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Tesis Doctoral

Mouli Chakraborty

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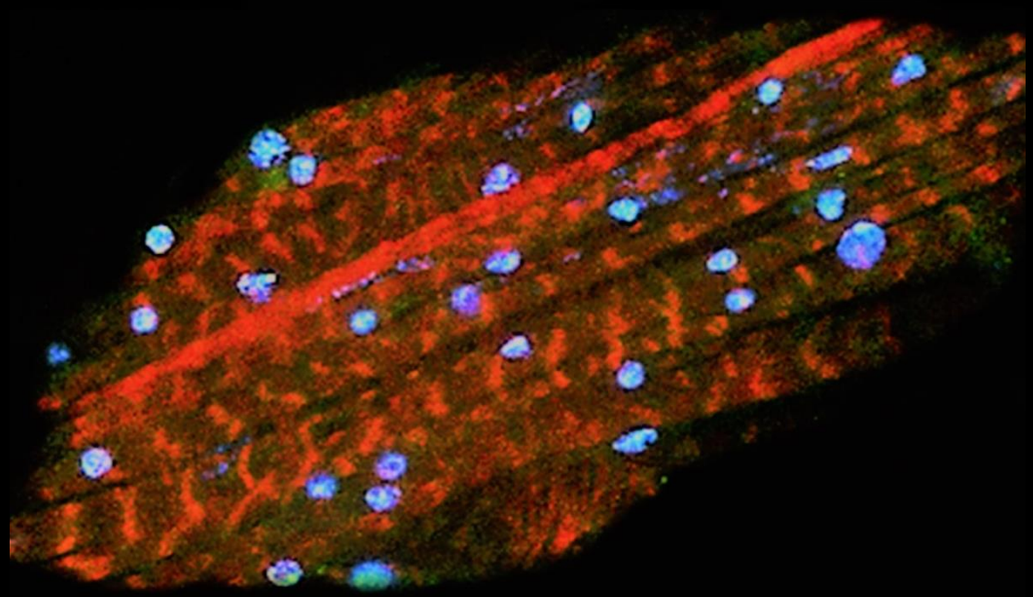
Generación y caracterización de modelos en *Drosophila* de disfunción cardiaca en Distrofia Miotónica



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2018

VNIVERSITAT DE VALÈNCIA

Facultad de Ciencias Biológicas
Programa de doctorado en Biomedicina y Biotecnología

Generación y caracterización de modelos en *Drosophila* de disfunción cardiaca en distrofia miotónica

Tesis Doctoral

Mouli Chakraborty
June 2018

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INFORMAN

Que Doña Mouli Chakraborty, licenciada en Fisiología humana por la universidad de Calcutta, ha realizado bajo nuestra supervision el trabajo de investigación original recogido en la presente memoria y artículos adjuntos titulada "Generación y caracterización de modelos en *Drosophila* de disfunción cardiaca en distrofia miotónica"

Revisado el presente trabajo, expresan su conformidad para la presentación del mismo en el Departamento de Genética de la Universidad de Valencia, por considerar que reúne los requisitos necesarios para ser sometido a discusión ante el Tribunal correspondiente, para optar al grado de Doctor por la Universidad de Valencia dentro del programa oficial de Doctorado en Biomedicina y Biotecnología.

En Valencia, a 8 de junio de 2018

Dr. D. Rubén D. Artero Allepuz

Dra. M. Beatriz Llamusi Troisi

TO MY PARENTS

-Acknowledgement-

This is a small tribute to my supervisors, colleagues and fellow workers who have contributed to the successful completion of the thesis. I solemnly express my heartfelt gratitude to all those who have helped me to fulfill my responsibility. At the outset, I take this opportunity to express my humble and sincere gratitude to my supervisors, Dr. Ruben Artero and Dr. Beatriz Llamusi, without whom the study would not have been possible. Their constant guidance, advice and constructive suggestions have enabled me to conduct this study successfully. They had always shown complete confidence in my abilities and tried to solve my problems which provided me with immense encouragement. I can only convey my deepest regards and respect to them in return.

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Índice general

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Introducción global

Introducción global:

La distrofia miotónica (DM) es la forma más común de distrofia muscular en adultos (Giovanni Meola and Cardani 2015). La DM es una enfermedad genética de herencia dominante que se caracteriza por manifestaciones multisistémicas tales como.miotonía progresiva, degeneración muscular preferentemente distal, cataratas de inicio temprano (menores de 50 años), defectos de conducción cardíaca, cambios en los parámetros neuropsicológicos y trastornos endocrinos (Harper 2001; Ashizawa and Sarkar 2011). La esperanza de vida de los pacientes con la forma más típica de la enfermedad está fuertemente reducida. Con base en la determinación clínica, la prevalencia mundial de DM se estimó en 1/8000, y en Europa, la prevalencia es 1/20000 (Harper 2001; Udd and Krahe 2012).

Actualmente se conocen dos tipos genéticamente distintos de DM. Distrofia miotónica tipo 1 (DM1; OMIM #160900) y distrofia miotónica tipo 2 (DM2; OMIM #602668). Ambos tipos son trastornos basados en la expansión de microsatélites en regiones no codificantes que afectan a diferentes genes. La entidad clínica de DM1 fue reconocida por primera vez por Steinert (Steinert 1909) y Batten y Gibb (Batten and Gibb 1909) en 1909. La DM1 es causada por una expansión de repeticiones de trinucleótidos CTG inestables en la región 3' no traducida (UTR) del gen de la proteína kinasa de la distrofia miotónica (DMPK; OMIM 605377) en la posición cromosómica19q13.3 (Brook et al. 1992; Fu et al. 1992; M. Mahadevan et al. 1992). Posteriormente, se identificó a otro grupo de pacientes en 1994 con un transtorno multisistémico diferente. Se describió con miotonía hereditaria dominante, debilidad muscular más proximal que distal y cataratas, pero sin el defecto del gen responsable de la enfermedad de Steinert (Ricker 1999; C A Thornton, Griggs, and Moxley 1994; G Meola and Sansone 1996; Udd et al. 1997). Esta enfermedad se denominó miopatía miotónica proximal (PROMM, OMIM*160900)(Ricker 1999) o distrofia miotónica tipo 2 (DM2) (C A Thornton, Griggs, and Moxley 1994). El tipo 2 de DM es causado por una expansión inestable del tetranucleótido CCTG en el intrón 1 del gen *CCHC-type zinc finger nucleic acid-binding protein* (CNBP) (anteriormente conocido como zinc finger 9 gene, ZNF9; OMIM 116955) en la

posición cromosómica 3q21.3 [Fig. I1](Ranum et al. 1998; Liquori et al. 2001; Bachinski et al. 2009).

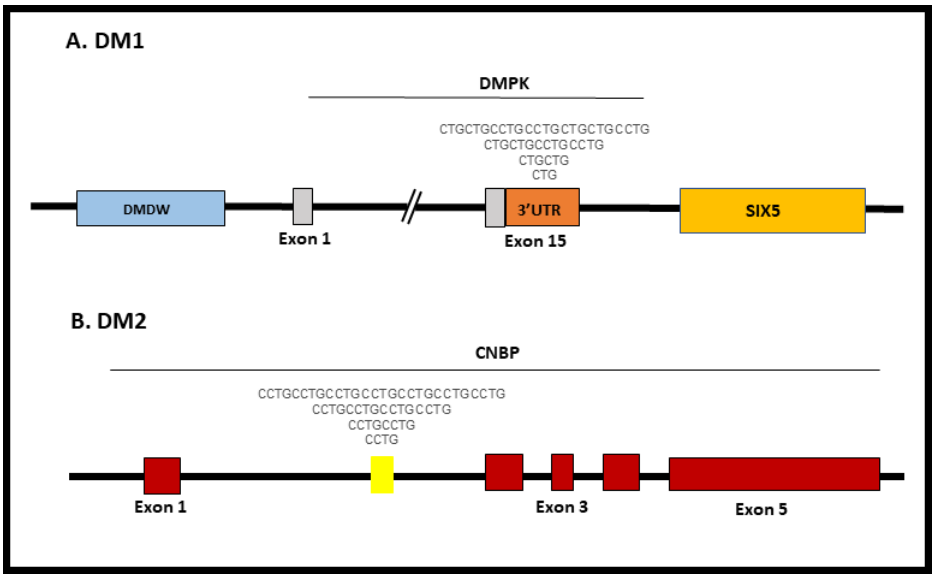

Fig. I1. Representación esquemática de las mutaciones causantes de DM: (A) En el caso de DM1, la mutación genética es la expansión de una repetición de CTG en la región 3 'UTR del exón 15 del gen DMPK. En la figura, está representado por el cuadro naranja. La región flanqueante a DMPK contiene los genes DMDW y SIX 5 (recuadro azul y el amarillo, respectivamente). (B) En el caso de DM2, la expansión de CCTG está presente en el primer intrón del gen CNBP que se representa con una caja amarilla.

En DM1, el número de repeticiones CTG en el gen DMPK es variable en la población general, estimándose entre 5 y 37 repeticiones. En pacientes con DM1, el tamaño de la repetición varía y longitudes entre 50 y 4000 (150-12.000 bp) se asocian con la enfermedad sintomática (Giovanni Meola and Cardani 2015). Longitudes de repeticiones de 38-50 se consideran alelos de premutación, mientras que 51-100 repeticiones se conocen como protomutaciones. Las mutaciones de expansiones CTG muestran dos fenómenos importantes. El primero es el mosaicismo somático, presentando una inestabilidad y variación en el tamaño de la expansión de las repeticiones dependiendo de diferentes tipos de tejidos y células (Morales et al. 2012; Higham et al. 2012). El segundo es la anticipación genética, donde el tamaño de las repeticiones CTG parece aumentar en las sucesivas generaciones y la

enfermedad tiene una manifestacion clinica más garve. En CNBP, los individuos normales llevan menos de 26 repeticiones mientras que los alelos con 27-74 repeticiones se consideran como una “zona gris”, ya que tienen una asociación poco clara con la enfermedad (Kamsteeg et al. 2012). El tamaño patogénico más pequeño informado varía entre 55-75 CCTG pero puede llegar hasta aproximadamente 11,000 repeticiones en pacientes con DM2 (Liquori et al. 2001; Bachinski et al. 2009; Giovanni Meola and Cardani 2017). Al igual que las mutaciones de repetición CTG, el tamaño de las expansiones CCTG parece aumentar con el tiempo y estas repeticiones son inestables en las células somáticas. Contrariamente a DM1, en DM2, la anticipación no se ha observado. Por otra parte, DM2 se considera menos grave que DM1 (Giovanni Meola and Cardani 2017) y generalmente las expansiones de CCTG se contraen en la siguiente generación (Day et al. 2003). El espectro clínico de DM es extremadamente amplio, diverso y puede variar entre ambos tipos. En comparación con DM1, DM2 es clínicamente más leve y progresa más lentamente que DM1. En este sentido, en un informe reciente, se describe la participación de la proteína rbFOX como amortiguador del efecto de las repeticiones CCUG in vivo (Chantal S. et al. 2018). En la tabla I1 se comparan diferentes síntomas clínicos de ambos tipos de la enfermedad. Clínicamente, la DM1 es más diversa y contiene cuatro subtipos diferentes que pueden aparecer en diferentes etapas de la vida y pueden tener diferentes longitudes de repeticiones según el subtipo. En la Tabla I2, los diferentes subtipos DM1 se enumeran con sus respectivas longitudes medias de repetición y características cardinales asociadas (De Antonio et al. 2016; Yum, Wang, and Kalsotra 2017). Entre todos los subtipos de DM1, el de inicio en el adulto y el de inicio congénito son los subtipos más prevalentes y graves DM1, respectivamente (Harper 2001; Arsenault et al. 2006).

Tabla 1: Diferencias clínicas entre DM1 y DM2

Características clínicas	DM1	DM2
Herencia	Dominante	Dominante
Defecto genético	Cromosoma 19; expansion CTG en el 3'UTR del gen DMPK	Cromosoma 3; Expansión de CCTG en el intrón 1 del gen CNBP
Edad de inicio	Infancia a la edad adulta	Infancia hasta la edad adulta avanzada
Forma congénita	Presente	Ausente
Cataratas	Presente	Presente
Músculo esquelético		
Debilidad muscular	Incapacitante a los 50 años	Inicio después de los 20-40 años
Dolor muscular	Ausente o leve	Incapacitante en muchos
Miotonía clínica	Evidente en el comienzo del adulto	Presente en <50%
Miotonía EMG	Siempre presente	Ausente o variable en muchos
Cerebro		
Trastornos del sueño	Presente	Infrecuente
Deterioro cognitivo	Prominente	No es aparente
Problemas del SNC en la infancia	Frecuentemente presente	Ausente
Arritmia cardíaca	Presente	De ausente a grave en algunos grupos depacientes
Hipogonadismo masculino	Presente	Presente
Anticipación	Siempre presente	Presente excepcional y solo clínico
Esperanza de vida	Reducida	Normal

Adaptado de Meola G. & Cardani R. 2017

Tabla I2: Resumen de fenotipos clínicos en subtipos DM1 y su correlación con el tamaño de repeticiones CTG

Fenotipo	Síntoma clínico	Rango de repeticiones	Edad de inicio (años)	Esperanza de vida	Penetración y anticipación (Sí / No)
Premutación	Ninguno	38-49	-	Normal	-
Congénito	Defectos del desarrollo, hipotonía, insuficiencia respiratoria, defectos cardíacos, problemas cognitivos graves, dismorfia facial, disfagia	750–1400	Nacimieto	Mortalidad infantil	Penetrancia completa Sí
Comienzo en la infancia	Síntomas similares a la DM1 congénita pero menos grave	>800	1–10	Reducida	Penetrancia completa Sí
Inicio en adultos	Miotonía, arritmias cardíacas, hipersomnia, dificultades gastrointestinales, debilidad y desgaste muscular, cataratas, hipogonadismo masculino, resistencia a la insulina, problemas cognitivos, disfunción ventricular izquierda	250–750	20–40	Reducida	Penetrancia completa Sí
Incio Juvenil	Síntomas similares a DM1 en adultos pero más graves	400–800	10–20	Acortado	Penetrancia completa Sí
Inicio tardío	Cataratas, hipersomnia, miotonía	100–600	>40 año	Normal	Penetrancia completa Sí

Adaptado de De Antonio M. et al., 2016; Yum K. et al., 2017.

Manifestaciones cardíacas en DM:

Aproximadamente el 80% de los pacientes con DM1 desarrollarán enfermedad cardíaca en sus vidas, pero el riesgo de enfermedad cardíaca es más pronunciado en pacientes jóvenes (2-30 años) que en los ancianos (Lund et al. 2014). De hecho, las complicaciones cardíacas representan el 30% de las muertes de los pacientes y son la segunda causa más importante de fatalidades después de la insuficiencia respiratoria (Mathieu et al. 1999; Mankodi and Thornton 2002; Pelargonio et al. 2002). Se observan tres fenotipos cardíacos interrelacionados en individuos con DM1.

- El primero son los defectos de conducción, que son particularmente comunes y pueden progresar hasta provocar bloqueo cardíaco (Nguyen et

al. 1988). Estos defectos son el resultado de la degeneración del sistema de conducción cardíaca y son causados principalmente por la fibrosis miocárdica (Petri et al. 2014). La fibrosis miocárdica se debe a la hipertrofia de los miocitos, a la infiltración grasa focal y también a la infiltración linfocítica (Nguyen et al. 1988; McNally and Sparano 2011). Las alteraciones de la conducción son prevalentes y afectan al 40% de los pacientes con DM1 (Groh et al. 2008) 65% de los pacientes tiene un ECG anormal y las anormalidades típicas de ECG incluyen la prolongación del intervalo PR [período que se extiende desde el inicio de la despolarización auricular hasta el comienzo de la despolarización ventricular también conocido como complejo QRS] (>240 ms; 20-40% de pacientes) y la duración del QRS (>120 ms; 5-25% DM1 patients) (Lau et al. 2015).

- El segundo fenotipo cardíaco es el desarrollo de arritmias ventriculares y / o auriculares potencialmente fatales (Nigro, Papa, and Politano 2012; Benhayon et al. 2015). Las alteraciones de la conducción proporcionan un sustrato para el bloqueo de la conducción, la actividad ectópica y las arritmias reentrantes. Estas alteraciones pueden causar palpitaciones, síncope y muerte súbita cardíaca (Finsterer and Stöllberger 2012). Alrededor del 25% de los pacientes con DM1 muestran taquiarritmias auriculares (supraventriculares), específicamente fibrilación auricular y aleteo auricular (Pelargonio et al. 2002; Groh et al. 2008). Las arritmias ventriculares son menos comunes pero más graves y se consideran la principal causa de muerte súbita (Hermans et al. 2012; Bienias et al. 2017).
- El tercer fenotipo, aunque más raro, es la disfunción diastólica y / o sistólica mecánica que puede progresar a un fallo sistólico y diastólico combinado (Phillips and Harper 1997; Mathieu et al. 1999; Lazarus et al. 2002). La disfunción ventricular es más común, sin embargo, la dilatación auricular izquierda también puede ocurrir en el curso de la enfermedad (McNally and Sparano 2011). Otras manifestaciones cardíacas asociadas incluyen cardiopatía isquémica, en forma de angina (tanto estable como inestable) e infarto de miocardio. Los estudios de ECG también han encontrado que algunos individuos con DM1 tienen contractilidad del corazón reducida, como lo revela una fracción de eyección del ventrículo izquierdo inferior al 50% (Dhand, Raja, and Aggarwal 2013; Chaudhry and Frishman 2012). En

algunos pacientes con DM1 también se observó insuficiencia pulmonar (Schmacht et al. 2016).

En comparación con DM1, los problemas cardíacos en DM2 se consideraon menos graves y frecuentes (G. Meola et al. 2002; Flachenecker et al. 2003; Sansone et al. 2007). Sin embargo, estudios recientes indican que el riesgo general de enfermedad cardíaca en pacientes con DM2 es muy cercano al de los pacientes con DM1 (Giovanni Meola and Cardani 2017). Al igual que en DM1, las características cardíacas en DM2 incluyen defectos de conducción AV, arritmias y cardiomiopatía dilatada (T. M. Lee et al. 2012). La lesión miocárdica subclínica causa defectos de conducción y se correlaciona directamente con las anomalías del ECG encontradas en pacientes con DM2 (Schmacht et al. 2016). Los defectos de conducción también causan arritmias cardíacas graves y muerte súbita en pacientes con DM2 (Wahbi et al. 2009). A diferencia de DM1, los pacientes con DM2 no muestran insuficiencia pulmonar (Schmacht et al. 2016).

Patogénesis molecular:

Tanto DM1 como DM2 comparten un complejo mecanismo de fisiopatología. Las mutaciones encontradas en ambos tipos, que contienen expansiones CTG o CCTG, se transcriben y procesan en mRNA poliadenilados y procesados. Los RNA mutantes forman una estructura secundaria y se acumulan formando foci ribonucleares. El RNA mutante interfiere con proteínas de unión a RNA que actúan en trans, lo que conduce a cantidades aumentadas del primer miembro de la familia CUGBP / Elav (CELF1) y actividad reducida de las proteínas Muscleblind-like (MBNL) (Osborne and Thornton 2006; Klein, Gasnier, and Furling 2011; Fernandez-Costa et al. 2011). En el núcleo, la actividad de las proteínas MBNL (MBNL1-3 en vertebrados, Muscleblind en *Drosophila*) se ve disminuida debido a su secuestro en foci ribonucleares (Miller et al. 2000; Kino et al. 2004; Mankodi et al. 2001; Fardaei et al. 2002; Jiang et al. 2004; Cardani et al. 2006; LukáŠ et al. 2012). Por su parte los niveles de CELF1 se regulan positivamente mediante hiperfosforilación a través de diferentes quinasas de señalización (Kuyumcu-Martinez, Wang, and Cooper 2007; Salisbury et al. 2008; Jin et al. 2009; Huichalaf et al. 2010). La sobreexpresión cardíaca específica de CUGBP1 reproduce anomalías moleculares y funcionales de la distrofia miotónica tipo 1 (Kim et al. 2014). Estas dos proteínas juntas regulan múltiples eventos de procesamiento de RNA, incluyendo procesamiento alternativo,

traducción, poliadenilación, biogénesis de miRNA, estabilidad de mRNA y localización intracelular de mRNA (J. E. Lee and Cooper 2009; Batra et al. 2014; Giovanni Meola 2013; Rau et al. 2011; Adereth et al. 2005; E. T. Wang et al. 2012, 2015; Konieczny et al. 2017). Por lo tanto, en DM, la pérdida de función de MBNL por secuestro, combinada con la sobreexpresión de CELF1, conduce a la reprogramación del procesamiento del RNA hacia un patrón embrionario. Se han descrito a fondo y se han relacionado con síntomas específicos de la enfermedad varios defectos de procesamiento alternativo de pre-mRNAs concretos (Mankodi and Thornton 2002; Savkur, Philips, and Cooper 2001; Tang et al. 2012; Fugier et al. 2011; Freyermuth et al. 2016). Por ejemplo, el procesamiento incorrecto de *CLCN1*, el canal de calcio *CaV1.1* y el receptor insulina (*IR)* causa diferentes fenotipos de la enfermedad, como miotonía, debilidad muscular y resistencia a la insulina, respectivamente (Charlet-B. et al. 2002; Tang et al. 2012; Savkur, Philips, and Cooper 2001).

