of MBNL1 resulting from its accumulation in the nuclear CUGexp foci characteristic of DM1. However, with only small amounts of MBNL1 colocalising with nuclear CUGexp foci, and the failure of almost complete MBNL1 knockdown to recapitulate the magnitude of effects seen in cells expressing CUGexp RNA, MBNL1 sequestration in CUGexp foci is unlikely to provide a full explanation. Our observations point to a complex interplay between nuclear and cytoplasmic structures involved in mRNA metabolism, and suggests that further investigation is warranted regarding the contribution of both altered LLPS and autophagy towards cellular defects in DM1.

**MATERIAL AND METHODS**

**Plasmids and lentiviral vectors**

Plasmids pBI-Tet-CTG12 and pBI-Tet-CTG960 (Lee et al., 2012; Philips et al., 2009), to generate plasmids pLKO_MBNL1 and pLKO_CUGBP1. Lentiviral particles were produced using 293T cells and transduction of HeLa cells was performed as described by Wiederschain et al. (2009). Luciferase (control) plasmid was Tet-pLKO-puro_shLUC (Harwardt et al., 2016; a gift from Dr Christina Paulus, University of St Andrews). Following selection with puromycin, the mixed populations of transduced cells were grown at 37°C under 5% CO2 in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin. Following transduction with lentiviral vector, medium was supplemented with 250 ng/ml puromycin and 200 µg/ml G418 geneticin (G 418 disulfate salt, Sigma). To generate cell lines with reduced expression of MBNL1 or CUGBP1, lentiviral particles were produced using 293T cells and transduction of HeLa cells was performed as described by Wiederschain et al. (2009). Luciferase (control) plasmid was Tet-pLKO-puro_shLUC (Harwardt et al., 2016; a gift from Dr Christina Paulus, University of St Andrews). Following selection with puromycin, the mixed populations of transduced cells were grown at 37°C under 5% CO2 in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin. Following transduction with lentiviral vector, medium was supplemented with 250 ng/ml puromycin and 200 µg/ml G418 geneticin (G 418 disulfate salt, Sigma).

**Cell lines and cell culture**

Human lens epithelial cells (HLECs) from DM1 patients (DMCat1-4) and from age-matched controls (CCat1 and CCat2) have been described previously (Rhodes et al., 2006). Cells were grown at 37°C under 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 10% foetal bovine serum (FBS, Sigma), 1% penicillin/streptomycin (Sigma) and 1% L-glutamine (Sigma). HeLa cells were grown at 37°C under 5% CO2 in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin. Following transduction with lentiviral vector, medium was supplemented with 250 ng/ml puromycin and 200 µg/ml G418 geneticin (G 418 disulfate salt, Sigma).

**Drug treatment**

For SG formation, cells were treated with 0.25 mM NaAsO2 in DMEM for 20 min before fixation. For live-cell experiments, cells were maintained in s medium (DMEM, Sigma) supplemented with 10% foetal bovine serum (FBS, Sigma), 1% penicillin/streptomycin, and 150 µM sodium selenite. Drugs were added sequences. When required, expression of the constructs was induced using 1 µg/ml of the tetracycline equivalent doxycycline (Dox), using fluorescence in situ hybridisation (Cy3-CAG10 probe) and fluorescence microscopy (GFPMBNL1). These cell lines were maintained under conditions identical to those used for the parental HeLa cell line, with G418 geneticin and puromycin added periodically to ensure retention of the added sequences. When required, expression of the constructs was induced using 1 µg/ml Dox for 24, 48 or 72 h as indicated.
DMEM with or without NaAsO2. Where possible, downstream analysis was carried out with the researcher blinded to the identity of the sample.

**Western blotting**

Cells were lysed with ice-cold nuclear lysis buffer [50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1% IGEPA, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, complete protease inhibitor cocktail (Roche)] for 5 min, then passed through a QIAshredder (QIAGEN) during 2 min centrifugation at 12,000 g. Lysates were electrophoresed in a 10% acrylamide gel and transferred onto a nitrocellulose membrane (GE Healthcare) using a semi-dry blotter (BioRad). Skimmed milk powder (5%) in PBS was used as blocking solution. Protein detection using primary antibodies mouse anti-MBNL1 (1:50; gift from Glenn Morris; Holt et al., 2007); rabbit anti-GFP (Abcam ab290, 1:50); rabbit anti-CUGBP1 (Abcam ab129115, 1:1000); mouse anti-tubulin (Sigma; 1:500) and secondary antibodies goat anti-rabbit-AlexaFluor790 and goat anti-mouse-AlexaFluor680 (both Invitrogen; 1:20,000). Blots were imaged using a Licor Odyssey CLx.

**Immunocytochemistry**

Cells were seeded on 18 mm square glass coverslips and incubated in normal DMEM for 24 h minimum. Drug treatments or transient transfections using Effectene Reagent (Qiagen) according to the manufacturer’s instructions were carried out as required. Fixation was performed using 3.7% paraformaldehyde (PFA) (Sigma) in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2 pH 6.9) for 10 min at room temperature. Immunostaining was carried out essentially as described by Sleeman et al. (2003), with 1% goat serum in PBS as blocking buffer and antibody incubation for 1 h. Primary antibodies were MBlas mouse anti-MBNL1 (Holt et al., 2007) 1:15; rabbit anti-TIA1 (Proteintech #12133-2-AP) 1:50; rabbit anti-CUGBP1 (Abcam, #ab129115, 1:100); rabbit anti-GE1 (Cell Signalling Technology, #2548, 1:400). Secondary antibodies were goat-anti-rabbit AlexaFluor594, goat-anti-mouse AlexaFluor488, goat-anti-mouse and goat-anti-rabbit Cy5 (All Jackson Laboratories, 1:250). 1.6 μg/ml DAPI (Sigma Aldrich) in H2O was used to stain the nuclei. Coverslips were mounted using ProLong Gold Antifade (Invitrogen).