Además de las alteraciones de procesamiento alternativo, se han descrito otros mecanismos moleculares alterados en la progresión de la enfermedad. La redistribución o lixiviación inapropiada del factor de transcripción SP1 (Ebralidze et al. 2004) y la localización errónea del factor de transcripción SPEN (también conocido como SHARP) que se han observado en DM, se han relacionado con cambios en las concentraciones en el estado estacionario de varias moléculas de mRNA (Dansithong et al. 2011). La downregulación de microRNAs ha sido también implicada en ambos tipos de DM (Perbellini et al. 2011; Rau et al. 2011). En un estudio, se ha demostrado que MEF2, que está disminuído en el corazón de ratones modelo de DM1, regula microRNAs específicos del corazón, lo cual podría contribuir a la disfunción cardíaca específica de DM1 (Kalsotra et al. 2014). El papel de CUGBP1 también parece relevante en los fenotipos cardiacos DM1. La sobreexpresión de CUGBP1 humano en corazón de ratón adulto reprodujo cambios funcionales, electrofisiológicos y moleculares como se observó en pacientes con DM1 (Koshelev et al. 2010). Los estudios funcionales demostraron que la inhibición de PKC mejoró los defectos de conducción cardíaca y las anomalías de contracción encontradas en este modelo de ratón. El inhibidor también redujo la mala regulación de los eventos de corte y empalme regulados por CUGBP1, pero no los regulados por MBNL1, lo que sugiere papeles distintos para estas proteínas en la patogénesis cardíaca de DM1 (G. S. Wang et al. 2009). Sin

embargo, las consecuencias fisiológicas combinadas de las alteraciones en el procesamiento alternativo, la expresión génica y las alteraciones de miRNAs en el corazón aún no se han aclarado (Fernandez-Costa et al. 2013; Kalsotra et al. 2014; E. T. Wang et al. 2015). En los últimos años, el descubrimiento de la traducción no-AUG asociada a las repeticiones (RAN) (Cleary and Ranum 2014; Zu et al. 2017) en las enfermedades neurodegenerativas causadas por microsatélites, ha abierto la posibilidad de que también este mecanismo contribuya a las diferentes disfunciones sistémicas observadas en la DM. Sin embargo, esto aún no se ha establecido.

Modelos animales para estudiar la disfunción cardíaca en DM:

A pesar de la relevancia de la afectación cardiaca en la DM, existen muy pocos modelos animales disponibles para estudiar los mecanismos moleculares que la causan. Los ratones transgénicos conocidos como DMSXL (Huguet et al. 2012) y GFP-DMPK-(CTG) 5 (M. S. Mahadevan et al. 2006) son los únicos modelos disponibles para estudiar el efecto de las repeticiones en el desempeño del corazón. Estos ratones, sin embargo, tienen algunas limitaciones. Los ratones DMSXL reproducen las principales características clínicas observadas en la enfermedad humana, incluida la fuerza muscular reducida, menor rendimiento motor y deficiencias respiratorias. Sin embargo, para observar fenotipos cardiacos en los ratones DMSXL es necesario tratarlos con el agente antiarrítmico de clase I flecainida (Algalarrondo et al. 2015). Por su parte, los ratones GFP-DMPK- (CTG) 5 muestran toxicidad dentro del rango normal de 5 repeticiones CTG en ausencia de foci ribonucleares y tienen una alta tasa de mortalidad.

***Drosophila* como alternativa a los modelos de ratón para estudiar la disfunción cardíaca:**

El corazón de *Drosophila melanogaster* exhibe notables similitudes con el de los vertebrados en términos de origen morfogenético, estructura y mecanismos de regulación del desarrollo. Tanto en *Drosophila* como en embriones de vertebrados, el corazón se origina en dos hileras bilateralmente simétricas de células mesodérmicas que migran distalmente desde el punto de invaginación durante la gastrulación, se especifican como tejido cardíaco y finalmente se fusionan para formar un tubo en la línea media (Bodmer 1995). La formación

subsecuente de bucles y septos dota al corazón de múltiples cámaras en vertebrados, mientras que el corazón de *Drosophila* permanece tubular, aunque dividido por una válvula intracardíaca en una estructura anterior estrecha, llamada aorta, y una estructura posterior pulsátil de 1 mm, conocida como vaso dorsal o corazón (Ocorr, Vogler, and Bodmer 2014). La estructura y fisiología simple del tubo del corazón de la mosca proporcionan un sistema de ensayo *in vivo* adecuado para estudiar las disfunciones cardíacas (Fig. I2 A).

Las ventajas de la genética de invertebrados se han utilizado para estudiar el desarrollo cardíaco y desarrollar modelos de enfermedades humanas en la mosca. La principal herramienta genética de *Drosophila* es la capacidad de dirigir la expresión de un transgén dado a prácticamente cualquier tejido de mosca y a cualquier tiempo de desarrollo. Las moscas transgénicas se pueden obtener en un periodo de tiempo de 6-8 semanas. La expresión transgénica en la mosca se logra generalmente usando el sistema binario Gal4-Upstream Activating Sequence (UAS) derivado del control del metabolismo de la galactosa en la levadura (Brand and Perrimon 1993). En las moscas Gal4, la expresión específica de tejido del factor de transcripción Gal4 de levadura se consigue colocándole promotores específicos de tejido aguas arriba del mismo, mientras que las moscas UAS llevan transgenes de interés aguas abajo de una secuencia UAS específica. Típicamente, se cruza una linea Gal4 con un promotor específico con líneas de genes diana UAS y se examinan los efectos de la expresión génica específica de tejido en la progenie. Por ejemplo, en la descendencia F1, Hand-Gal4 dirige la expresión del transgén UAS al mesodermo cardiogénico embrionario (Han 2005) mientras que tinC-Gal4 impulsa la expresión de transgenes en cardioblastos (Lo and Frasch 2001). Una versión mejorada del controlador tinC-Gal4 es la GMH5-Gal4, que permite la expresión temprana en cardiomiocitos impulsada por un enhancer de *tinman* de 900 nt y mantiene la expresión a través de un bucle autorregulador UAS-Gal4 (Fig. I2 B) (Wessells and Bodmer 2004).

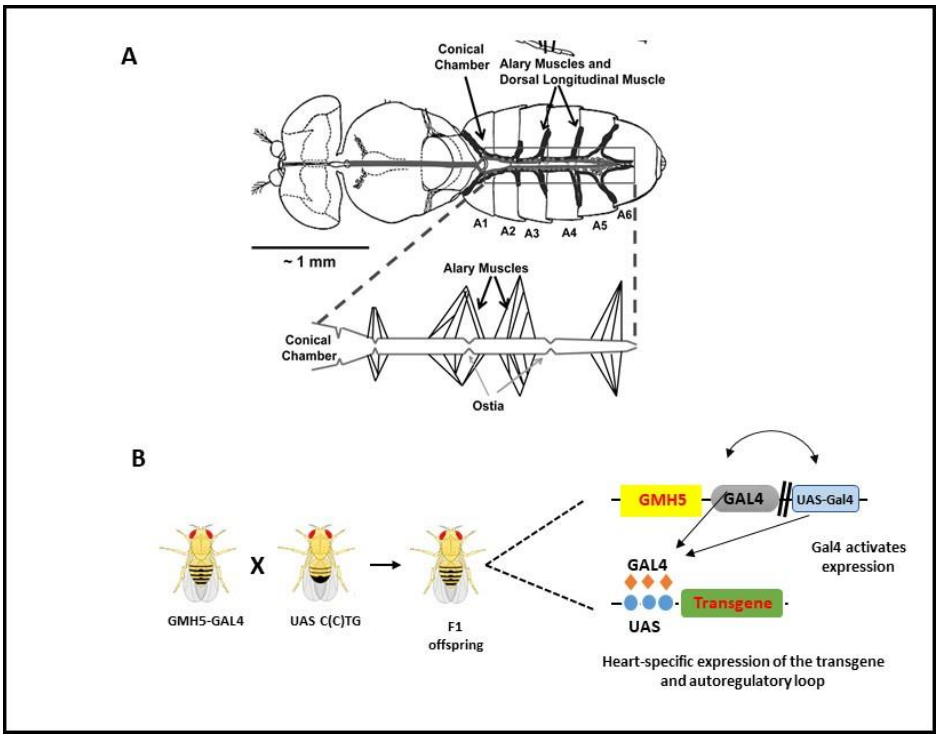

Fig. I2: Representación esquemática del corazón de la mosca y el sistema Gal4-UAS: (A) Representación esquemática del corazón de *Drosophila* o vaso dorsal (Wolf and Rockman 2011). El corazón se extiende desde el segmento abdominal A2 hasta A6. La cámara cónica está ubicada al comienzo del vaso dorsal. Los ostia, que son las aperturas en el corazón, ayudan a redistribuir la hemolinfa del corazón a la cavidad del cuerpo. Los músculos alares conectan el corazón con la cutícula.

Estrategias terapéuticas experimentales:

Hasta ahora, no está disponible para la DM ningún tratamiento efectivo , pero varios enfoques han sido probados experimentalmente en modelos de DM *in vivo* e *in vitro*. Se han desarrollado varias herramientas terapéuticas nuevas con el objetivo de abordar diferentes procesos de la fisiopatología de la DM que incluyen: (1) silenciamiento transcripcional, (2) silenciamiento postranscripcional, (3) inhibición de interacciones entre MBNL y RNA tóxico, o (4) rutas alteradas aguas abajo del RNA tóxico [Fig. I3] (Charles A Thornton, Wang, and Carrell 2017; Gourdon and Meola 2017; Konieczny et al. 2017).

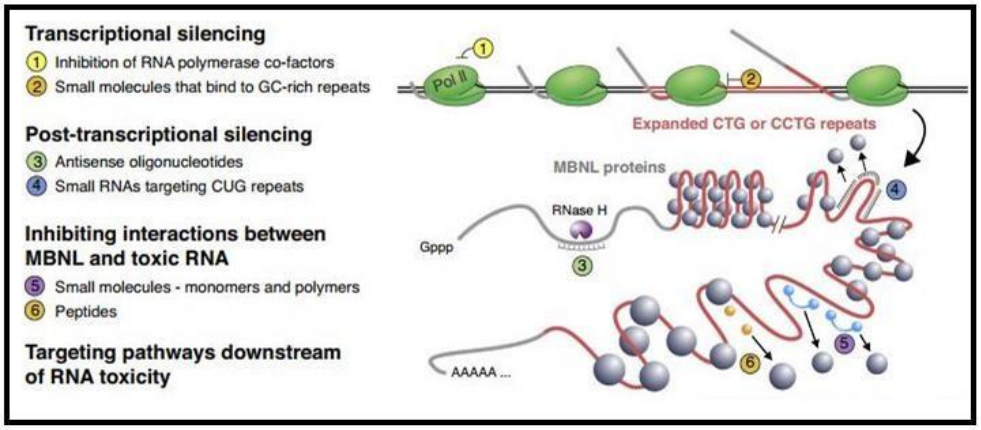

Fig I3: Diferentes estrategias para tratar la distrofia miotónica (Tomado de Thorton C.A. et al., 2017)

En los últimos años, las pruebas de concepto terapéuticas se han centrado en dirigirse a la parte más aguas arriba de la patología de DM para bloquear el comienzo de la cascada tóxica. Aunque la inhibición de la transcripción no se considera una estrategia general robusta para tratar las mutaciones de ganancia de función, existen informes de diferentes compuestos que pueden reducir la expresión de las repeticiones tóxicas a través de la inhibición de la transcripción. Por ejemplo, en Coonrod et al. han demostrado que la pentamidina y los antibióticos relacionados se pueden unir a las repeticiones CTG.CAG y reducir la expresión del RNA con repeticiones CUG (Coonrod et al. 2013).

En el caso de DM1, se han usado varias tecnologías basadas en oligonucleótidos (por ejemplo, oligonucleótidos antisentido - ASO) para destruir el RNA tóxico a nivel postranscripcional. Por ejemplo, los ASO que activan RNasa H1 causan una reducción marcada del RNA con CUGs en el músculo esquelético, la liberación de proteína MBNL de foci, la corrección de errores de procesamiento, la eliminación de miotonía y la mejora de la fisiología muscular (Wheeler et al. 2012). Varios hallazgos estimularon los esfuerzos para desarrollar un ASO optimizado para DMPK y en diciembre de 2014 se lanzó la primera fase de un ensayo clínico utilizando estos ASO optimizados (gapmer-ASO) con el objetivo de destruir los transcritos de DMPK en los músculos (Pandey et al. 2015).

Varios grupos han seguido un enfoque alternativo para tratar los fenotipos relacionados con la enfermedad utilizando el diseño racional o rastreos de alto rendimiento para identificar moléculas pequeñas que regulan positivamente MBNL1 e inhiben la interacción MBNL: CUG^{exp} o dispersan los foci de RNA(Ketley et al. 2014; Garcia-Lopez et al. 2011; Rzuczek, Southern, and Disney 2015; Nakamori et al. 2016; Cerro-Herreros et al. 2016). En comparación con los ASO, este enfoque es cada vez más favorable para el desarrollo de fármacos, ya que los ASO presentan dificultades en su distribución al músculo.

Otra estrategia terapéutica es dirigirse a las vías de señalización que se activan después de la expresión de CUG^{exp}. Estas vías aguas abajo incluyen la proteína quinasa C (PKC), la glucógeno sintasa quinasa 3 beta (GSK3beta) y la proteína quinasa activada por AMP (AMPK)/ mTOR (Charles A Thornton, Wang, and Carrell 2017). La inhibición de PKC por moléculas pequeñas conduce a la normalización de los niveles alterados de proteína CELF, rescatando el fenotipos cardíacos en modelos de ratón (G. S. Wang et al. 2009). Recientemente, la normalización de la vía AMPK / TOR mediante la administración de 5-aminoimidazol-4-carboxamida ribonucleótido (AICAR), un activador de AMPK o rapamicina, un inhibidor de mTOR, redujo el tiempo de relajación muscular después de la estimulación tetánica en DM1 (Brockhoff et al. 2017). En los últimos tiempos, Tideglusib, un inhibidor de GSK-3 se está estudiando en ensayos clínicos de Fase II como tratamiento para la DM1 congénita / juvenil (AMO pharmaceuticals; Konieczny et al. 2017).

Objectivos

Aunque algunos modelos murinos están disponibles para investigar las complicaciones cardíacas en DM, el complicado manejo de estas líneas por su baja tasa reproductiva y reducida longevidad, y el hecho de que solo presenten fenotipos cardiacos al ser expuestas a ciertas drogas o a condiciones de estrés, desaconseja su uso para conocer la causa molecular de los fenotipos cardiacos en DM.

Además de los diferentes modelos en ratón, en el campo de la DM, varios investigadores han desarrollado moscas transgénicas varias líneas de Drosophila para imitar los fenotipos multisistémicos, p.ej. en los músculos y los ojos. Además, se ha demostrado previamente en la literature que los moscas modelos también se pueden utilizar para comprender diferentes alteraciones cardiacas que se producen en humanos debido a la elevada similitud con el desarrollo del corazón humano.

Por lo tanto, el objetivo de este estudio fue generar y caracterizar modelos en Drosophila de disfunción cardiaca en DM para dilucidar los mecanismos patogénicos moleculares y testar moléculas que puedan revertirla. Para cumplir con el objetivo general de este estudio nos propusimos los siguientes objetivos específicos:

Objetivo 1: Estudio de la fisiopatología y las alteraciones moleculares relacionadas con la disfunción cardíaca producida al expresar repeticiones CTG expandidas en el corazón de *Drosophila*.

Objetivo 2: Estudio de las alteraciones cardiacas producidas al expresar las repeticiones CCTG en el corazón de *Drosophila* y su comparación con las producidas por la expression de repeticiones CTGs.

Objetivo 3: Testar el potencial terapéutico de diferentes candidatos químicos sobre el fenotipo de disfunción cardiaca en DM.

Objetivo 4: Caracterización del papel de diferentes modificadores genéticos en el progreso de la enfermedad cardiaca en DM.

Resultados principales

Resultados principales:

La sección de resultados principales incluye trabajos de tres artículos publicados que describen la generación y caracterización de fenotipos cardiacos en modelos en *Drosophila* de disfunción cardiaca en DM1 y DM2 y posibles estrategias terapéuticas para rescatar estos fenotipos. Para obtener modelos adecuados de ambos tipos de fenotipos cardiacos DM en moscas, se generaron líneas de moscas transgénicas UAS-CTG y UAS-CCTG que portaban 250 CTG o 1100 repeticiones puras no codificantes de CCTG, respectivamente (Chakraborty et al. 2015; Cerro-Herreros et al. 2017). Estas expansiones se encuentran dentro del rango patológico de repeticiones e imitan el tamaño de expansión al menos 4 veces mayor en pacientes con DM2 en comparación con DM1 (Schoser and Timchenko 2010; Warf and Berglund 2007). Como controles, se generaron moscas con 20x repeticiones. Las líneas de moscas UAS se cruzaron con moscas GMH5-Gal4 que promovían la expresión específica en corazón de las repeticiones (Wessells and Bodmer 2004). Este cepa también incluía el reportero UAS-GFP, que permite el marcaje de los tejidos en los que se expresa Gal4. Las moscas F1 que expresan repeticiones en el corazón se analizaron a varios niveles para caracterizar fenotipos relacionados con la DM.

1. Caracterización de la toxicidad de C(C)UG en el corazón de *Drosophila*:

Esta sección incorpora mi trabajo publicado en dos artículos (Chakraborty et al. 2015 y Cerro-Herreros et al. 2017). En estos trabajos se describe en detalle el efecto de la expresión de las repeticiones expandidas CTG y CCTG en el corazón de la mosca. En concreto, en el manuscrito Cerro-Herreros et al. 2017 del que soy co-primera autora, mi contribución se centró en describir el modelo de disfunción cardiaca en DM2.

1.1 La expresión de expansiones C(C)UG produce el secuestro de Muscleblind en el corazón de mosca y altera eventos de procesamiento alternativo dependientes de Muscleblind:

A nivel molecular, se ha demostrado que las proteínas MBNL están secuestradas en foci ribonucleares y juegan un papel crucial en la manifestación de la enfermedad. En nuestros estudios, hemos demostrado por primera vez la presencia de Muscleblind en el corazón de la mosca. En moscas control, Muscleblind no se detectó en el corazón embrionario (Artero et al. 1998) pero

en cardiomiocitos adultos, se detectó claramente. Inmunohistoquímica con un anticuerpo anti-Muscleblind (Houseley et al. 2005) muestra una expresión difusa de Muscleblind tanto en el núcleo como en el citoplasma de los cardiomiocitos de la mosca (Chakraborty et al. 2015). La hibridación fluorescente in situ (FISH) seguida de inmunofluorescencia reveló que, tras la expresión de repeticiones expandidas de CUG o CCUG en las células del corazón, Muscleblind quedaba secuestrada en foci ribonucleares. Por el contrario, las moscas que expresan un tamaño de repeticiones en el rango no patológico tanto en el caso de las repeticiones CUG como en las CCUG no mostraron ningún foci o acumulación de Muscleblind (Chakraborty et al. 2015; Cerro-Herreros et al. 2017). De acuerdo con la literatura, el secuestro de Muscleblind conduce a errores en el procesamiento alternativo de varios transcritos, lo cual origina fenotipos de la enfermedad. En los corazones de moscas modelo que expresan repeticiones largas, la inclusión del exón 13 del gen Serca y el exón 16´ del gen Fhos se encontraron alterados significativamente. Estos datos confirmaron que el secuestro de Muscleblind en foci ribonucleares observado en corazones de moscas DM1 y DM2 conduce a una reducción funcional de la proteína en el tejido cardíaco adulto (Cerro-Herreros et al. 2017).

1.2 La expresión de repeticiones expandidas en el corazón de *Drosophila* induce la autofagia:

Se ha demostrado previamente que la expresión de repeticiones largas de CTG induce la activación patológica de autofagia causando atrofia muscular en las moscas. Entre los diferentes genes relacionados con la autofagia, se encontró que la expresión de Atg4, Atg7 y Atg12 están significativamente aumentados en los músculos de la mosca que expresan repeticiones (Bargiela et al. 2015). La expresión de estos genes también se encontró aumentada en el caso de que las repeticiones expandidas de CUG o CCUG se dirigieran al corazón de *Drosophila*, en comparación con las moscas control que expresan GFP o las repeticiones cortas (Cerro-Herreros et al. 2017). Estos datos sugerían, por primera vez, un papel potencial de la desregulación de la vía de la autofagia en la disfunción cardíaca de DM tras la expresión de repeticiones expandidas. Curiosamente, la expresión de repeticiones CCUG largas en el corazón provoca una regulación al alza más fuerte de los factores relacionados con la autofagia que las repeticiones CUG, lo cual sugiere la existencia de factores desconocidos

dependientes del tejido que pueden modular la toxicidad de las repeticiones CUG y CCUG. Sin embargo, todavía falta la conexión mecanística entre la autofagia y los defectos cardíacos en las moscas. Para comprender la relación entre la activación de diferentes genes de la autofagia y los parámetros cardíacos, esta regulación al alza debe confirmarse detectando la presencia de lisosomas y alteraciones en las otras vías aguas abajo.

1.3 La expresión de repeticiones CUG o CCUG expandidas en el corazón de la mosca reduce la supervivencia y altera el rendimiento cardíaco:

Estudios poblacionales han encontrado tasas más altas de mortalidad y morbilidad, y una correlación positiva entre la edad al inicio y la edad al momento de la muerte, en individuos afectados por DM (Breton and Mathieu 2009). Como resultado de la expresión de repeticiones CUG o CCUG expandidas en el corazón, la supervivencia media y la vida máxima en las moscas modelo también se redujeron significativamente (casi a la mitad) en comparación con los controles mantenidos a 29°C (Chakraborty et al. 2015; Cerro-Herreros et al. 2017). Este hallazgo en archivos DM1 se correlaciona con informes de humanos afectados (Breton and Mathieu 2009; Petri et al. 2012). Es de destacar que la curva de supervivencia de las moscas que expresan repeticiones cortas de CUG o CCUG fue similar a la de las moscas control.

Para estudiar la función cardíaca, corazones de moscas adultas de 1 semana de edad fueron disecados en hemolinfa artificial y grabados con una cámara de video digital. Las grabaciones de video se analizaron usando un método semiautomático de análisis óptico del ritmo cardíaco (SOHA) para cuantificar los diferentes parámetros funcionales del corazón. Los trazados en *M-mode* obtenidos a partir de los clips de película proporcionaron detalles de las posiciones de los bordes de la pared del corazón (eje y) a lo largo del tiempo (eje x), ilustrando la ritmicidad y la dinámica de las contracciones del corazón (Ocorr, Akasaka, and Bodmer 2007). El proceso lo describimos en detalle en una publicación en la revista electrónica “Protocols Exchange” (Selma-Soriano et al. 2018). El *M-mode* permite la cuantificación de los siguientes parámetros cardiacos: fase de relajación y contracción (DI, y SI, por intervalo diastólico y sistólico), período cardíaco (HP, equivalente a intervalo diastólico más sistólico), índice de arritmia (AI; obtenido al dividir la desviación estándar del HP por su mediana), diámetro sistólico final (ESD), diámetro diastólico final (EDD) y el

porcentaje de acortamiento fraccional (%FS, FS = EDD - ESD / EDD × 100), que es una medida de la contractilidad del corazón. Usando este enfoque, se ha observado que la expresión de ambas repeticiones CUG o CCUG en cardiomiocitos causa alteraciones en diferentes parámetros cardíacos (Chakraborty et al. 2015; Cerro-Herreros et al. 2017). Se observó un aumento significativo de HP y AI en las moscas modelo, que imita el problema de conducción observado en pacientes con DM (Groh et al. 2008; McNally and Sparano 2011; Petri et al. 2012). Es importante destacar que ambos intervalos se vieron más afectados por las repeticiones de CCUG que por la expresión de repeticiones de CUG (Cerro-Herreros et al. 2017). Además, las moscas modelo tanto de DM1 como de DM2 mostraron una reducción de porcentaje de FS, que se correlaciona con una fracción de eyección ventricular disminuida observada en pacientes con DM (McNally and Sparano 2011; Hermans et al. 2012; Giovanni Meola and Cardani 2015). Curiosamente, en el tejido cardíaco, la expresión de repeticiones cortas produjo una prolongación leve pero significativa en el SI, que fue más pronunciada en el caso de las moscas que expresan CCUG (Chakraborty et al. 2015; Cerro-Herreros et al. 2017). La expresión de 20 repeticiones de CUG / CCUG no indujo el secuestro de Muscleblind en foci. Por lo tanto, los fenotipos observados en estas moscas podrían ser independientes del secuestro de Muscleblind, y los factores que originan el fenotipo parecen ser más sensibles a la toxicidad por repeticiones CCUG. En ensayos de velocidad de escalada y de vuelo las moscas modelo se comportaron como los controles sugiriendo que la reducción en el porcentaje de FS de moscas que expresan C(C)TG no afectó a la demanda de carga de trabajo aguda (vuelo y escalada), pero sí tuvo un efecto acumulativo perjudicial sobre la supervivencia (Cerro-Herreros et al. 2017).

2. Prueba de dos candidatos terapéuticos en el modelo de disfunción cardíaca DM1 de *Drosophila*:

Durante mi trabajo, hemos estudiado los efectos de dos compuestos en los fenotipos cardiacos del modelo de DM1 en *Drosophila* que ya están publicados (Chakraborty et al. 2015 y Chakraborty et al. 2018). A continuación se explican a fondo.

Moléculas pequeñas que inhiben la interacción Muscleblind-CUG tóxica muestran una actividad anti-DM1 relevante (Konieczny et al. 2017). La utilidad

de las moscas modelo como herramienta *in vivo* para buscar posibles compuestos terapéuticos contra la disfunción cardíaca en DM1 se verificó testando el efecto de un compuesto inhibidor de la interacción MBNL-CUGs ya conocido, la pentamidina. Posteriormente, usamos estas moscas modelo para testar el efecto anti-DM1 de una nueva molécula con potencial terapéutico la daunorubicina hidrocloruro.

La pentamidina, que es un conocido compuesto anti-DM1, inhibe significativamente la interacción MBNL-CUG, reduce la formación de foci ribonucleares y libera MBNL de los foci en las células tratadas. *In vivo*, también se ha encontrado que la pentamidina rescata parcialmente los defectos de procesamiento alternativo de dos pre-mRNA en ratones que expresan CUG^{exp} (Warf et al. 2009). Para probar el efecto de este compuesto en el fenotipo cardiaco de moscas modelo, se añadió pentamidina diluida en dimetilsulfóxido (DMSO) al medio nutritivo de las moscas hasta una concentración final de 1 µM (Chakraborty et al. 2015). Los parámetros moleculares y fisiológicos se compararon entre las moscas modelo alimentadas con pentamidina y las alimentadas solo con el solvente. En las moscas modelo tratadas con pentamidina, el rendimiento del corazón mejoró notablemente; se observó una reducción significativa en la arritmicidad (AI) y una recuperación importante de la contractilidad (% FS) en comparación con las moscas modelo tratadas solo con el solvente. Sin embargo, el SI y el DI afectados, que recuerdan a la disfunción sistólica y diastólica presente en pacientes, no fueron rescatados completamente por la pentamidina, aunque se observó una tendencia conspicua hacia los parámetros normales. Curiosamente, la mejora de los parámetros cardíacos fue suficiente para rescatar los valores medios de supervivencia de las moscas modelo tratadas con pentamidina. A nivel molecular, no se observaron diferencias significativas en el nivel de transcripción de las repeticiones en las moscas que tomaban pentamidina y las control. Además, hibridación in situ e inmunofluorescencia para detectar CUG RNA y Muscleblind, respectivamente, mostraron que los foci ribonucleares estaban ausentes en los núcleos de los cardiomiocitos y Muscleblind estaba disperso por todo el núcleo en las moscas modelo que tomaban pentamidina.

En colaboración con el laboratorio del Dr. Charlet-Berguerand (IGBMC, INSERM, University of Strasbourg) y mediante la realización de un rastreo basado en

polarización de fluorescencia, descubrimos que el fármaco antitumoral, daunorubicina hidrocloruro, compite con MBNL por unirse a las repeticiones CUG *in vitro* (Chakraborty et al., 2018). La daunorubicina es un agente intercalante en presencia de dsRNA y dsRNA y se une competitivamente a las repeticiones de CUG e inhibe la unión de MBNL1 con una CI50 *in vitro* de alrededor de 100 nM. La técnica de fluorimetría de barrido diferencial (DSF) reveló que este compuesto estabiliza la estructura bicatenaria de las repeticiones de CUG *in vitro* y limita la accesibilidad de MBNL1 a las estructuras de ssRNA libres. La daunorubicina se probó en moscas en las mismas condiciones que la pentamidina. Este compuesto produjo una mejora notable en el rendimiento cardíaco de las moscas modelo (Chakraborty et al., 2018). A diferencia de la pentamidina, este compuesto mejoró todos los parámetros cardíacos, incluidos SI y DI. Es importante destacar que la mejora del rendimiento cardíaco también fue suficiente para recuperar los valores medios de supervivencia de las moscas que toman daunorubicina. A nivel molecular, este compuesto no modificó el nivel de expresión de las repeticiones. Además, la doble FISH/inmunofluorescencia mostró que los foci ribonucleares estaban ausentes en los núcleos de los cardiomiocitos y que Muscleblind se distribuyó por todo el núcleo en las moscas modelo que tomaban daunorubicina. Es importante destacar que esta liberación de Muscleblind mejoró significativamente el defecto de procesamiento de los transcritos Fhos y Serca en moscas modelo que tomaban daunorubicina (Chakraborty et al., 2018).

Todos estos resultados apoyaban una mecanismo de acción de ambos compuestos basado en inhibir la interacción Muscleblind- RNA CUG, en lugar de en la inhibición de la transcripción. Esta liberación de Muscleblind podría ser la razón por la que los parámetros cardíacos y las alteraciones moleculares se recuperan en las moscas modelo. De hecho, el grado de recuperación fue diferente dependiendo del fármaco, p.ej la pentamidina no rescató completamente los intervalos sistólicos o diastólicos, pero la daunorubicina rescató ambos. Aunque es especulativo, es tentador sugerir que las diferencias en el grado de recuperación pueden originarse a partir de una mayor liberación de Muscleblind por la daunorubicina que por la pentamidina.

3. Efecto de la sobreexpresión de Muscleblind en el modelo de disfunción cardíaca de DM1:

Finalmente, en mi tercera publicación (Chakraborty et al., 2018) describí el efecto de la sobreexpresión de Muscleblind en fenotipos de corazón en moscas modelo DM1.

Los resultados anteriores sugieren fuertemente que el secuestro de Muscleblind contribuye a la disfunción cardíaca. Para abordar específicamente esta cuestión, la isoforma C de Muscleblind [la isoforma mejor conservada evolutivamente] (Garcia-Casado, Artero and Perez-Alonso 2002) fue sobreexpresada junto con repeticiones CUG en cardiomiocitos de *Drosophila*. Como moscas control, en este caso se emplearon moscas modelo de DM1 que sobreexpresan GFP, tras haber demostrado que la expression del reportero GFP era inocua en el corazón y no alteraba los parámetros detectados en las moscas modelo. En contraste con la GFP, la expresión de MblC en moscas modelo corrige la disfunción cardiaca. Es importante destacar que el HP mejoró principalmente debido a una reducción del SI. La AI, el % FS y la contractilidad también se recuperaron significativamente en las moscas DM1 que sobreexpresaban Muscleblind. Todos los parámetros fueron recuperados excepto el intervalo diastólico que tal vez requiera una sobreexpresión más alta o la presencia de otras isoformas de proteína. Además, la recuperación del rendimiento cardíaco también mejoró la supervivencia media y vida máxima de las moscas modelo que sobreexpresaban MblC (Chakraborty et al., 2018).

Discusión y perspectivas de futuro

Discusión y perspectivas de futuro:

Durante mi trabajo de tesis he generado y caracterizado, por primera vez en el campo de la distrofia miotónica, sendos modelos de disfunción cardiaca en *Drosophila*. En este trabajo se describieron los fenotipos cardiacos de moscas que expresan repeticiones C(C)UG largas y cortas. Los diferentes parámetros dinámicos del corazón, incluidos la frecuencia cardíaca, la ritmicidad, los intervalos sistólicos y diastólicos (SI y DI) y %FS se encontraron alterados en las moscas DM que expresaban expansiones largas en el corazón. La relevancia del modelo de DM está respaldada por las similitudes entre el fenotipo cardíaco de las moscas modelo de DM y las documentadas en individuos con DM (McNally and Sparano 2011).

Es interesante destacar que SI y DI se vieron más afectados por las expansiones CCUG que por las CUG. De la misma forma, en el tejido cardíaco, la expresión de 20 repeticiones produjo una prolongación leve, pero significativa, en el intervalo sistólico, que fue más pronunciada en el caso de las moscas que expresan CCUG. Estas observaciones establecen paralelismos con ratones que sobreexpresan agudamente un número de repeticiones CTG dentro del rango normal en DMPK 3' UTR (5 repeticiones) y que reproducen características cardinales de la DM1, incluida la miotonía, anomalías de la conducción cardíaca, histopatología y defectos de empalme de RNA en ausencia de inclusiones nucleares detectables (Storbeck et al. 2004; M. S. Mahadevan et al. 2006). Los autores plantearon la hipótesis de que la sobreexpresión de DMPK 3 'UTR con un número pequeño de repeticiones podría comportarse como la expresión más débil de un RNA portador de expansiones largas generando fenotipos patológicos equivalentes. En las moscas modelo se observó un nivel de expresión similar tanto para los transgenes que expresan repeticiones cortas como largas, pero es posible que el grado de expresión sea suficientemente elevado como para generar fenotipos. Igual que en ratón, la expresión de 20 repeticiones CTG no conduce a la formación de foci ribonucleares que retienen Muscleblind, pero es suficiente para inducir algunos fenotipos cardiacos.

Los mecanismos patogénicos subyacentes a las diferencias fenotípicas entre DM1 y DM2 aún se desconocen. Varios estudios han confirmado que la frecuencia y la gravedad de la afectación cardíaca y de la debilidad muscular son más limitadas en DM2 en comparación con DM1 y la progresión es más lenta y

menos grave en DM2. Esto sugiere que otras vías celulares y moleculares están involucradas además de la toxicidad al RNA C(C)UG, que es un mecanismo común. Se ha demostrado que tres factores influyen en el nivel de toxicidad de RNAs portadores de repeticiones expandidas: nivel de expresión, longitud, y secuencia. Asumiendo misma expresión, las secuencias más largas tienden a causar una patogénesis más grave pero, dependiendo de la secuencia, los factores de unión al RNA pueden verse afectados diferencialmente. Es importante destacar que, en pacientes con DM2, la gravedad de la enfermedad no se ha correlacionado directamente con el número de repeticiones, solo se ha establecido una relación entre tamaño de expansión y la tasa de secuestro de MBNL1. Los fenotipos similares en las moscas que expresan repeticiones CUG o CCUG expandidas sugieren la existencia de modificadores desconocidos en humanos, lo que podría mitigar la toxicidad del RNA en pacientes con DM2. Recientemente se ha demostrado que la proteína de unión a RNA rbFOX1 se une con expansiones CCUG. Esta proteína compite con MBNL1 por unirse a las repeticiones CCUG y libera MBNL1 de foci en células musculares DM2. Curiosamente, esta proteína no se une a las repeticiones CUG, lo cual explica bien la diferente presentación clínica de las dos enfermedades. De hecho, la sobreexpresión de rbFOX1 en *Drosophila* rescata la desregulación del splicing de transcritos musculares así como la atrofia muscular en un modelo en *Drosophila* (Sellier C et al. 2018).

El rescate de los parámetros cardíacos utilizando pentamidina y daunorubicina respalda la especificidad del fenotipo de disfunción cardíaca y confirma el efecto deseable de ambos compuestos en un modelo *in vivo*. Ambos compuestos lograron una mejora a nivel fisiológico y molecular al rescatar diferentes parámetros cardíacos, disminuir la retención de Muscleblind en foci y corregir el procesamiento alternativo alterado de transcritos concretos. Es importante destacar que la pentamidina no restableció por completo todos los parámetros cardíacos. La función diastólica y sistólica se mantuvo alterada en las moscas tratadas. Por el contrario, la daunorubicina rescató completamente todos los parámetros cardíacos alterados en las moscas modelo DM1.

Perspectivas de futuro:

La extensa descripción y comparación que hemos realizado de los modelos de mosca de DM1 y DM2, permitirá el empleo de los mismos para el descubrimiento de modificadores genéticos que afecten diferencialmente a estas enfermedad pudiendo ser causantes de las diferencias clínicas entre los fenotipos de las mismas (ej. rbFOX), contribuyendo a aumentar el conocimiento sobre sus vías de patogénesis y hacia el desarrollo de nuevos tratamientos.

Otro aspecto que todavía requiere mucha atención es la relevancia del secuestro de proteínas Muscleblind en la patología cardíaca. El trabajo con *Drosophila* indica claramente que la reducción de Muscleblind por secuestro contribuye a la patología, pero esto aún no se ha demostrado en modelos mamíferos. La transcriptómica del corazón de la mosca puede arrojar luz sobre este problema al descubrir los diferentes eventos moleculares que se alteran a consecuencia de dicho secuestro y cómo estas alteraciones pueden contribuir a los diferentes parámetros de disfunción cardíaca.

Aunque *Drosophila* se comporta del modo esperado en cuanto a reproducir fenotipos de conducción cardiaca, desgraciadamente no se conocen bien los mecanismos subyacentes al origen del potencial del marcapasos en *Drosophila* lo que impide avanzar en la base celular y molecular de estas alteraciones. Otros estudios electrofisiológicos avanzados como p. ej. estudios de potenciales extracelulares e intracelulares y técnicas mecánicas como la microscopía de fuerza atómica (AFM) son necesarios para describir más de cerca el funcionamiento del corazón de la mosca normal y enferma.

Respecto a la translacíon clínica de la daunorubicina, ésta debe tomarse con precaución debido a los efectos genotóxicos de este compuesto en tratamientos a largo plazo, por lo que se necesitarían estudios posteriores en modelos murinos de la enfermedad para determinar si podría considerarse un enfoque terapéutico adecuado para la DM1. En este sentido debe destacarse que los modelos aquí descritos permiten el testeo *in vivo* fácil y asequible de nuevas terapias experimentales en fenotipos cardiacos tales como el hexapeptido ABP1, previamente identificado por nuestro grupo (Garcia-Lopez et al. 2011), y otros que pudieran encontrarse en el futuro.

Conclusiones:

Las diferentes conclusiones que pueden extraerse de este trabajo son las siguientes:

1. La expresión de repeticiones largas de CTG / CCTG en los corazones de *Drosophila* reproduce varios aspectos de la enfermedad humana tales como defectos de conducción, arritmias y defectos de contractilidad.

2. A diferencia de lo que occure en humanos, el efecto de la sobreexpresión prolongada de CCUG en el corazón de la mosca fue muy similar al efecto de la expresión de repeticiones CUG .

3. El RNA con repeticiones expandidas de C(C)UGs secuestra a Muscleblind en foci en núcleos de cardiomiocitos de *Drosophila* produciendo errores en el procesamiento alternativo de los transcritos de SERCA y Fhos en el corazón de la mosca.

4. La sobreexpresión de Muscleblind es capaz de rescatar diferentes parámetros cardíacos a excepción de la disfunción diastólica. Por tanto, igual que en la musculatura esquelética, las expansiones C(C)TG provocan falta de función de Muscleblind también en tejido cardiaco.

5. La expresión prolongada de repeticiones CUG o CCUGs en corazón produce desregulación de diferentes genes relacionado con la ruta de la autofagia. Estas alteraciones abren otro posible mecanismo de disfunción cardíaca en DM.

6. Las moscas modelo de DM son una importante herramienta *in vivo* para probar diferentes terapias candidatas y llenar un vacío en los modelos animales disponibles. En este sentido la pentamidina, un conocido compuesto anti-DM1, ha rescatado los problemas cardíacos de DM1 y la daunorubicina, identificada en el marco de este trabajo, es un fármaco capaz de rescatar problemas cardíacos de la DM1 en moscas modelo.

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-Anexos-

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RESEARCH ARTICLE

Pentamidine rescues contractility and rhythmicity in a *Drosophila* model of myotonic dystrophy heart dysfunction

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ABSTRACT

Up to 80% of individuals with myotonic dystrophy type 1 (DM1) will develop cardiac abnormalities at some point during the progression of their disease, the most common of which is heart blockage of varying degrees. Such blockage is characterized by conduction defects and supraventricular and ventricular tachycardia, and carries a high risk of sudden cardiac death. Despite its importance, very few animal model studies have focused on the heart dysfunction in DM1. Here, we describe the characterization of the heart phenotype in a *Drosophila* model expressing pure expanded CUG repeats under the control of the cardiomyocyte-specific driver *GMH5-Gal4*. Morphologically, expression of 250 CUG repeats caused abnormalities in the parallel alignment of the spiral myofibrils in dissected fly hearts, as revealed by phalloidin staining. Moreover, combined immunofluorescence and *in situ* hybridization of Muscleblind and CUG repeats, respectively, confirmed detectable ribonuclear foci and Muscleblind sequestration, characteristic features of DM1, exclusively in flies expressing the expanded CTG repeats. Similarly to what has been reported in humans with DM1, heart-specific expression of toxic RNA resulted in reduced survival, increased arrhythmia, altered diastolic and systolic function, reduced heart tube diameters and reduced contractility in the model flies. As a proof of concept that the fly heart model can be used for *in vivo* testing of promising therapeutic compounds, we fed flies with pentamidine, a compound previously described to improve DM1 phenotypes. Pentamidine not only released Muscleblind from the CUG RNA repeats and reduced ribonuclear formation in the *Drosophila* heart, but also rescued heart arrhythmicity and contractility, and improved fly survival in animals expressing 250 CUG repeats.

KEY WORDS: *Drosophila*, Heart dysfunction, Myotonic dystrophy, Muscleblind, Pentamidine

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is the most frequently inherited neuromuscular disease in adults. Aside from skeletal muscle symptoms, multi-organ involvement is also common and typically affects cardiac, endocrine and central nervous system tissues (Thornton, 2014). DM1 [Online Mendelian Inheritance of Man (OMIM) 160900] has been identified as an autosomal-dominant disorder associated with the presence of an abnormal CTG trinucleotide repeat expansion in the 3′ untranslated region (UTR) of the gene encoding myotonic dystrophy protein kinase (*DMPK*) on chromosome 19. Whereas 5-34 CTG repeats are observed in normal alleles, their number can reach up to between 50 and 2000 in DM1 (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). The best-characterized effect of the expanded *DMPK* RNA (CUG^{exp} RNA) is disruption of the function of RNA-binding proteins, including muscleblind-like 1 (MBNL1) and CUGBP Elav-like family member 1 (CELF1), which regulate multiple RNA-processing events, including alternative splicing, translation, polyadenylation, miRNAs biogenesis, mRNA stability and mRNA intracellular localization (Lee and Cooper, 2009; Batra et al., 2014; Meola et al., 2013; Rau et al., 2011; Adereth et al., 2005; Wang et al., 2012; Wang et al., 2015). CUG^{exp} RNA impairs normal postnatal alternative-splicing transitions regulated by MBNL1 and CELF1. Whereas MBNL1 is sequestered to the CUG repeats, the toxic effect of mutant RNA on CELF1 activity is very complex, and involves increased CELF1 protein levels as a result of its stabilization in the nucleus (Kuyumcu-Martinez et al., 2007; Kim et al., 2014; Timchenko, 2013; Timchenko et al., 2001). As a result of disrupting the function of these proteins, several mis-splicing defects have been described and have been linked to specific symptoms of the disease (Mankodi et al., 2002; Savkur et al., 2001; Tang et al., 2012; Fugier et al., 2011). However, the physiological consequences of alternative splicing, gene expression and microRNA alterations in the heart are yet to be clarified (Phillips et al., 1999; Rau et al., 2011; Kalsotra et al., 2014; Zu et al., 2011; Lopez Castel et al., 2011; Moseley et al., 2006; Perbellini et al., 2011; Fernandez-Costa et al., 2013; Wang et al., 2015). In general, cardiac involvement, which often precedes the skeletal muscle one, occurs in 80% of individuals with DM1 and represents the second most common cause of death of such individuals, after respiratory failure (Vinereanu et al., 2004). Several studies have reported an overall positive association between CTG-repeat size and cardiac involvement, and between the degree of neuromuscular and cardiac dysfunction (Petri et al., 2012; Groh et al., 2002; Dello Russo et al., 2006).