**RNA fluorescence in situ hybridisation**

For RNA fluorescence in situ hybridisation (FISH), cells were seeded on 18 mm square glass coverslips and incubated in normal DMEM or DMEM supplemented with 1 μg/ml Dox as appropriate. Cells were fixed using 3.7% PFA (Sigma) in diethylpyrocarbonate (DEPC)-treated PBS and permeabilised with 70% ethanol in DEPC-treated H2O for 1 h. A Cy3 dye-labelled CAG10 DNA probe (5′-5Cy3(CAG)10, Integrated DNA Technologies) was diluted in hybridisation buffer (2% SSC, 10% w/v dextran sulphate; 10% formamide) to a final concentration of 200 nM and incubated with the samples for 4 h at 37°C. Samples were washed with 2× SSC, 3× 5 min. 1.6 μg/ml DAPI (Sigma Aldrich) in H2O was used to stain the nuclei. Coverslips were mounted using ProLong Gold Antifade (Invitrogen).

**Microscopy and image processing**

Fixed samples were imaged using a 1.35na 100× objective on an Olympus DeltaVision RT microscope (Applied Precision) with 2.5 s binning using for weaker samples and 0.2 μm sectioning for z-stacks. Exposure times using DAPI, FITC, TRITC and Cy5 filter sets were chosen to aim for maximum intensities of 3600. Where indicated, deconvolution was carried out by using Velocity 6.3 image analysis software (Quorum Technologies) with calculated point-spread functions. Object identification for quantitative analyses was carried out using the quantitation module in Velocity 6.3, as illustrated in Fig. S4.

**Images capture and analysis**

Images were captured using the Zeiss LSM 880 laser scanning microscope using the Airyscan detector with either the ×63 or ×100 objectives. This detector increases the resolution of the final image from a theoretical maximum of 0.2 μm to 0.1 μm (Huff, 2015). Correlation parameters were collected from the images by using a custom programme written in the Volocity quantitation module (Quorum Technologies); GFPMBNL1-positive structures were identified using the Otsu method (Otsu, 1979), objects in the nucleus were removed by identifying those intersecting with DAPI-labelled regions. All remaining cytoplasmic structures were investigated for the correlation between GFPMBNL1 and CUGBP1 intensities.

**Prediction of prion-like domains**

The amino acid sequences of MBNL1 and CUGBP1 were analysed using the Prion-like Amino Acid Composition (PLAAC) program (http://plaac.wi.mit.edu; Lancaster et al., 2014). The parameters used were ‘core length: 30’ and ‘background frequency: homo sapiens’.

**Live-cell imaging**

Cells were seeded onto 40 mm round coverslips (Intracel) for 24 h before transfections or transductions (where appropriate) were performed. After an additional 24 h, the coverslips were placed into a POC-R imaging chamber (Zeiss) within an environmental incubator (Solenet Scientific) on an Olympus DeltaVision RT microscope (Applied Precision), with a quantifiable laser module including 488 nm and 405 nm lasers, and either maintained at 37°C with 5% CO2 or incubated in CO2-independent medium (Life Technologies). z-stacks of images separated by 500 nm were collected at a minimum of 3-min intervals by using a 1.35NA 100× objective. Exposure times were optimized to give a maximum intensity value of ~1000 in each z-stack or in the first time-point of each time-lapse series.

**Fluorescence recovery after photobleaching**

Fluorescence recovery after photobleaching (FRAP) was carried out using an Olympus DeltaVision RT microscope using a 1.35NA 100× objective, with the chamber heated to 37°C. A 488 nm laser at 100% power was used to photobleach GFP for 2.5 s, aiming to obtain ~50% bleaching efficiency. A time-course applying the FITC filter was taken by taking three images before and 25 images after the laser fire, using adaptive time intervals as implemented in the DeltaVision software. Where appropriate, P-bodies were identified using pmCherry-Dep1A, viewed through the TRITC filter before FRAP. Image analysis was carried out using Velocity 6.3 image analysis software (Quorum Technologies). The photobleached region was indicated by a point and the intensity value measured against time. The values were corrected for background signal and photobleaching, and normalised by setting the intensity immediately after the firing event as 0% and the highest of the pre-fire images as 100%. A one-phase association curve analysis was used in Prism 8 (GraphPad) to determine the mobile fraction and half-time of recovery for each data set.

\[ Y = Y_0 + (Plateau - Y_0) \times e^{-K \times x} \]

with \( Y \) referring to the relative fluorescence intensity, \( Y_0 \) to the value when \( x = 0 \), plateau to the \( Y \) value at infinite times, \( K \) to the rate constant and \( x \) to time.

**Statistical analyses**

All statistical analyses, including ANOVA, t-tests, Mann–Whitney U test, D’Agostino–Pearson omnibus K2 normality test and removal of outliers as appropriate were carried out using Prism 8 (GraphPad). For analyses where all relevant datasets showed normal distribution, a one-way ANOVA test was used. For analyses where some or all datasets failed the D’Agostino–Pearson omnibus K2 normality test, Mann–Whitney U test was used. Optimal sample sizes to detect a medium (0.25) effect size at significance level <0.05 with 90% power were determined using R.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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Data availability

References