Three interrelated cardiac phenotypes are observed in individuals with DM1. The first is conduction defects, which are particularly common and can progress to complete heart blockage (Nguyen et al., 1988). The second is the development of potentially fatal ventricular and/or atrial arrhythmias (Nigro et al., 2012; Benhayon et al., 2015). The third phenotype, although rarer, is mechanical diastolic and/or systolic dysfunction that can progress to combined systolic and diastolic heart failure (Phillips and Harper, 1997;

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TRANSLATIONAL IMPACT

Clinical issue

Cardiac involvement is a common complication of the skeletal muscle disorder myotonic dystrophy type 1 (DM1), occurring in 80% of DM1 cases. Heart dysfunction is the second most common cause of fatality associated with the disease, after respiratory distress. DM1 is caused by the expansion of an unstable CTG repeat in the 3′ untranslated region (UTR) of the *DMPK* gene, which encodes myotonic dystrophy protein kinase. The expanded CUG repeats form a hairpin that sequesters the RNA-binding protein muscleblind-like 1 (MBNL1) and other nuclear factors into ribonuclear foci in a manner that is proportional to the CUG expansion size. Sequestration has been proposed to cause depletion of these proteins, leading to defects in splicing that underlie some of the clinical symptoms of DM1. Despite the central involvement of heart failure in DM1, very few studies have focused on the molecular cause of cardiac dysfunction in this disease and fewer have tested the effect of potential anti-DM1 compounds on this phenotype using suitable animal models.

Results

In this study, Beatriz Llamusi and colleagues generated and characterized a *Drosophila* model expressing pure expanded CUG repeats under the control of the cardiomyocyte-specific driver *GMH5-Gal4*. Supporting the suitability of this model to investigate cardiac dysfunction in DM1, the authors noted key similarities between the cardiac phenotype in DM1 model flies and those documented in individuals with DM1. First, they observed a reduction of median survival in model flies, which correlates to that reported in humans with DM1. Second, they observed a significantly increased heart period and arrhythmia index in the fly model, in line with heart conduction abnormalities that are common in DM1. Thirdly, they observed systolic and diastolic dysfunction reminiscent of that reported in affected humans. DM1 individuals with cardiac abnormalities generally show various extents of heart chamber dilation and hypertrophy, which result in decreased ventricular ejection. This phenotype was also mimicked in the fly model, which demonstrates reduced fractional shortening. Providing proof-of-concept, the authors also reported the efficacy of a known anti-DM1 compound, pentamidine, to partially rescue these heart phenotypes. Adult DM1 model flies fed with pentamidine showed reduced arrhythmicity and improved contractility, allowing a rescue of cardiac output that translated into a median survival that did not differ from control flies expressing 20× CUG repeats. However, the heart-rate dysfunction observed in the DM1 model flies was not completely rescued by pentamidine.

Implications and future directions

A better understanding of the molecular mechanisms altered by expansion of CTG repeats, and of the molecular interactions of the repeat sequence *in vivo*, is crucial for deciphering the origin of the symptoms of DM1 and to generate appropriate treatments. This work describes, for the first time, the toxic effects of long CUG RNA on cardiac function in a *Drosophila* model, paving the way for further studies to elucidate the molecular alterations underlying cardiac involvement in DM1. Moreover, the ability to detect changes in the phenotype in response to treatment with a known anti-DM1 compound confirms the specificity of the phenotype and its ability to respond to therapeutic intervention. Because several aspects of DM1 pathogenesis are still unclear, this model could be used to provide a more detailed description of heart involvement in DM1 and allow the identification of potential genetic modifiers of the heart alterations. Importantly, the model can also be used to test the efficacy of different therapeutic approaches that so far have only been tested in skeletal muscle.

Mathieu et al., 1999; Lazarus et al., 2002; Groh et al., 2008). The majority of individuals with DM1 show abnormal electrocardiography (ECG) assessments, with prolonged time of conduction of the sinoatrial impulse to the ventricles (PR interval) (20-40% of affected individuals) and ventricular depolarization (QRS complex) widening (5-25%) (McNally and Sparano, 2011). Moreover, echocardiogram studies have also found that some individuals with DM1 have reduced heart contractility, as revealed by a lower left ventricular ejection fraction (LVEF less than 50%) (Dhand et al., 2013; Chaudhry and Frishman, 2012).

Despite the relevance of heart involvement in DM1, the molecular mechanisms causing the abnormalities in electric conduction or contractility are not well understood. In previous inducible DM1 mouse models, conduction disturbances appeared a few days after inducing acute expression of either a short stretch of five CTG triplets or long interrupted CTG repeats (Wang et al., 2007; Mahadevan et al., 2006). These studies pointed to alterations in cardiac conduction and excitability properties as an early event in the appearance of DM1-associated cardiomyopathies. Another mouse model was generated more recently that carries the human DM1 locus constitutively expressed under the regulation of its own promoter and its *cis*-regulatory elements (DMSXL). These mice constitute a good model of slow and steady-state expression of the triplet expansion, as is observed in individuals with DM1. However, cardiac abnormalities (reduced ventricular myocardium cell excitability) were not observed in baseline conditions; rather, they were only revealed after injection of the sodium-channel blocker flecainide (Algalarrondo et al., 2015).

Adult *Drosophila* possess an open circulatory system consisting of a dorsal vessel, which is the 1-mm-long pulsatile heart tube, and an anterior aorta that extends through the thorax and into the head (for a review of *Drosophila* heart development and assessment see Ocorr et al., 2014). The simple structure and physiology of the *Drosophila* heart tube, together with its readily available genetics, provide a suitable *in vivo* assay system for studying cardiac dysfunctions. Here, we report the first *Drosophila* DM1 heart-dysfunction model, generated by overexpression of long pure CUG repeats {250 CUG repeats [CUG(250)×]} under the control of the cardiomyocyte-specific driver *GMH5-Gal4*. We have detected CUG ribonuclear foci and Muscleblind sequestration (the main molecular features of the disease in humans) in the *Drosophila* heart cell nuclei, and a shortened median survival and lifespan in DM1 flies. We also measured several *Drosophila* heart parameters and found that these also resemble the heart dysfunction found in DM1 humans. Importantly, we confirmed that oral administration of pentamidine to flies expressing long CUG repeats releases Muscleblind from these repeats and prevents foci formation in cardiac cell nuclei, also rescuing a subset of heart-dysfunction phenotypes. Our data suggest that *Drosophila* represents an appropriate DM1 heart-dysfunction model for physiopathological studies and supports the utility of this model for the heart-specific testing of potential therapeutic compounds.

RESULTS

Generation and characterization of a DM1 heart-dysfunction model in *Drosophila*

To develop a heart-dysfunction model of DM1 in flies, we have generated UAS-CTG transgenic lines carrying 20 [CUG(20)×] or 250 [CUG(250)×] pure CTG repeats and crossed them with the cardiac-specific driver *GMH5-Gal4* (Wessells et al., 2004), which includes the UAS-GFP reporter, allowing the labeling of the tissues in which Gal4 is expressed. The level of expression of the repeats was assessed by qPCR analysis using primers against the common SV40 terminator (Fig. S1), and the transgenes were confirmed to express the expected number of repeats (Fig. S2).

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Hearts of flies expressing CUG repeats under the *GMH5-Gal4* driver were dissected for immunohistological and morphological assessment. Given the crucial involvement of Muscleblind protein in DM1 pathogenesis, it was highly relevant to confirm its expression in the *Drosophila* adult heart. Previous studies of Muscleblind expression in *Drosophila* have focused on adult skeletal muscle (Llamusi et al., 2013) or in the embryo (Artero et al., 1998). In the current study, using an anti-Muscleblind antibody (Houseley et al., 2005), we observed Muscleblind expression in the adult heart cardiomyocytes. Muscleblind displayed a diffuse expression not only in the nucleus but also in the cytoplasm of cardiomyocytes, in both control (OrR) (not shown) and short-repeat-expressing flies (Fig. 1A-F). In contrast, Muscleblind was found concentrated in CUG ribonuclear foci in the nuclei in flies expressing long CUG expansions. Muscleblind sequestration is one of the main features of DM1. Ribonuclear foci were only present in the nuclei of heart cells in long-CUG-expressing flies (Fig. 1G-I).

We also assessed the heart structure by staining actin, a structural component of the contractile machinery of muscles. *Drosophila* heart tubes have two types of muscle fibers, each with distinct myofibrillar structures (Mery et al., 2008; Taghli-Lamallem et al., 2008): (1) spirally or transversely oriented myofibrils that represent the contractile 'working' myocardium; and (2) longitudinally oriented myofibrils that are found along the ventral surface of the tube (Molina and Cripps, 2001). In young flies, both types of myofibrils exhibit a tight and well-aligned arrangement. Cardiac myofibrils have been reported to stain uniformly along the entire length of the thin filament with phalloidin (Ao and Lehrer, 1995), so it can be used to visualize both types of myofibrils.

Phalloidin staining of actin did not reveal gross structural abnormalities in the heart tube but found slight differences between flies expressing short or long CUG repeats in the areas surrounding the ostia. Cardiac myofibrils were tightly arranged and well aligned in short-repeat-expressing flies at 1 week of age. However, age-matched flies expressing long CUG repeats in heart showed abnormalities in the parallel alignment of transverse myofibrils, which showed a remarkable spiral disposition and less organized and compact arrangement of the myocardial myofibrils. These alterations have been also reported in aged fly hearts (Paternostro et al., 2001; Wessells et al., 2004; Taghli-Lamallem et al., 2008) (Fig. 1J-K).

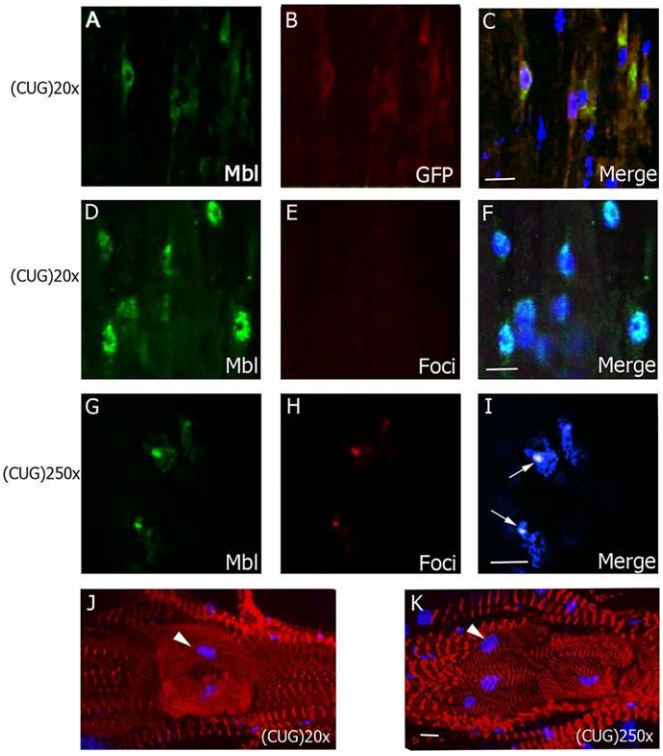

Fig. 1. Characterization of the DM1 heart-dysfunction phenotype in flies. Representative fluorescent confocal images of adult heart cells from flies expressing short [CUG(20)×] or long [CUG(250)×] repeats under the control of *GMH5-Gal4*. (A-C) In cardiomyocytes expressing CUG(20)×, revealed by anti-GFP antibody (red, B), Muscleblind signal (green, A) was dispersed in the nuclei and cytoplasm. (D-I) Combined immunodetection of Muscleblind (green, D and G) and FISH to detect ribonuclear foci (red, E and H) revealed dispersed expression of Muscleblind and absence of foci in flies expressing short CUG repeats (D-F). However, in flies expressing long CUG repeats (G-I), Muscleblind colocalized with ribonuclear foci (arrows). (J,K) Representative confocal stacks of phalloidin (red)-stained spiral fibers in the region surrounding the ostia in adult hearts (posterior A2-anterior A3 segment) reveals details of the heart structure, in particular increased fiber disorganization in GMH5-Gal4›CUG(250)× flies. Arrowhead points to ostia-associated nuclei. Merged images in C,F,I,J and K include DAPI (blue) counterstaining of the nuclei. All images are from 7-day-old flies. Scale bars: 10 µm.

DM1 flies show a median survival reduction and arrhythmicity

Population studies have reported higher mortality and morbidity rates, and a positive correlation between age at onset of DM1 and age at death in affected individuals (Breton and Mathieu, 2009). Similarly, we found that, as a result of long-CUG-repeat expression in heart, median survival and lifespan of flies were reduced at 29°C. The analysis of the survival curves showed that expression of long CUG repeats caused a significant reduction in the median survival of flies. From 47 days in control (*GMH5-Gal4 UAS-GFP*) and 41 days in short-repeat-expressing [*GMH5-Gal4* UAS-CUG(20)×] flies, median survival was reduced to only 25 days in flies expressing long CUG repeats [*GMH5-Gal* UAS-CUG(250)×] (Fig. 2A). Of note, lifespan and median survival of short-repeat-expressing flies was not significantly reduced in comparison to controls (Fig. S3).

To study heart function, adult fly hearts dissected in artificial hemolymph were recorded with a digital video camera. Because previous studies have reported that heart function changes with age, we selected 1-week-old flies for this study. Cardiac contractions were analyzed using a semi-automatic optical heartbeat analysis (SOHA) method to quantify the fly heart functional parameters. M-mode traces of movie clips provided details of the heart wall edge positions (*y*-axis) over time (*x*-axis), illustrating the rhythmicity and the dynamics of the heart contractions (Ocorr et al., 2007).

For the characterization of the cardiac phenotype of long-CUG-expressing flies, we compared their dynamic parameters with short-repeat-expressing flies to reveal the effect of the repeat length, and to two different controls: (1) F1 flies from crossing *GMH5-Gal4* and w^- flies (abbreviated w^-) to take into account potential contributions of the driver to the phenotype; and (2) F1 flies from crossing *GMH5-Gal4* to *UAS-GFP* flies (abbreviated GFP), accounting for the dose of the *UAS* transgenes.

The exposed and largely denervated heart in control and short-repeat-expressing flies showed rhythmic contractions; however, the contractions were clearly arrhythmic in hearts from flies expressing long repeats. We also observed morphological constrictions in small regions along the heart tube where no relaxation phase was observed (see Movie 1). Quantification of the heart period length (HP, defined as the diastolic plus systolic interval) showed that long-repeat-expressing flies exhibited a significantly increased HP compared to control or short-repeat-expressing flies (Fig. 2B).

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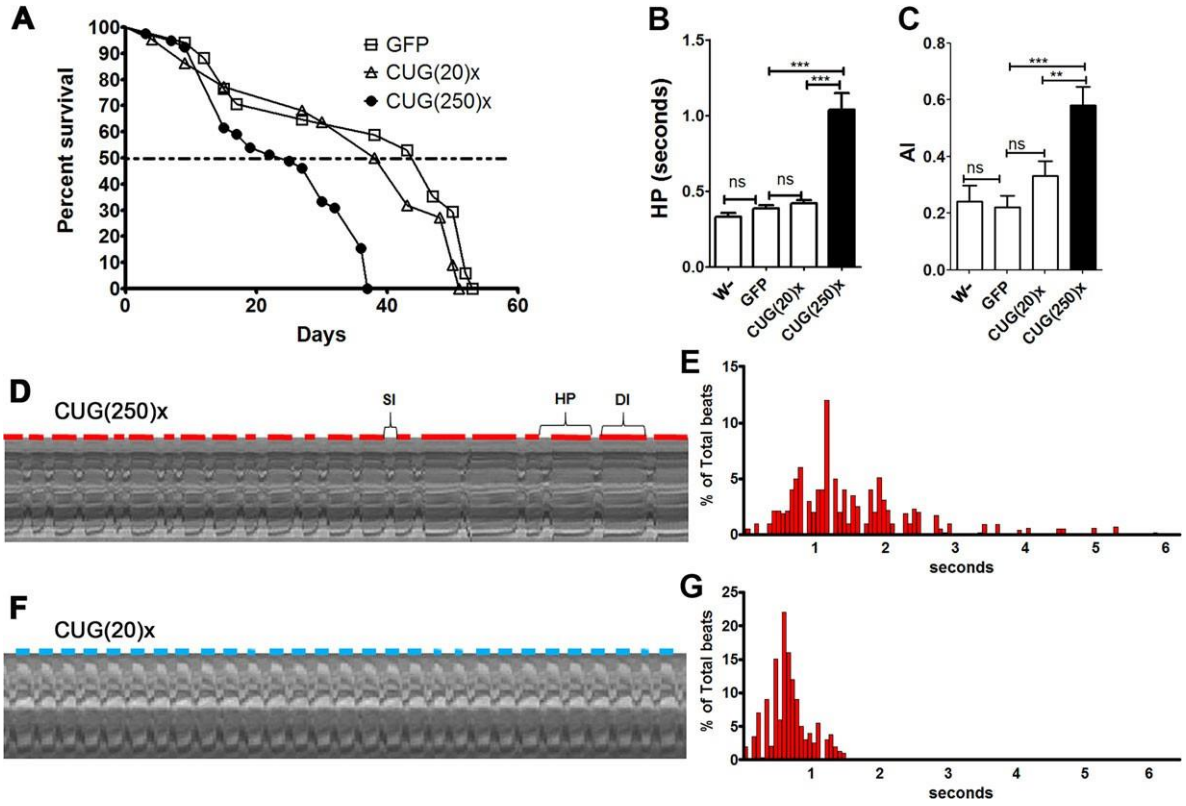

Fig. 2. Flies expressing long CUG repeats in *Drosophila* cardiomyocytes have a shortened median survival and increased arrhythmicity. (A) Average percentage of live flies, with the genotypes indicated, versus age (in days). Horizontal dotted line marks the median survival. Whereas control and short-repeat-expressing flies had a similar median survival of 47 and 40.5 days, respectively [GFP, n=40 and CTG(20)×, n=50], long-CUG-expressing flies lived a median of only 25 days [CTG(250)×, n=45). Differences in survival curves were highly significant (P<0.0001, log-rank test). (B,C) Heart period mean (HP, B), and arrhythmia index (AI, C) from flies expressing long and short CUG repeats and two types of control flies (F1 from crosses between the *GMH5-Gal4* driver and w^- or *UAS-GFP*). Both parameters are significantly increased in flies expressing long repeats. The bars on the graph show mean values and their standard errors. **P<0.01, ***P<0.001, ns, not significant. (D-G) Representative M-modes (20 s) (D,F), with their respective histogram showing the percentage of beats and their duration (E,G), taken from movies of semi-intact fly hearts expressing long (D,E) or short (F,G) repeats. Red and blue horizontal lines represent the diastolic interval (DI) duration of CUG(250)×- and CUG(20)×-expressing flies, respectively. The systolic interval (SI) and the heart period (HP) length are also indicated in D. The HP histograms, plotted as individual data points (n=21, E; n=29, G), illustrate the variability of the HP within a group of flies expressing long (E) and short (G) repeats.

The distribution of all of the measured HP for all flies of a specific genotype was represented in a histogram format, which revealed that the HPs clustered relatively tightly in the short-repeat flies, and that this distribution broadened in long-CUG-expressing flies, emphasizing the increased variability in the HP (compare Fig. 2D,E and 2F,G). The variability in the heart periodicity can be quantified as an 'arrhythmia index' (AI) obtained by dividing the standard deviation of the HP by its median (Fig. 2C). Flies expressing long CUG repeats showed approximately a 50% increase in AI compared to control and short-repeat-expressing flies.

Model flies display systolic and diastolic dysfunction, and reduced contractility

Alterations of systolic and diastolic function, as well as decreased ventricular ejection fraction, have previously been reported in individuals with DM1 (Dello Russo et al., 2006). To test similar alterations in model flies, we measured the heart rate (HR), the diastolic and systolic intervals (DI and SI, respectively), and the end-diastolic and end-systolic diameters (EDD and ESD, respectively), and calculated the resulting percentage of fractional shortening (% FS), and compared them to control and short-repeat-expressing flies. We found that the increased mean HP, and the correspondingly reduced HR (HP=1/HR) observed in flies expressing long CUG repeats, were caused by systolic and diastolic dysfunction, because both SI and DI (contraction and relaxation period, respectively) were significantly prolonged in comparison to control and short-repeat-expressing flies. To note, the HP of CUG(20)× flies (Fig. 2B) was not significantly different to controls because the slight increment in SI observed was compensated by a decreased DI (Fig. 3B,C). Image analysis of heart contractions also provided cardiac chamber parameters, including EDD and ESD. In addition, the proportional decrease in heart wall diameter during contraction provides an indication of the cardiac output. Control flies displayed an average EDD of about 70 µm and an ESD of 50 µm, and the average FS was higher than 30%. In long-repeat-expressing flies, we observed a significant decrease in EDD (to 50 µm), and also a reduced FS of only 20% (Fig. 3D-F). These data revealed that heart tube volume is reduced and there is a dysfunction of the contractile properties in hearts expressing long CUG repeats. Interestingly, flies expressing short CUG repeats showed both reduced ESD and EDD but normal FS, suggesting that contractile dysfunction resulting in a reduced cardiac output was exclusive to long-repeat-expressing hearts.

Pentamidine rescues survival, rhythmicity and contractility in the heart-dysfunction model

To assess whether model flies could be used as an *in vivo* tool to search for potential therapeutic compounds against cardiac dysfunction in

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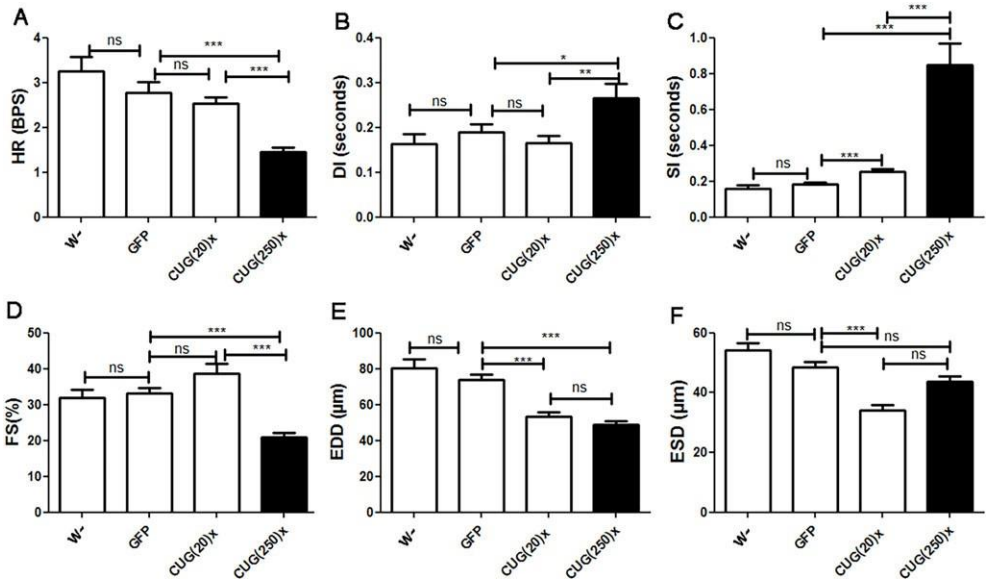

Fig. 3. DM1 model flies displayed systolic and diastolic dysfunction, decreased diastolic diameter and contractility defects. Flies expressing long CUG repeats showed reduced heart rate [HR, A; expressed in beats per second (BPS)], increased diastolic (DI, B) and systolic (SI, C) intervals, reduced fractional shortening (FS, D), and decreased end diastolic diameter (EDD, E). The reduced contractility did not affect the end systolic diameter (ESD, F), which was not significantly different from controls. Short-repeat-expressing flies displayed an increased SI in comparison to control flies and had a reduced ESD and EDD, without any alteration of FS, suggesting that they have no contractility alteration. Graph bars show mean values and their standard errors (*n* used was between 14 and 29). **P*<0.05, ***P*<0.01, ****P*<0.001, ns, not significant.

DM1, we tested the effect of a known anti-DM1 compound on the *Drosophila* heart phenotype. Small molecules designed to inhibit the toxic MBNL1-CUG repeat interaction had shown relevant anti-DM1 activity (Wong et al., 2014). Concretely, pentamidine significantly reduces the formation of ribonuclear foci, and releases MBNL1 from the foci in treated cells. Furthermore, pentamidine has been found to

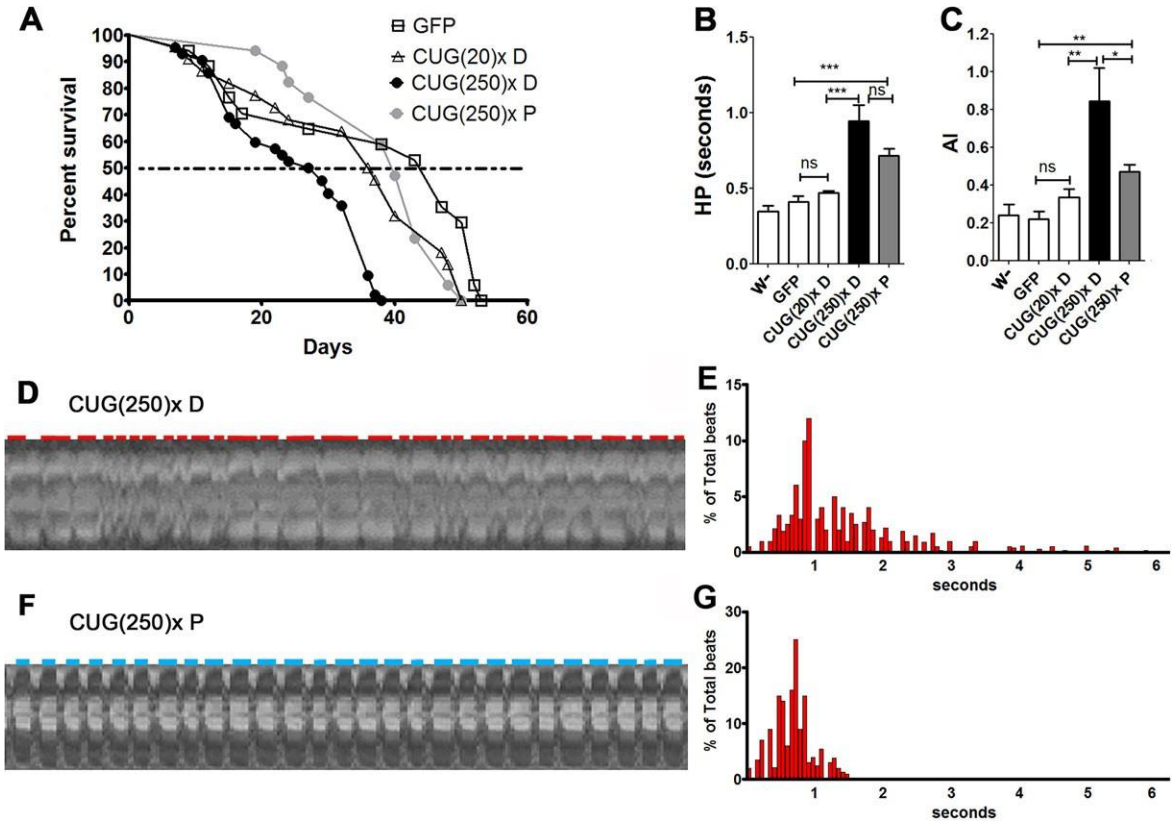

Fig. 4. Pentamidine rescued lifespan, median survival and arrhythmicity in DM1 model flies. (A) The average percentage of live flies, with the genotypes indicated, versus age (in days). Horizontal dotted line marks median survival. Model flies taking pentamidine [CUG(250)× P] had 40 days of median life, in comparison to only 28 days for long (CUG)250×-expressing flies fed with DMSO [CUG(250)× D]. The survival curves of model flies fed with pentamidine and control flies expressing short repeats fed with DMSO [CUG(20)× D] were not statistically different. (B) The heart period (HP) mean was not significantly altered by pentamidine administration, although a clear trend towards reduction was observed, whereas the arrhythmia index (AI) was strongly reduced (C). Graph bars show the mean values and their standard errors. **P*<0.05, ***P*<0.01, ****P*<0.001, ns, not significant. (D-G) Representative M-modes (20 s) (D,F) with their corresponding histograms (E, *n*=25; G, *n*=30) of percentage of beats of a given duration taken from movies of semi-intact flies expressing long repeats fed either with DMSO (D,E) or with pentamidine (F,G). Red and blue horizontal lines denote the diastolic interval (DI).

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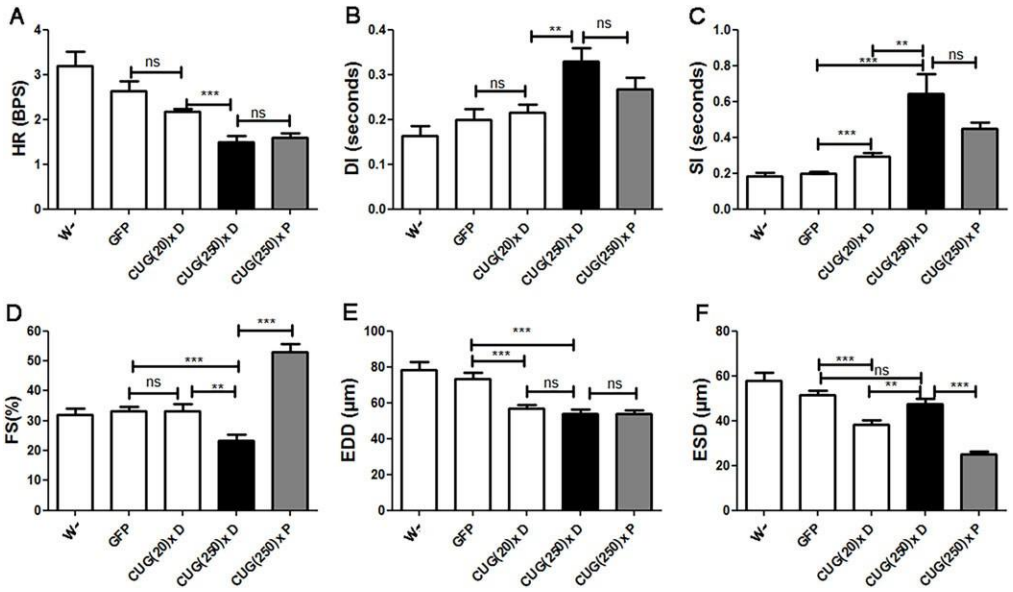

Fig. 5. Pentamidine improved contractility in model flies. Cardiac function parameters of short- and long-CUG-expressing flies fed either with DMSO [CUG(20)× D and CUG(250)× D] or pentamidine [CUG(250)× P], in comparison to controls. Pentamidine did not modify heart rate (HR, A), diastolic interval (DI, B), systolic interval (SI, C) nor end diastolic diameter (EDD, E) in model flies but strongly reduced end systolic diameter (ESD, F) in these flies, resulting in a relevant increase of fractional shortening (FS, D). Graph bars show the mean values and their standard errors (*n* used was between 14 and 30). ***P*<0.01, ****P*<0.001, ns, not significant.

partially rescue the splicing defects of two pre-mRNAs in mice expressing expanded CUG repeats (Warf et al., 2009). To test the effect of this compound in model flies, we added pentamidine diluted in dimethyl sulfoxide (DMSO) to the nutritive media to a final concentration of 1 µM. We tested the effect of DMSO in heart performance prior to these experiments and confirmed that, at the concentration used, it does not alter the cardiac parameters of *Drosophila* (Fig. S4). In comparison to model flies fed with DMSO, which have a median survival of 28 days, the median survival of model flies fed with pentamidine increased up to 40 days, which comes very close to the mean 47-day survival of control flies (Fig. 4A). Moreover, there was a significant reduction in arrhythmicity (see Movies 1, 2 and Fig. 4C). Although in long-repeat-expressing flies fed with pentamidine [CUG(250)× P] mean HP was not significantly reduced (Fig. 4B), there was a clear reduction in the deviation of the HP values, which reflected in a more constrained and grouped HP histogram pattern in comparison to the long-CUG-expressing flies taking DMSO [CUG(250)× D] (compare Fig. 4D,E and F,G). The altered HR, SI and DI detected in the model flies, reminiscent of the systolic and diastolic dysfunction reported in affected humans, were not rescued by pentamidine, although we did observe a conspicuous trend towards normal parameters (Fig. 5A-C). An important recovery of heart contractile properties was observed in pentamidine-treated flies. We observed a decreased ESD and unchanged EDD, resulting into an increased FS (Fig. 5D-F).

Pentamidine reduces foci and releases Muscleblind in cardiomyocytes of flies expressing long CUG repeats

In order to address the mechanism of action of pentamidine, we performed fluorescence in situ hybridization (FISH) and immunofluorescence to detect foci and Muscleblind, in hearts of long-CUG-expressing flies that were fed 1 µM pentamidine. As previously reported in DM1 cells in culture (Warf et al., 2009), ribonuclear foci were absent in cardiomyocyte nuclei and Muscleblind was distributed throughout the nucleus (Fig. 6A-C). Moreover, because biochemical experiments and cell and mouse model studies suggest that pentamidine and related compounds might bind the CTG.CAG repeat DNA and inhibit transcription (Coonrod et al., 2013), we measured expression levels of CUG^{exp} RNA in model flies fed with pentamidine or DMSO, detecting no significant difference (Fig. 6D). These data confirmed that the rescue of the cardiac-dysfunction phenotype achieved by pentamidine was mediated by releasing Muscleblind sequestration rather than reducing toxic RNA expression level.

DISCUSSION

Here we described, for the first time, the characterization of the cardiac phenotypes of flies expressing either long or short CUG repeats as a DM1 heart-dysfunction model. We measured changes

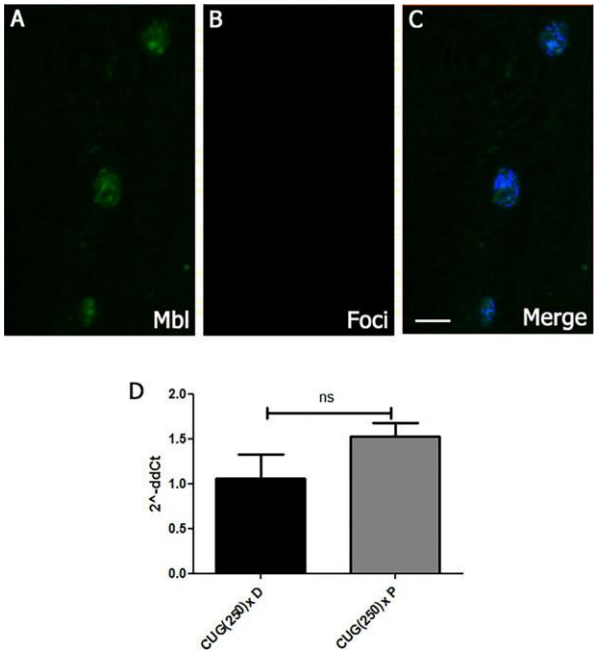

Fig. 6. Pentamidine mechanism of action. (A-C) Representative fluorescent confocal images of adult heart cells from long-CUG-expressing flies fed with pentamidine. Combined immunostaining of Muscleblind and CUG RNA FISH showed Muscleblind release (A) and no detectable foci (B) in the nucleus of cardiomyocytes of these flies. (C) Merge of A, B and DAPI; counterstaining of nuclei shows dispersed Mbl localization in nuclei. (D) Graph bar represents average fold changes of CUG(250)× expression in logarithmic scale, calculated by the $2^{-\Delta\Delta Ct}$ method, and their confidence intervals. Pentamidine did not significantly alter expression level of CUG repeat RNA in model flies. ns, not significant. Scale bar: 10 µm.

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in the heart dynamic parameters, including heart rate, rhythmicity, systolic and diastolic diameters and intervals (ESD, EDD, SI and DI), and FS. Moreover, we show that pentamidine, a compound with previously reported anti-DM1 activity, has the ability to modify the reported cardiac disease phenotypes.

The relevance of the model is supported by the similarities between the cardiac phenotype in DM1 model flies and those documented in individuals with DM1. First, we observed a reduction in the median survival in model flies, which correlates with reports for affected humans (Petri et al., 2012). Cardiac mortality in individuals with DM1 usually occurs because of progressive left ventricular dysfunction, ischemic heart disease, pulmonary embolism, or as a result of unexpected sudden death (SD) associated with the corrected prolonged QT interval (period including electrical depolarization and repolarization of the ventricles) (Park et al., 2013). Second, heart conduction abnormalities are common in individuals with DM1 (Groh et al., 2008; McNally and Sparano, 2011). Similarly, we observed a significantly increased HP and arrhythmia index in our fly model. Although several arrhythmias have been reported in individuals with DM1, recent studies found that atrial fibrillation (AF) and atrial flutter (AFL) are frequent in DM1 and are linked to increased mortality (Brembilla-Perrot et al., 2014). Thirdly, the altered SI and DI observed in DM1 flies are reminiscent of systolic and diastolic dysfunction reported in humans with the disease (Penisson-Besnier et al., 2008; Hermans et al., 2012). Recently, echocardiography-Doppler found an increase of the mean left-atrial diameter and an increase of the mitral deceleration time in DM1 individuals, suggesting diastolic abnormalities (Fayssoil et al., 2014). Left ventricular systolic dysfunction (LVSD) has also been reported in 7.2% of affected individuals (Petri et al., 2012). Moreover, individuals with DM1 with cardiac abnormalities generally show various extents of heart chamber dilation and hypertrophy, which results in a decreased ventricular ejection fraction (Dhand et al., 2013; Chaudhry and Frishman, 2012; McNally and Sparano, 2011; Pelargonio et al., 2002; Hermans et al., 2012) and correlates with the reduced FS we observed in model flies.

We have also found cardiac defects in flies expressing short CUG repeats; mainly, slightly increased SI and reduced cardiac tube diameters. Interestingly, model mice acutely overexpressing a normal-length *DMPK* 3′ UTR mRNA reproduced cardinal features of myotonic dystrophy, including myotonia, cardiac conduction abnormalities, histopathology and RNA splicing defects in the absence of detectable nuclear inclusions (Storbeck et al., 2004; O'Cochlain et al., 2004; Mahadevan et al., 2006). Authors hypothesized that the effects of overexpressing many *DMPK* 3′ UTR transcripts with a small repeat number might be pathogenically equivalent to expressing mutant transcripts with hundreds of CUGs. In our flies, we measured a similar level of expression of the repeats in short- and long-expressing flies but used a potent promoter for overexpression (*GMH5-Gal4*). This driver includes a *UAS-GAL4* element, allowing strong and continuous expression after induction. According to our data, controlled by this driver, expression of 20 CTG repeats does cause the formation of ribonuclear foci retaining Muscleblind but is enough to induce some cardiac phenotypes. Because MBNL loss in mice has been recently proven to be enough to cause cardiac pathology (Dixon et al., 2015), the finding of cardiac pathology in the absence of Muscleblind sequestration in our model flies becomes highly relevant because it shows a cardiac dysfunction mechanism that might be Muscleblind-independent in CUG-expressing flies.

The rescue of the cardiac parameters using pentamidine supports the specificity of the heart-dysfunction phenotype and confirms the therapeutic effect of pentamidine in an *in vivo* model. Interestingly, pentamidine did not completely re-establish all cardiac parameters. Diastolic and systolic function remained altered, suggesting that either the effect of the pentamidine is limited, or the defect itself is not susceptible to therapeutic recovery in adults. This could be the case for alterations occurring early in development because the *Drosophila* heart is one of a few structures that persist during pupal morphogenesis, although it undergoes extensive remodeling (Rizki and Rizki, 1978). Similarly, in humans, CUG RNA toxicity during development could cause alterations in heart physiology or anatomy that cannot be modified by treatment in adults. This situation is not reproduced by inducible models expressing the CUG expansions after birth, only in models with constitutive CUG-repeat expression. Another heart-dysfunction feature that is not rescued by pentamidine is the reduced EDD found in the model flies. Of note, EDD was equally reduced in both flies expressing short and long repeats, suggesting that it might not require Muscleblind sequestration. Therefore, pentamidine might not be able to modify this parameter. Importantly, the rescue achieved by pentamidine was enough to increase FS, which correlates with hemolymph volume ejected, and corrected the heart arrhythmia, both of which are thought to be the most prevalent causes of sudden death in individuals with DM1.

The long-repeat-expressing flies recapitulate many of the pathological and molecular features of DM1, including reduced survival, arrhythmias, systolic and diastolic dysfunction, and Muscleblind retention into ribonuclear foci. The rescue obtained by pentamidine treatment confirms that the DM1 model described has a sensitized phenotype that is suitable to unravel the mechanism of heart dysfunction in DM1 and to test potential therapeutic approaches in future studies.

MATERIALS AND METHODS

Drosophila strains

Self-priming (CTG)20× and (CAG)20× synthetic oligonucleotides were cloned into the linearized pUAST vector to generate pUAST-CTG(20)×. PUAST-CTG(250)× was constructed by subcloning 500 uninterrupted CTG repeats from the pcDNA-CTG(500)× vector, which was a kind gift from Dr Partha Sarkar (Department of Neurology, University of Texas Medical Branch, TX). After cloning into pUAST and amplification into the STBL3 (Invitrogen) *Escherichia coli* strain at 20°C, the 500 pure CTG repeats contracted to 250 CTG units. Transgenic flies carrying 250 or 20 pure repeats were generated by standard P-mediated transgenesis (BestGene Inc., Chino Hills, CA, USA). Transgenic lines carrying both long and short repeats were selected on the basis of moderate transgene expression and reproducibility of the phenotypes studied. In the fly lines used, the transgenes were located by inverse PCR to chromosome 2. The cardiomyocyte-specific driver *GMH5-Gal4* (kindly provided by the laboratory of Dr Rolf Bodmer in the Burnham Institute, CA, USA) is a 900 bp heart enhancer fragment 73 from the *tinman* gene that was cloned into the P{GaWB} vector upstream of the *Gal4* sequences. This driver was enhanced with multiple copies of a *UAS-Gal4* element allowing stronger myocardial expression and a *UAS-GFP* element allowing detection of the expression tissue (Wessells et al., 2004). All fly lines were maintained at 25° C with standard *Drosophila* food and standard day-night cycle.

Quantification of CUG-repeat expression level

Total RNA was extracted from ten flies per genotype using Trizol reagent (Sigma). DNase I treatment and reverse transcription were performed as previously reported (Llamusi et al., 2013). To quantify the expression of CUG RNA, the common SV40 terminator in the pUAST vector was used as target of the primers (F: 5′-GGAAAGTCCTTGGGGTCTTC-3′, R: 5′-GGAACTGATGAATGGGAGCA-3′). Expression levels were normalized to the reference gene *rp49* (F: 5′-ATGACCATCCGCCCAGCATAC-3′, R: 5′-ATGTGGCGGGTGCGCTTGTTC-3′) using the SYBR® Green mixture

(Roche) under $2^{-\Delta\Delta Ct}$ method. For each genotype, three biological samples were used and three technical replicates were performed.

Detection of CUG-repeat length

To confirm the length of the repeats in the *UAS*-CTG(20)× and *UAS*-CTG(250)× transgenes, 40 ng of genomic DNA was used as a template for the PCR amplification with KAPA HiFi (BIOSYSTEMS) and the primers: F 5′-GCAACTACTGAAATCTGCCAAGA-3′ and reverse-5′-GTTGAGAGT-CAGCAGTAGCC-3′, which flank the repeats. The region amplified by the primers includes the short repeats (60 bp) and 375 bp of the CTG(20×) plasmid, and the long repeats (750 bp) and 428 bp of the CTG(250)× plasmid. PCR amplification was performed under the following conditions: 95°C for 2 min, followed by 30 cycles of 98°C for 20 s, 65°C for 30 s, 72°C for 1 min and final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis at 110 V in 1.5% agarose gels.

Pentamidine treatment

Pentamidine was added to the standard food to a final concentration of 1 µM in 0.1% DMSO (Applichem). The control group of flies was fed with 0.1% DMSO. Flies were transferred every 3 days to new fresh food media, with or without pentamidine for the duration of their whole lifespan in life survival experiments or every 7 days in the case of the group used for cardiac analysis.

Survival analyses

For survival analyses, a minimum of 40 female flies from the corresponding genotypes were collected and kept at 29°C. Flies were transferred to new fresh nutritive media every second day and scored for deaths daily. Statistical analysis was performed with a log-rank test using the GraphPad Prism5 software.

Cardiac physiological analysis

For the physiological analysis, female flies were collected just after eclosion and were maintained for 7 days at 29°C. For the heart beat recordings, semi-intact heart preparations were made as previously described (Ocorr et al., 2007; Magny et al., 2013). An inverted Leica DM Irbe microscope, connected to a DFC450C Leica digital camera, was used to take 20 s recordings at 29 frames/s. Different cardiac parameters were measured using Fly_heart_analysis (SOHA) software based on Matlab R2009b (MathWorks, Natick, MA, USA) (Ocorr et al., 2007). For the statistical analysis, Student's *t*-test was used with Welch's correction when the variances were different.

Fluorescent immunofluorescence analysis

Fly hearts were dissected from 7-day-old females, fixed for 20 min in 4% paraformaldehyde, and washed in PBT (PBS containing 0.3% Triton X-100). Muscleblind staining and FISH to detect ribonuclear foci were performed as previously described (Llamusi et al., 2013). For double Muscleblind and GFP staining, dissected hearts were washed in PBT and incubated in blocking buffer (PBS containing 0.3% Triton X-100, 5% donkey serum and 0.5% bovine serum albumin) for 30 min prior to overnight incubation at 4°C with primary antibodies sheep-anti-Muscleblind (Houseley et al., 2005) and rabbit anti-GFP (#G10362, Invitrogen) diluted 1/500 in blocking buffer. After several PBT washes, the tissue was incubated for 45 min with biotin-conjugated secondary antibodies (#31840, Thermoscientific) at 1:200 dilution and then incubated with ABC solution (ABC kit, VECTASTAIN) for 30 min at room temperature, followed by washes and 45 min incubation with anti-rabbit FITC (#F9887-5ML, Sigma) secondary antibody and streptavidin–Texas-red (1:1000, #SA5006, VECTOR). For phalloidin staining, phalloidin (#P1951, Sigma) was diluted 1:1000 in PBT and tissues were incubated for 20 min. Samples were mounted in Vectashield (Vector) as described before (Alayari et al., 2009). All confocal images were taken in an Olympus FV1000 microscope.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

B.L. and R.A. conceived and designed the experiments. B.L., M.C. and E.S.-S. performed the experiments and analyzed the data. N.C.-B. contributed the plasmids carrying the CTG repeats. M.P.-A. helped in interpretation of results. J.P.C. and E.M. contributed methods and protocols for data collection and analysis. B.L., R.A., M.C. and E.S.-S. wrote the paper with contributions from all the authors.

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Supplementary information

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Expanded CCUG repeat RNA expression in *Drosophila* heart and muscle trigger Myotonic Dystrophy type 1-like phenotypes and activate autophagocytosis genes

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Myotonic dystrophies (DM1–2) are neuromuscular genetic disorders caused by the pathological expansion of untranslated microsatellites. DM1 and DM2, are caused by expanded CTG repeats in the 3′UTR of the *DMPK* gene and CCTG repeats in the first intron of the *CNBP* gene, respectively. Mutant RNAs containing expanded repeats are retained in the cell nucleus, where they sequester nuclear factors and cause alterations in RNA metabolism. However, for unknown reasons, DM1 is more severe than DM2. To study the differences and similarities in the pathogenesis of DM1 and DM2, we generated model flies by expressing pure expanded CUG ([250]×) or CCUG ([1100]×) repeats, respectively, and compared them with control flies expressing either 20 repeat units or GFP. We observed surprisingly severe muscle reduction and cardiac dysfunction in CCUG-expressing model flies. The muscle and cardiac tissue of both DM1 and DM2 model flies showed DM1-like phenotypes including overexpression of autophagy-related genes, RNA mis-splicing and repeat RNA aggregation in ribonuclear foci along with the Muscleblind protein. These data reveal, for the first time, that expanded non-coding CCUG repeat-RNA has similar *in vivo* toxicity potential as expanded CUG RNA in muscle and heart tissues and suggests that specific, as yet unknown factors, quench CCUG-repeat toxicity in DM2 patients.

Myotonic dystrophy type 1 (DM1) and type 2 (DM2) are dominantly-inherited multi-systemic genetic disorders. DM1 (OMIM: 160900) is caused by an unstable expansion of a CTG trinucleotide repeat motif located in the 3′ untranslated region (UTR) of the *dystrophia myotonica protein kinase* (*DMPK*) gene[1]. Unaffected individuals carry fewer than 37 triplet-repeats, whereas expansions ranging between 50 and 4000 CTG repeats have been found in affected individuals. DM2 (OMIM: 602668), initially named proximal myotonic myopathy due to the greater weakness of proximal compared to distal muscles[2], is caused by a tetranucleotide (CCTG) expansion in intron 1 of the CCHC-type zinc finger nucleic acid binding protein gene (*CNBP*, *also known as ZNF9*)[3]. Healthy individuals carry fewer than 30 tetra-nucleotide repeats, whereas repeat lengths found in affected patients are significantly longer than in DM1 (between 55 and 11000)[3]. In contrast to DM2, which does not have a congenital form, very large (>1,000 repeat) *DMPK* CTG mutations also cause congenital DM1 (CDM) characterized by neonatal hypotonia (floppy baby) and intellectual disability[4]. The expansions are transcribed into (CUG)n and (CCUG)n-containing RNA, respectively, which form secondary structures and sequester RNA-binding proteins, such as the RNA processing factors Muscleblind-like proteins (MBNL1-3 in vertebrates, Muscleblind in *Drosophila*), forming nuclear aggregates known as foci[5–11]. Additional splicing factors, such as CUGBP Elav-like family member 1 (CELF1), are also disrupted, leading to the mis-splicing of a large number of downstream genes[12–14]. Among them, the alteration in the splicing pattern of *CLCN1*, *INR*, *PKM*, *CACNA1S*, and *BIN1*

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pre-mRNAs has been associated with myotonia, insulin resistance, perturbed glucose metabolism and muscle weakness, respectively, which are all symptoms of DM[15–19]. Importantly, the repeat-length extensively correlated with disease severity in DM1[20] and with the amount of MBNL sequestered in both types of DM[5,21]. Although for DM2 the correlation between repeat length and disease severity in humans is less clear-cut, expression of non-coding CCUG-expanded RNA in flies has been shown to cause length-dependent toxicity in *Drosophila* eyes[22].

Clinically, DM2 patients generally experience a milder phenotype than DM1 patients, including slower and less severe progression of the disease, reduced severity of the cardiac involvement with a significant reduction in arrhythmicity and prophylactic pacing requirements, lack of prominent late respiratory or facial and bulbar muscle weakness, less evocable myotonia, and preserved social and cognitive abilities[23–26]. However, the molecular origin of these milder phenotypes in DM2 is unknown. Indeed, several studies have reported that DM2 individuals tend to carry significantly more (75 to approximately 11,000, with a mean of 5,000 CCTG) repeats in mutant alleles compared to patients with CTG expansions (classic DM1 range is 100–1000 repeats)[3] and, according to different sources *CNBP* is 4 to 8-fold more expressed in human muscles than the *DMPK* gene[27–29]. In addition, MBNL binds to CCUG with higher affinity than to CUG repeats[6,30], resulting in larger ribonuclear inclusions in DM2 patients, which sequester more MBNL[21]. Considering that CNBP is expressed at higher levels than DMPK in muscles, and that expanded alleles tend to carry more CTG repeats, as well as the fact that MBNL proteins have higher affinity for CCUG repeats than for CUG RNA, DM2 symptoms should be more severe, rather than milder, than DM1.

To investigate this paradox, we reasoned that the phenotypes brought about by both expansion types in *Drosophila* tissues might be informative. Significantly, weaker phenotypes are expected for CCUG expansions should they be intrinsically less toxic than CUG repeats, whereas similar phenotypes are expected if toxicity is modulated in humans by CCUG-specific factors. With this aim, we generated and characterized *Drosophila* models of DM1 and DM2 expressing pure CUG or CCUG repeats, respectively, in muscular and cardiac tissues. We found common pathogenic events between CUG and CCUG repeat toxicity, such as Mbl sequestration in foci, mis-splicing and increased autophagy in both tissues. Importantly, the severity of the phenotypes in the DM2 flies reveals that CCUG repeat expansions are potentially as toxic as CUG repeats in muscle and heart. Our study therefore suggests that unknown molecular RNA-toxicity modifiers account for the milder symptoms of DM2.

Results

Expression of either CUG or CCUG-expanded repeats sequester Muscleblind in ribonuclear foci in muscle and cardiac tissue. To accurately model DM1 and DM2 in flies, we generated *UAS-CTG* and *UAS-CCTG* transgenic fly lines carrying either 250 CTG (CTG (250)×) or 1100 CCTG (CCTG (1100)×) pure repeats, which are within the pathological range of repeat lengths and mimic the, at least 4 times longer, expansion size in DM2 patients compared with DM1[25,31]. As controls, we generated flies carrying short versions of the repeats (CTG (20)× or CCTG (20)×). In order to express the repeats in different tissues, we crossed the UAS fly lines with the muscle-specific driver myosin heavy chain *Mhc-Gal4*[32] or the cardiac-specific driver *GMH5–Gal4*[33]. The expression level of the repeats was assessed by qPCR using primers against the common SV40 terminator contained in these vectors (Fig. S1).

Fluorescent *in-situ* hybridization (FISH) to detect ribonuclear foci showed that they were present in the nuclei of indirect flight muscle (IFM) and heart cells expressing long CUG or CCUG repeats, but not in flies expressing the short versions of the repeats (Figs 1 and S2). Because Muscleblind sequestration is one of the main features of the disease, we studied Muscleblind subcellular localization in our model flies. As we previously reported, *Drosophila* Muscleblind is found in sarcomeric bands in adult muscle tissue and dispersed throughout the nuclei of cardiomyocytes[34,35]. Muscleblind immunodetection in muscle and heart tissue in flies expressing the short versions of the CUG or CCUG repeats showed that Muscleblind localization was the same as that described in control samples. In contrast, Muscleblind was concentrated in CUG or CCUG ribonuclear foci in muscle and heart cells from flies expressing long CUG or CCUG repeats (Fig. 1). Thus, both expanded CUG and CCUG arrays originate ribonuclear foci and Muscleblind sequestration in *Drosophila* muscle and heart tissue, which are both histological hallmarks of DM.

Muscleblind-dependent splicing is altered in flies expressing expanded CUG or CCUG repeats. To test whether the confirmed Muscleblind retention in foci was enough to cause splicing misregulation, we studied the percentage of exon retention ("*percentage spliced in*", PSI) of the *Drosophila formin* (*Fhos*) gene exon 16′, which has a highly conserved ortholog in human, with altered splicing in DM1 patients[36]. *Fhos* has 19 exons, which produce nine different transcripts (Ensembl Genome browser, release 83). We recently reported that exon 16′ (132 nt) is preferentially included in DM1 model flies expressing 480 interrupted CUG repeats (i(CUG)480) in muscle[37]. Importantly, this splicing event was shown to be Muscleblind-dependent. In control flies, the PSI of *Fhos* exon 16′ was around 50%. However, in flies expressing i(CUG)480 in muscle, this percentage increased to nearly 95%. Consistent with the milder toxic effects reported in DM1 individuals and in animal models with shorter CUG repeats, the inclusion percentage dropped to close 75% in flies expressing 250 CUG repeats in muscle. In the case of flies expressing expanded CCUG repeats in muscle, we also observed increased *Fhos* exon 16′ inclusion, which reached 85%. Importantly, flies expressing short versions of either CUG or CCUG repeats, showed no significant changes in exon usage (Fig. 2A and C). In cardiac tissue, the 50% exon inclusion found in control or short-repeat-expressing flies, increased to 75% in both expanded CUG and CCUG-expressing flies (Fig. 2B and D). We also quantified the inclusion of exon 13 of the Mbl-dependent *Serca* gene, which decreased 50% in the flies expressing the long repeats in muscle, while in heart, resulted into a 50% increase. Accordingly, in previous studies the expression of 480 interrupted CUG repeats in adult flies using the

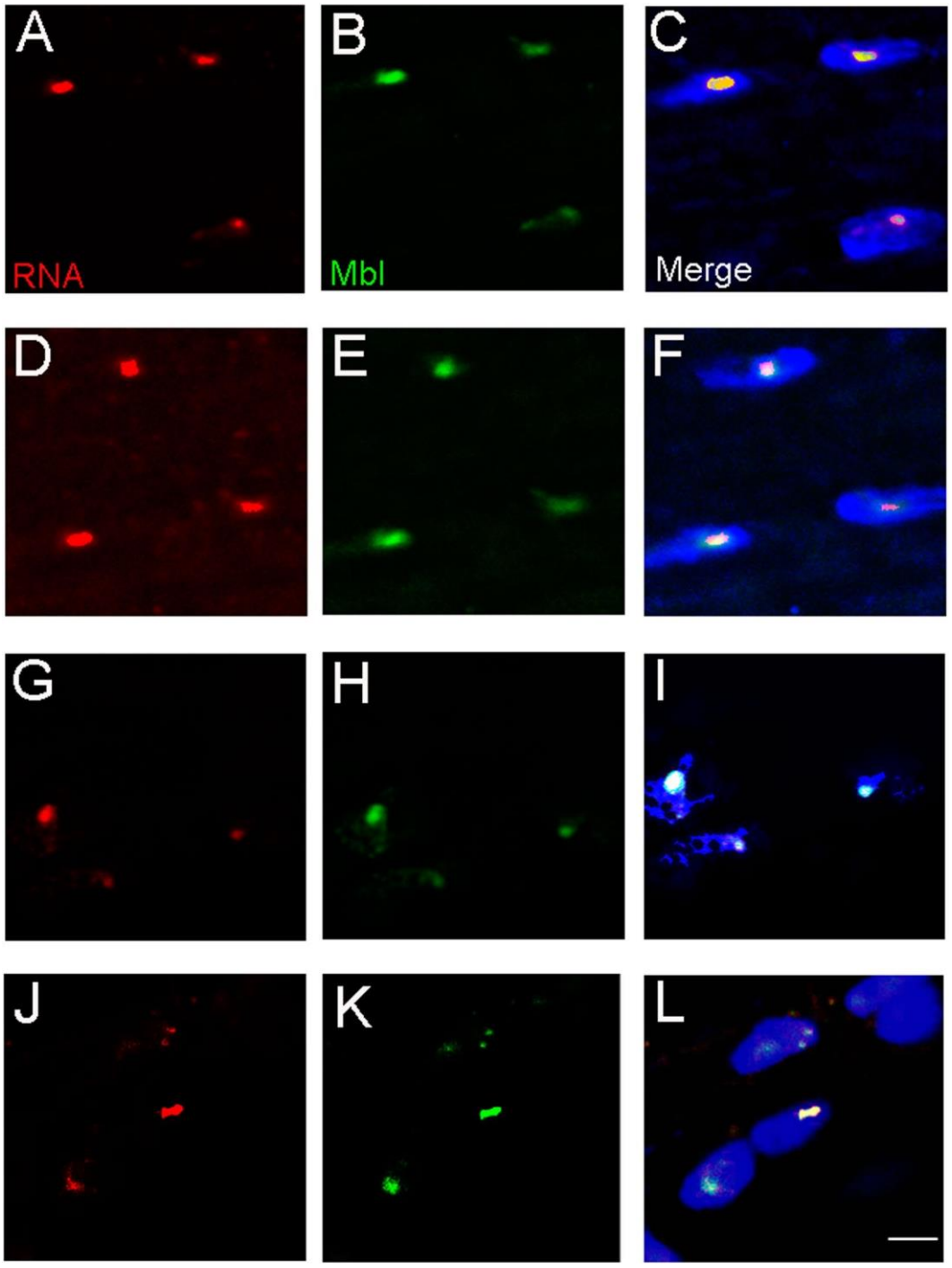

Figure 1. Muscleblind is retained in ribonuclear foci in flies expressing expanded CUG or CCUG repeats. Representative fluorescent confocal images of IFMs (**A**–**F**) and heart cells (**G**–**L**) from flies expressing expanded CUG (**A**–**C** and **G**–**I**) or CCUG (**D**–**F** and **J**–**L**) repeats under the control of the Mhc-Gal4 and GMH5-Gal drivers, respectively. Ribonuclear foci retaining Muscleblind were present in flies expressing long CUG or CCUG repeats. Merged images in (**C**,**F**,**I** and **L**) include DAPI (blue) counterstaining of the nuclei. Scale bar = 10 μm.

late muscle driver Mhc-Gal4 induced a 2.4-fold reduction of *Serca* transcripts with exon 13[37], while the expression of 960 CUG repeats using the Mef-Gal4 driver resulted in increased expression[38], suggesting a remarkable developmental-dependent regulation of this event in flies (Fig. 2E and F).

These data confirmed that the Muscleblind sequestration in ribonuclear foci observed in fly models of DM1 and DM2, led to a functional depletion of Muscleblind in adult muscle and heart tissue.

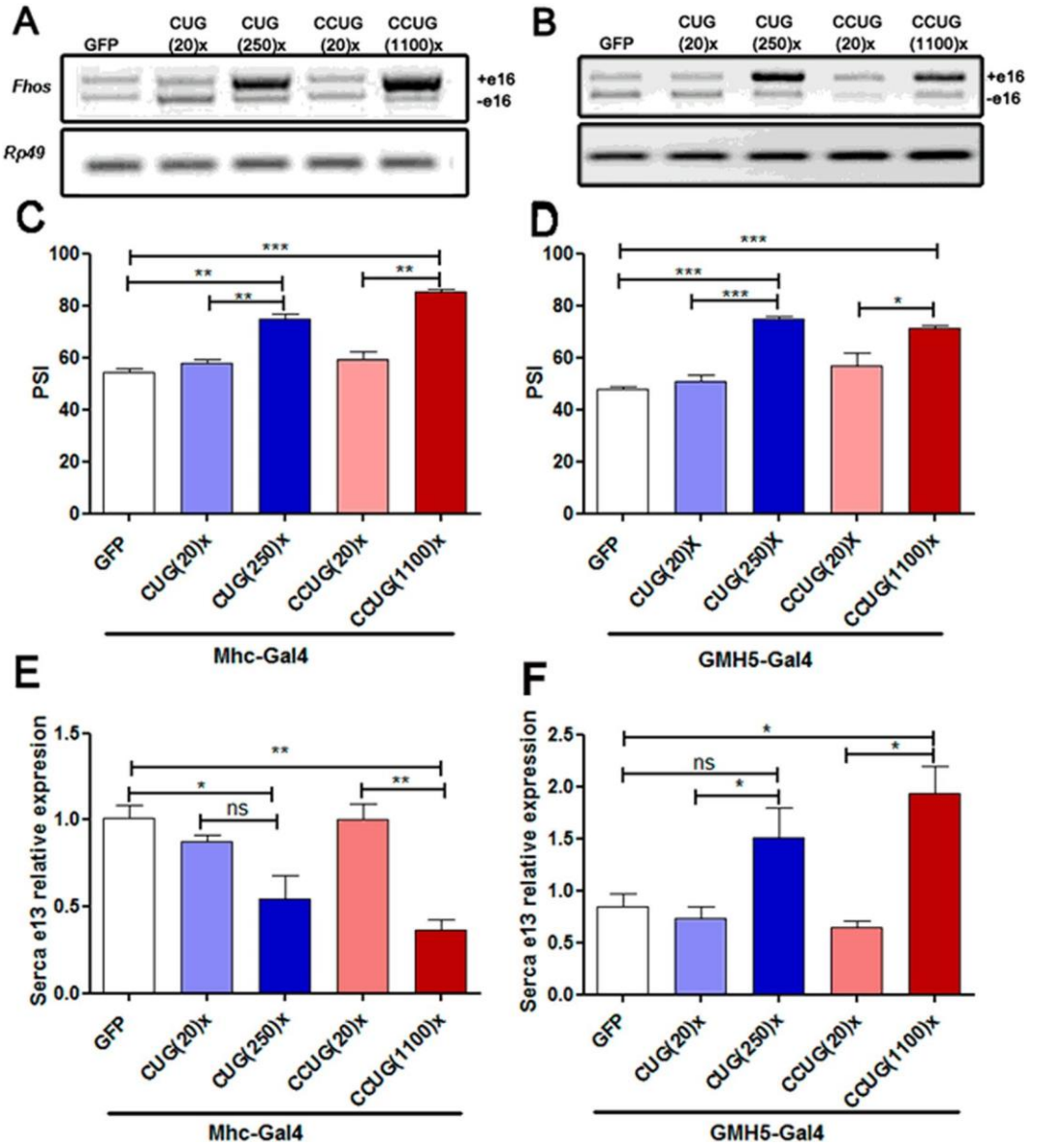

Figure 2. CUG and CCUG expansions cause Muscleblind-dependent missplicing. (**A**,**B**) Representative semi-quantitative RT-PCR showing inclusion of *Fhos* exon 16′ in flies expressing the indicated constructs in muscle (A) or heart (**B**) under the control of Mhc-Gal4 or GMH5-Gal4, respectively. Endogenous *Rp49* was used for normalization. Percentage of exon 16′ inclusion, revealed that expression of long CUG or CCUG repeats in the fly muscle (**C**) or heart (**D**), favored increased use of this exon. qRT-PCR results of *Serca* exon 13 expression relative to *Rp49 expression*, confirmed that the use of this exon in the flies expressing the expanded repeats and the control flies is significantly different in muscle (**E**) and heart tissues (**F**). The histograms show the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t-test).

The expression of autophagy-related genes is increased in muscular and cardiac tissues in DM1 and DM2 model flies. Several studies have reported a pathological over-activation of the autophagy-lysosome pathway in DM1 models. Apoptotic activation and increased presence of autophagy markers has been reported in primary human cell lines from adult-onset DM1 patients[39,40] and in human DM1 embryonic stem cells-derived neural stem cells[41]. In addition, pathway analysis on global PolyA-seq studies of human DM skeletal muscle[42] and brain[43] identified enriched terms associated with ubiquitin-mediated proteolysis and the mTOR pathway. More recently, studies performed in a murine model of DM1 have reported that targeting deregulated AMPK/mTORC1 pathways improves muscle function in DM1[44]. Accordingly, we have previously demonstrated over-activation of apoptosis and autophagy by inducible expression of 480 interrupted CUG repeats in *Drosophila* adults and a rescue of muscle atrophy by silencing the expression of the autophagy-related genes Atg4, Atg7, Atg8a and Atg9[34]. To study the expression of autophagy-related genes in our DM1 and DM2 *Drosophila* models, we performed qPCRs with cDNAs from heart and thorax samples of flies expressing short and long versions of the CUG or CCUG repeats in heart and muscle (Fig. 3). In general, we found that expression of Atg4,

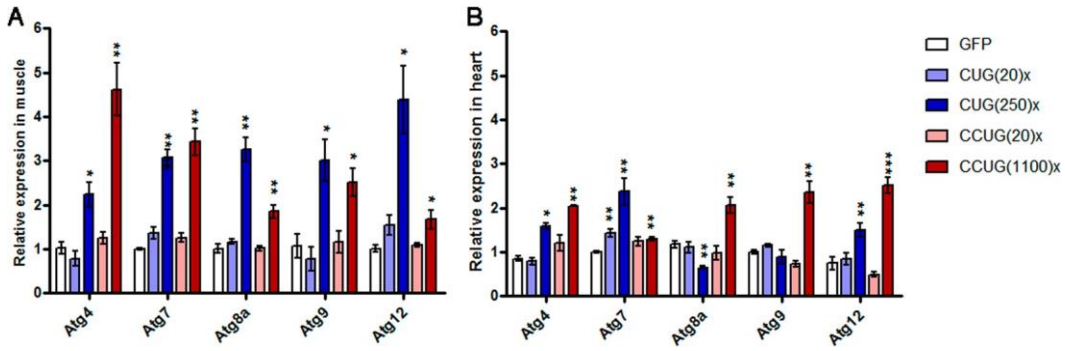

Figure 3. The expression of autophagy-related genes is upregulated in flies expressing expanded CUG or CCUG repeats in muscle or heart. Relative expression levels of *Atg4*, *Atg7*, *Atg8a*, *Atg9* and *Atg12* measured by qRT-PCR in muscle (Mhc-Gal4 driver; **A**) and heart samples (GMH5-Gal4 driver; **B**), showed a significant upregulation of these autophagy-related genes in flies expressing expanded CUG (CUG(250)×) or CCUG repeats (CCUG(1100)×). The histograms show the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student's t-test).

Atg7, Atg8a, Atg9 and Atg12 were significantly upregulated in flies expressing either expanded CUG or CCUG repeats in muscle, compared to control flies expressing GFP or short repeats. Of note, the expression levels of these genes in flies expressing short CUG or CCUG repeats were similar to the levels in control flies that did not express the repeats (Fig. 3A). In comparison, the expression of the repeats in heart caused a moderate upregulation of *Atg* genes expression, in the case of flies expressing the long repeats. Upregulation of Atg genes mediated by the repeats was higher in the flies expressing CCUGs compared to those expressing CUG repeats (Fig. 3B). Consistent with these findings, we observed upregulation of *AKT2*, *AKT1S1* and *ATG4* mRNAs in human patient skeletal muscle[34]. These data support a role of autophagy activation in DM pathogenesis not only in DM1, as we previously reported, but also in DM2. In addition, our results highlight the relevance of the activation of this pathway in different tissues affected by repeat expression.

Both expanded CUG and CCUG repeat RNA reduced cross-sectional muscle area and fly survival. Despite the fact that MBNL1 is sequestered in CCUG foci and it is expected that the longer CCUG repeat expansions will have a greater inhibitory effect on MBNL1 in DM2 cells, visible muscle atrophy in DM2 muscle is actually milder than in DM1 patients[45]. To investigate how *Drosophila* muscle responds to expanded CCUG repeat RNA, we quantified the cross-sectional muscle area of IFMs from adult flies at different ages that expressed 250 CUG or 1100 CCUG repeats, or controls expressing 20 units or the GFP reporter, under the control of the Mhc-Gal4 driver. We observed a significant reduction in muscle area in 3-day-old flies expressing long CUG or CCUG repeat RNA, whereas cross-sectional muscle area in flies expressing the short versions of the repeats were not significantly different from control GFP-expressing flies. Importantly, flies expressing either long CUG or CCUG repeats showed similar muscle phenotype, which reached up to a 50% reduction in muscle area in both cases (Fig. 4A–E and K). Similarly, muscle area in aged flies (30-day-old flies) expressing expanded repeats was reduced in comparison to aged GFP flies. The decrease in the muscle area in young and aged flies was similar in all the genotypes studied (around 20%) suggesting that the strong muscle reduction observed in the model flies had an important developmental component. Nevertheless, we observed vacuolization, splitting muscles and occasional absence of muscle packages, characteristic of degenerating muscles[46], which were only present in aged flies expressing the expanded CUG or CCUG repeats (Fig. 4F–K). Taken together, these results suggests that toxic RNAs interfere with both muscle development and muscle maintenance.

Population studies have reported higher mortality and morbidity rates, and a positive correlation between the age at onset of DM1 and age at death in patients[47,48]. Similarly, we observed that the lifespan and mean survival of flies expressing expanded CUG or CCUG repeat RNA was significantly reduced in comparison to control flies expressing only GFP, whereas the lifespan of flies expressing 20 units of the repeats was not significantly different from the control flies (Fig. 5A). These results are consistent with our previous description of muscle loss, degeneration and reduced viability of flies expressing i(CTG)480 throughout the fly musculature[34,49]. Taken together our data indicate that the expression of expanded CUG or CCUG repeats in muscle causes similar defects in the IFMs of young and aged flies, and in the viability of *Drosophila*.

Locomotor performance is compromised in flies expressing expanded CUG or CCUG repeat RNA in muscle. To test whether the muscle loss observed in the model flies was of functional relevance, we assessed the flight and climbing ability of flies expressing the expanded repeats and compared them to control flies expressing GFP or short repeats. Climbing velocity and landing distance were only reduced in flies expressing the expanded versions of the repeats and no significant differences were observed between DM1 and DM2 model flies. Of note, these functional parameters were not altered in flies expressing the short versions of the repeats compared to the controls. In the case of climbing velocity, flies expressing the long CUG or CCUG repeats retained 70% of the control-fly climbing speed, and there was no significant difference in velocity between these two genotypes (Fig. 5B). The average landing height was reduced to 25% compared to control flies expressing

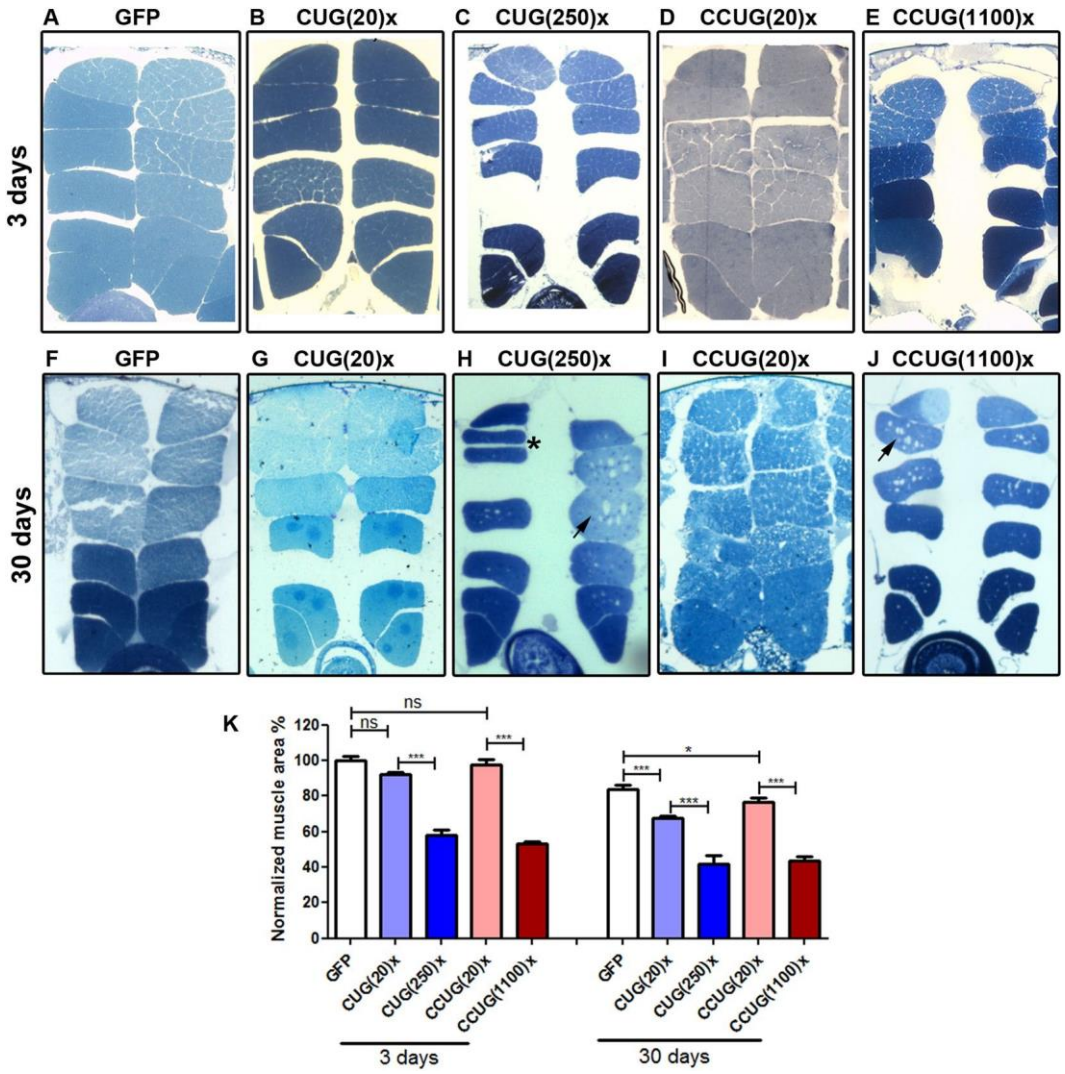

Figure 4. Expression of expanded CUG or CCUG repeats in muscle induces similar levels of muscle area reduction and degeneration. (**A**–**J**) Dorsoventral sections of resin-embedded fly thoraces. In all images the dorsal side is displayed at the top. Mhc-Gal4 was used to drive the expression of the indicated constructs in muscle. (**K**) Quantification of the mean percentage of muscle area per genotype relative to the muscle area of the control flies (GFP), which is considered as 100%. While young flies (3 day-old, in **A**–**E**) expressing 20 CUG or CCUG repeats were not different from control flies expressing GFP, flies expressing expanded CUG or CCUG repeats have a 50% reduction in IFM muscle area. All aged flies (30 day-old, in **F**–**J**) displayed reduced muscle area compared to young flies of the same genotype. However, vacuolization (arrows) and occasional muscle splitting (asterisk) characteristic of degenerating muscles were present only in muscles expressing expanded repeats. The graph shows the means ± *SEM*. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t-test).

GFP or 20 units of the repeats, and was similar in flies expressing either expanded CUG or CCUG repeat RNA (Fig. 5C). Thus, in contrast to human patients, where DM2 muscle disability is milder than in DM1, these data indicate that expression of long CUG or CCUG repeat RNA in muscle tissue has a similar effect on locomotion in flies.

Heart dysfunction in both DM1 and DM2 model flies includes systolic and diastolic alterations, arrhythmia, and contractility defects. Cardiac alterations, characterized by conduction delays, arrhythmia, and heart blockage are the second most common cause of death in DMs[50]. In DM2, cardiac abnormalities have been reported to be similar to those described in DM1 but less frequent and severe[24]. To study heart function in the *Drosophila* DM models, adult fly hearts were dissected in artificial hemolymph and recorded with a high-speed video camera. Cardiac contractions were analyzed using a semi-automatic optical heartbeat analysis (SOHA) method to quantify the fly heart functional parameters[51]. The study of heart function in DM2 model flies revealed that expression of long CCUG repeats in fly heart caused lengthening of the heart period (HP), and extension of the systolic and diastolic intervals (SI and DI, respectively). Heart contraction, measured as a percentage of fractional shortening (%FS), and arrhythmicity measured using the arrhythmia index (AI), were

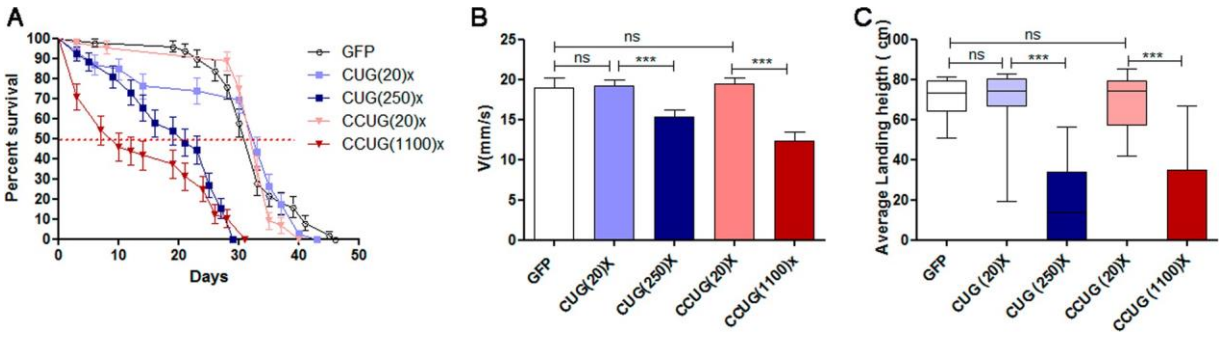

Figure 5. Survival and locomotor function were reduced in flies expressing expanded CUG or CCUG repeats in muscle. (**A**) Average percentage of live flies versus age (in days). The Mhc-Gal4 driver was used to induce the expression of the indicated constructs in muscle. The horizontal dotted line marks the median survival. Whereas control and short-repeat-expressing flies had similar median survival (GFP; n = 90, CUG(20)×, n = 100 and CCUG(20)×, n = 95), long CUG and CCUG-expressing flies have reduced survival (CUG(250)×; n = 95 and CCUG(1100)×; n = 100). Differences in the survival curves were highly significant ($p < 0.0001$, log-rank test). (**B**) Histogram showing the climbing speed as the mean speed ± *SEM* in mm/s. Flies expressing long CUG or CCUG repeats had reduced climbing velocity compared to control flies or flies expressing the short versions of repeats. (**C**) Notched box plot showing the median and the distribution of the average landing height data obtained in the flight assay with the relevant genotypes. Flight disability was observed in flies expressing long CUG or CCUG repeats. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t-test).

significantly altered compared to controls. In these model flies, the %FS was reduced to 20% and AI increased by around 3-fold (Fig. 6D,E). Importantly, the expression of short CCUG repeats did not affect %FS or AI but it increased the SI compared to controls, and resulted in a significantly increased HP (Fig. 6A–C). Similarly, we previously reported, that overexpression of expanded CUG repeats *in Drosophila* heart results in an increased HP with prolonged DI and SI, a reduction in %FS, and increased AI. In contrast, the expression of short CUG repeats only produced a slight increase in the SI duration[35].

Expression of expanded CUG or CCUG repeat RNA in fly heart reduces survival but does not affect locomotion. We previously reported that overexpression of long CUG repeats in fly heart results in a reduction in mean survival and lifespan[35]. The mean survival in control flies expressing GFP was 29 days which was reduced to about half in the DM1 model flies. The survival curve for flies expressing CCUG repeats in heart tissue was also significantly reduced compared to the GFP control flies. Of note, the survival curve of flies expressing short CUG or CCUG repeats was similar to that of control flies (Fig. 7A). These data suggest that the cardiac alterations in our DM1 and DM2 models affect the survival of flies.

To assess whether the expression of repeats in heart affects locomotor performance in flies, we analyzed the climbing velocity and landing distance of flies expressing CUG or CCUG repeats and found that neither the expression of short nor long versions of CUG or CCUG repeats affected these abilities (Fig. 7B,C). Thus, the reduction in %FS did not affect acute workload demands (flight, and climbing), but did have an accumulative detrimental effect on survival.

Materials and Methods

Drosophila strains. Pure expanded CTG and CCTG repeats were generated by PCR amplification of self-priming single-stranded CTG and CAG or CCTG and CAGG oligonucleotides as previously described[52]. Synthesized DNA duplexes were electrophoresed, size fractionated, purified using a DNA gel extraction kit (Qiagen), 5′-phosphorylated with T4 polynucleotide kinase, and cloned into the *Eco*RV site of pUAST. The recombinant plasmids containing uninterrupted stretches of CTG or CCTG repeats were amplified in STBL3 *E. coli* (Invitrogen) at 20 °C. Plasmid DNA was purified using a Qiagen plasmid DNA purification kit and sequenced from both ends to ensure the sequence integrity of the clones. Transgenic flies were generated by injecting the plasmids into w^{1118} embryos by BestGene Inc. following the method described in ref. 53. UAS-GFP strain was obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). The cardiomyocyte-specific driver *GMH5–Gal4* was kindly provided by Dr. Bodmer from the Sanford Burnham Institute, California, USA[33]. The Mhc-GAL4 line was previously described[32]. Mhc-Gal4 drives expression in terminally differentiated muscle under the control of endogenous myosin heavy chain regulatory regions, while GMH5-Gal4 is expressed in cardiomyocytes initially driven by a 900 nt *tinman* heart enhancer and later maintained by a *UAS-Gal4* autoregulatory loop[33]. All the fly lines were maintained in standard *Drosophila* food. The flies were grown at 25 °C to study the effect of expressing repeats throughout the musculature and at 29 °C to study the cardiac defects. Expression levels of the different transgenes were assessed as previously described[35].

Cardiac physiological analysis. For the physiological analysis, female flies were collected just after eclosion and were maintained for 7 days at 29 °C. For the heart-beat recordings, semi-intact heart preparations were made as previously described[54,55]. An Leica DFC 450C microscope, connected to an ORCA Flash (Hamamatsu)

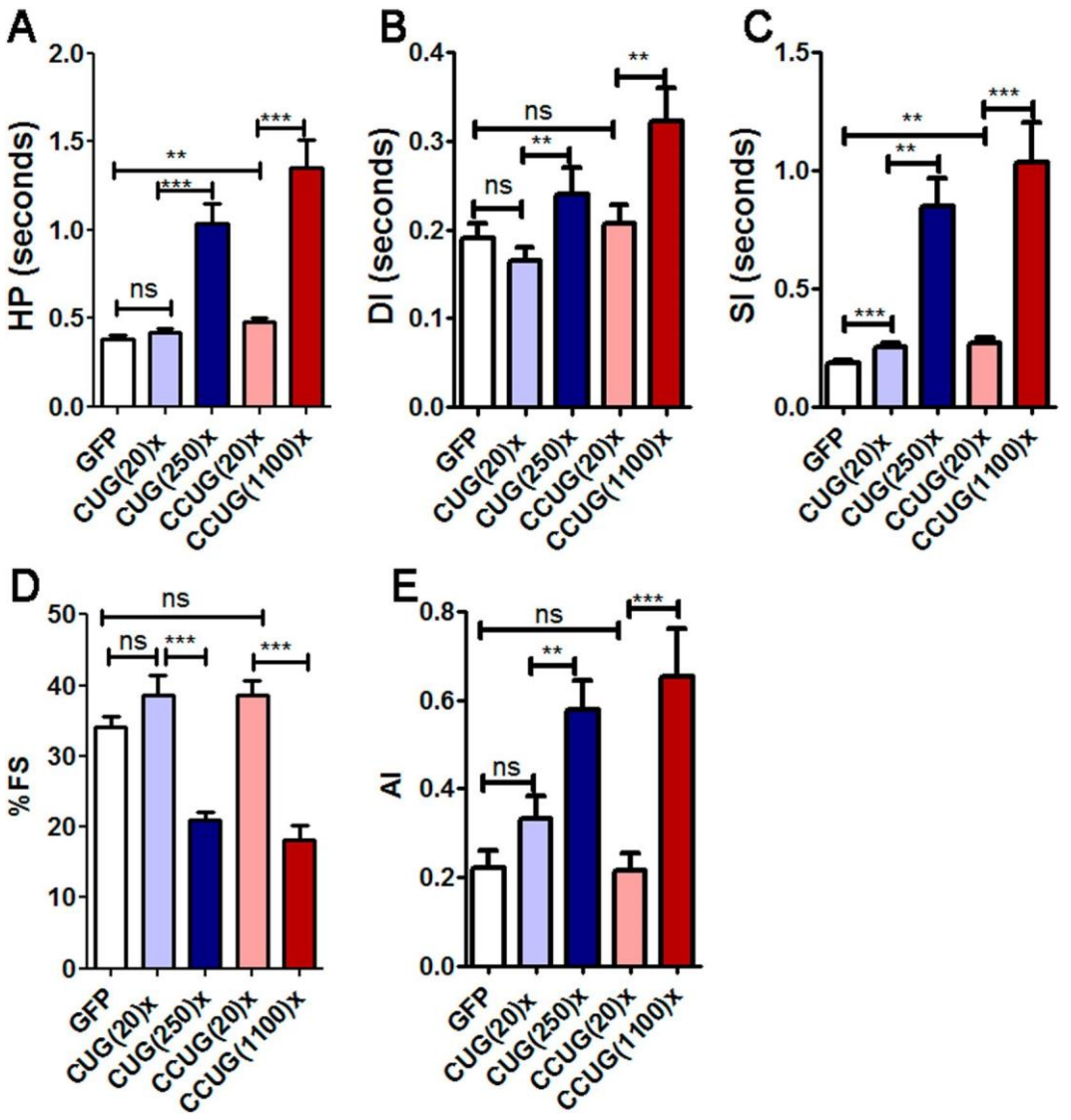

Figure 6. Cardiac dysfunction in DM1 and DM2 model flies includes diastolic and systolic elongation, increased arrhythmicity, and reduced contractility. The mean heart period (HP, in **A**) was significantly increased in flies expressing expanded CUG or CCUG repeats in heart. This increase was caused by a prolongation of both diastolic and systolic intervals (DI, in **B** and SI, in **C**) in the model flies. Heart tube contractility and cardiac rhythm were also affected in these flies, because the percentage of fractional shortening (%FS in **D**) was reduced to only 20% and arrhythmia, measured as the arrhythmicity index (AI in **E**), was significantly increased to similar levels in both DM1 and DM2 model flies. Graph bars show the mean values and their standard errors (n= 18 to 29). *p< 0.05, ** $p < 0.01$, *** $p < 0.001$ (Student's t-test).

high-speed digital camera was used to take 20 s recordings at a minimum speed of 150 frames/s. Different cardiac parameters were measured using SOHA software[51].

Histological analysis. Analysis of the IFM area in *Drosophila* thoraces was performed as previously described[56]. Briefly, six thoraces from three-day or thirty-day-old (aged group) females were embedded in Epon following standard procedures. After drying the resin, semi-thin 1.5 µm-sections were obtained using an ultramicrotome (Ultracut E, Reichert-Jung and Leica). Images were taken at 100× magnification with a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany). To quantify the muscle area, five images containing IFMs per fly were converted into binary images. Considering the complete image as 100% of the area, we used ImageJ software to calculate the percentage occupied by pixels corresponding to the IFMs. The percentage of pixels occupied by muscle in the control GFP flies were considered as 100%, and the percentage of muscle area of the rest of genotypes were normalized to these control flies.

For immunofluorescence analysis, dissected fly hearts or *Drosophila* thorax longitudinal sections were fixed for 20 min in 4% paraformaldehyde, and washed in PBT (PBS containing 0.3% Triton X-100) before staining. Muscleblind staining, and FISH to detect ribonuclear CUG foci, were performed as previously described[56]. The

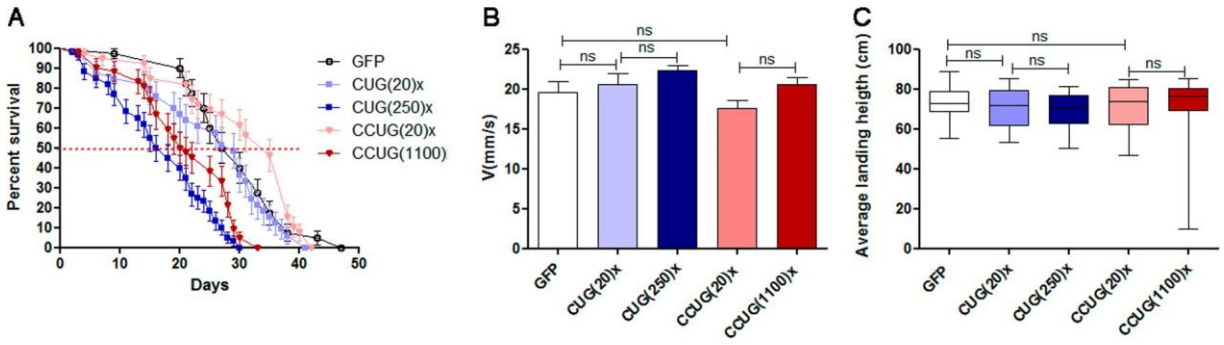

Figure 7. Expression of expanded CUG or CCUG repeats in fly heart alters survival but not locomotion. (**A**) Average percentage of live flies, with the indicated genotypes, versus age (in days). The *GMH5-Gal4* driver was used to induce expression of the indicated genotypes in cardiomyocytes. Horizontal dotted line marks the median survival. Flies expressing expanded CUG (CUG(250)×; n = 100) or CCUG (CCUG(1100)×; n = 97) repeats had a reduced lifespan compared to control flies (GFP; n = 100) or flies expressing the short versions of the repeats (CUG(20)×; n = 95 and CCUG(20)×; n = 99). The differences in survival curves were highly significant ($p < 0.0001$, log-rank test). (**B**) Histogram showing the climbing velocity of flies as the mean speed ± *SEM* in mm/s. Expression of long CUG or CCUG repeats in heart did not modify climbing velocity compared to control flies or flies expressing the short versions of repeats. (**C**) Notched box plot showing the median and the distribution of the average landing height data obtained in the flight assay using flies with the genotypes indicated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t -test).

specificity of the anti-Mbl antibody has been previously tested in ref. 56. To detect CCUG foci a variation of the FISH protocol was implemented using a locked nucleic acid (LNA) probe with 7 CAGG repeats (Sigma), which was hybridized at 55 °C. All the confocal images were taken with an Olympus FV1000 microscope.

RNA extraction, RT-PCR and qRT-PCR. For each biological replicate, total RNA was extracted using Trizol (Sigma) from 10 five-day old adult males for the muscle studies and 20 seven-day old adult female hearts for the cardiac studies. One microgram of RNA was digested with DNase I (Invitrogen) and reverse-transcribed with SuperScript II (Invitrogen) using random hexanucleotides. 20 ng of cDNA were used in a standard PCR reaction with GoTaq polymerase (Promega) and specific primers to analyze *Fhos* exon 16′ inclusion (Table S1). *Rp49* was used as endogenous control using 0.2 ng of cDNA. qRT-PCR to analyze *Serca* exon 13 and *Atg 4*, *Atg7*, *Atg8a*, *Atg9* and *Atg12* expression levels was carried out from 2 ng of cDNA template with SYBR Green PCR Master Mix (Applied Biosystems) and specific primers (Table S1). For reference gene, *Rp49*, qRT-PCR was carried out from 0.2 ng of cDNA. Thermal cycling was performed in Step One Plus Real Time PCR System (Applied Biosystems). Three biological replicates and three technical replicates per biological sample were carried out. Relative expression to endogenous gene and the control group was obtained by the $2^{-\Delta\Delta Ct}$ method. Pairs of samples were compared using two-tailed t-test ($\alpha = 0.05$), applying Welch's correction when necessary.

Survival curves. Survival experiments were performed independently twice with a minimum of 45 flies each time. Flies were maintained at 25 °C for experiments involving Mhc-Gal4 and at 29 °C for the GMH5-Gal4 driver. The flies were transferred to new fresh nutritive media every second day and scored for deaths daily.

Flight and climbing functional assays. Given the heterogeneity generally found in the functional assays performed with female flies, we only used males in these experiments. Flight assays were performed on day five as described previously[57] using 100 flies per group. To assess climbing velocity, groups of 15, five-day-old males were transferred into 25 cm long, 1.5 cm diameter pipettes, after a period of 24 h without anesthesia. The height reached from the bottom of the vial by each fly in a period of 10 s was recorded with a camera. For each genotype, approximately 30 flies were tested.

Statistical analysis. Statistical analysis was performed using GraphPad Prism5 software. Pairs of samples were compared using a two-tailed Student's t-test ($\alpha = 0.05$), applying Welch correction when necessary. The survival curves used a minimum of 90 individuals and a log-rank test was used to assess whether there were any significant differences between them. The flight assay data are represented as a notched box plot, which includes the median and the distribution of the average landing heights obtained; the horizontal lines inside the boxes represent the median values, the bottom and top edges of the boxes represent the 25th and 75th percentiles, and bottom and top whiskers reach the 10th and 90th percentiles, respectively.

Discussion

A significant feature of DM is that two different microsatellite expansions in two unrelated genes cause a clinically similar disease. The histological features of skeletal muscle biopsies taken from DM1 and DM2 patients are very similar[50,58]. In both diseases, affected muscles show central nuclei, a reduction in the number and diameter of specific fiber types, fibrosis and adipose deposition. DM2 is specifically characterized by the presence of atrophic fibers with nuclear clumps even before the muscle weakness appearance as well as by a predominant type 2 fiber

atrophy[59, 60]. In DM2, cardiac abnormalities have also been reported to be similar to those described in DM1, including conduction disturbances, cardiac arrhythmias and sudden death[26, 61–63]. Similarly, the characteristic features of DM that we describe in our DM2 (CCUG-repeat bearing) flies, including muscle and locomotor defects, cardiac dysfunction and reduced survival, were very similar to the characteristics of flies expressing CUG repeats. Interestingly, the phenotypic similarities between our DM1 and DM2 model flies go beyond phenotypes to the pathogenesis mechanisms. We showed that Mbl retention in foci resulting in missplicing, and autophagy activation are common to both diseases. We report that autophagy-related genes are upregulated not only in muscle, but also in heart in both models, suggesting that this is not a tissue-specific mechanism of repeat toxicity. Some important differences between both models are also highlighted in our study. The effect of the expression of long CCUG repeats in heart was more pronounced than that of long CUG repeats, and correlated with stronger upregulation of autophagy-related factors. These data suggest the existence of unknown tissue-dependent factors that might modulate the toxicity of CUG and CCUG repeats. The difference between the expression level of autophagy-related factors in control or expanded repeat-expressing flies was higher in muscle compared to heart samples, suggesting that autophagy is importantly involved in pathogenesis in this tissue. The autophagy activation in the DM1 and DM2 model flies coincides in muscle with strong muscle area reduction in the flies expressing the long versions of the repeats. These data are consistent with our previous results in the model flies expressing 480 CUG repeats[34]. Moreover, our experiments with young and aged flies have shown that muscle defects caused by expanded CCUG repeats have not only a developmental contribution but may also impinge on adult muscle maintenance and/or degeneration, as we have previously shown with heat-shock-induced expression of CUG expansions exclusively in adult muscle[34]. Importantly, this is the first DM2 animal model showing obvious muscle phenotypes.

As a result of expanded repeats expression in heart, we observed systolic and diastolic dysfunction, reduction of the fractional shortening and increased arrhythmicity in DM2 model flies, which resembled the DM1-like phenotype previously described in flies[35] and in DM patients[64]. Importantly, SI and DI were more affected by CCUG repeats than by CUG repeats expression. Accordingly, in heart tissue, the expression of short repeats produced a slight but significant prolongation in the systolic interval, which was more pronounced in the case of CCUG-expressing flies. Remarkably, the expression of short versions of repeats did not induce Muscleblind sequestration in foci in IFM or heart tissue. Therefore, the phenotypes observed in these flies might be independent of Muscleblind, and the factors originating the phenotype seem to be more sensitive to CCUG repeats than to CUG repeats.

An open question in the field of DM is to clarify the pathomechanisms underlying the phenotypic differences between DM1 and DM2. Several studies have confirmed that the frequency and severity of cardiac involvement and of muscle weakness are reduced in DM2 compared to DM1 and that progression is slower and less severe in DM2[24, 26]. This suggests that other cellular and molecular pathways are involved besides the shared toxic-RNA gain of function in the human disease phenotype. Three factors have been shown to influence the level of toxicity of expanded repeats in the RNA; expression level, length, and sequence[21, 65, 66]. Longer sequences tend to cause severe pathogenesis but depending on the sequence, RNA binding factors might be differentially affected. Importantly, in DM2 patients, the severity of the disease has not been directly correlated with the repeat number, only a relationship between repeat lengths and MBNL1 rate of sequestration has been established[21]. In flies, however, a previous report showing the effect in eye of the expression of pure, uninterrupted CCUG-repeat expansions ranging from 16 to 720 repeats in length, has shown a nice correlation between length and toxicity of the CCUG repeats[22]. We believe that this previous observation, and our own reports of similar phenotypes in flies expressing either expanded CUG or CCUG repeats, suggest the existence of unknown modifiers in humans, which might quench RNA toxicity in DM2 patients.

In our flies expressing 250 CUG repeats we observed very similar phenotypes but milder than the ones previously reported by expressing 480 interrupted CUG repeats in muscle[34, 49]. Our data suggests that these phenotypes are sensitive to CUG repeat length, a main feature of DM1, and suggest that the phenotypes described in the previous model were not significantly affected by interrupting sequences.

The experiments expressing the CUG or CCUG repeats in a non-human context in *Drosophila* provide evidence of the strong toxicity potential of the CCUG repeats, as the phenotypes we report in the DM2 model flies expressing the repeats in muscle or heart, are as strong as the phenotypes obtained from expressing the CUG repeats. Disease-specific manifestations may then result from factors that are extrinsic to the repeats and previous evidence suggested several hypotheses. Disease-specific manifestations may result from differences in spatial and temporal expression patterns of *DMPK* and *CNBP* genes. Similarly, changes in the expression of neighboring genes may define disease-specific manifestations. It was recently reported that CUGBP1 protein is overexpressed in muscle biopsies from patients affected by the adult classical form of DM1 but not in muscle from DM2 patients, suggesting that CUGBP1 overexpression in DM1 might be an additional pathogenic mechanism not shared by DM2[67]. Another possible explanation for the clinical differences between the two DM forms is the reduction of DMPK or ZNF9 protein levels in DM1 and DM2 respectively[68–70]. However, *Dmpk* knockout young mice do not develop a multisystemic phenotype mimicking myotonic dystrophy[71]. On the contrary, reduction of *CNBP* levels is sufficient to produce multiorgan symptoms resembling those of DM as observed in heterozygous Cnbp +/− knockout mice[72] implying that CNBP may well play a role in DM2 pathology[21]. According to different sources, *CNBP* is 4 to 8-fold more expressed in human muscles than the *DMPK* gene[27–29], which makes it difficult to explain the phenotypic differences between DM1 and DM2 based on the small reductions in *CNBP* expression reported in DM2 patients. Another important difference between CUG and CCUG expansions is that MBNL has been reported to bind CCUG repeats with a stronger affinity compared to CUG repeats[6, 30]. In addition, the ribonuclear inclusions in DM2 patients appear to be larger than in DM1 patients, and sequester more MBNL[21]. Accordingly, our results in the DM model flies show that, at least in muscle, flies expressing expanded CCUG repeats tend to have higher levels of missplicing, suggesting a reduced activity of Mbl. However, the muscle

defects in DM1 and DM2 model flies were similar, suggesting that Mbl involvement in muscle phenotype is already limiting in DM1 model flies and decreasing levels of Mbl would not result in stronger phenotype.

In conclusion, through this demonstration of CUG and CCUG repeat-induced toxicity in different fly tissues we have gained a useful insight into the differences and similarities in the mechanism of DM pathogenesis in these tissues. The dual system we report (DM1 vs DM2 fly model) with well-characterized repeat expression, resulting phenotypes and molecular alterations, will also be useful to compare the effect of potential chemical or genetic modifiers of RNA toxicity on each of these diseases. The potential discovery of genetic modifiers that affect only one of the components in flies, either CUG or CCUG toxicity, could explain the clinical differences between both human diseases, contributing to increase the knowledge about their pathogenesis pathways and towards the development of new treatments.

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Author Contributions

B.L.L., R.A. and M.P.A. conceived and designed the experiments. B.L.L., E.C.H., and M.C. performed the experiments and analyzed the data. B.L.L. and R.A. wrote the paper with contributions from all the authors.

Additional Information

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CORRECTION

Correction: Daunorubicin reduces MBNL1 sequestration caused by CUG-repeat expansion and rescues cardiac dysfunctions in a *Drosophila* model of myotonic dystrophy (doi: 10.1242/dmm.032557)

Mouli Chakraborty, Chantal Sellier, Michel Ney, Pascal Villa, Nicolas Charlet-Berguerand, Ruben Artero, Beatriz Llamusi

There was an error published in *Dis. Model Mech.* 11, dmm032557 (doi: 10.1242/dmm.032557)

The name of the co-author Pascal Villa was presented incorrectly. It has now been corrected and the original article changed correspondingly.

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RESEARCH ARTICLE

Daunorubicin reduces MBNL1 sequestration caused by CUG-repeat expansion and rescues cardiac dysfunctions in a *Drosophila* model of myotonic dystrophy

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ABSTRACT

Myotonic dystrophy (DM) is a dominantly inherited neuromuscular disorder caused by expression of mutant myotonin-protein kinase (*DMPK*) transcripts containing expanded CUG repeats. Pathogenic *DMPK* RNA sequesters the muscleblind-like (MBNL) proteins, causing alterations in metabolism of various RNAs. Cardiac dysfunction represents the second most common cause of death in DM type 1 (DM1) patients. However, the contribution of MBNL sequestration in DM1 cardiac dysfunction is unclear. We overexpressed *Muscleblind* (*Mbl*), the *Drosophila MBNL* orthologue, in cardiomyocytes of DM1 model flies and observed a rescue of heart dysfunctions, which are characteristic of these model flies and resemble cardiac defects observed in patients. We also identified a drug – daunorubicin hydrochloride – that directly binds to CUG repeats and alleviates *Mbl* sequestration in *Drosophila* DM1 cardiomyocytes, resulting in mis-splicing rescue and cardiac function recovery. These results demonstrate the relevance of Mbl sequestration caused by expanded-CUG-repeat RNA in cardiac dysfunctions in DM1, and highlight the potential of strategies aimed at inhibiting this protein-RNA interaction to recover normal cardiac function.

KEY WORDS: Daunorubicin, *Drosophila*, Muscleblind, Myotonic dystrophy, Trinucleotide repeat disorder

INTRODUCTION

Myotonic dystrophy type 1 [DM1; Online Mendelian Inheritance of Man (OMIM) 160900] is the most common muscular dystrophy in adults (Harper, 2001; Smith and Gutmann, 2016). Presently, DM1 has no effective treatment, but several therapeutic options are being explored (Rzuczek et al., 2017; Gao et al., 2016; Bisset et al., 2015; Konieczny et al., 2017). Although DM1 mainly affects skeletal muscle, cardiac involvement occurs in 80% of DM1 patients and represents the second most common cause of death after respiratory failure (Vinereanu et al., 2004). Three interrelated cardiac phenotypes are observed in individuals with DM1. The first are conduction defects, which can progress to complete heart blockage (Nguyen et al., 1988). The second is the development of potentially fatal ventricular and/or atrial arrhythmias (Nigro et al., 2012; Benhayon et al., 2015). The third type of cardiac dysfunction observed in DM1, although rarer, is mechanical diastolic and/or systolic dysfunction that can progress to combined systolic and diastolic heart failure (Phillips and Harper, 1997; Mathieu et al., 1999; Lazarus et al., 2002; Groh et al., 2008). The genetic cause of DM1 is an expansion of CTG repeats in the 3′UTR of the *DMPK* gene (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). This microsatellite expansion is transcribed into mutant *DMPK* mRNA that contains hundreds to thousands of CUG repeats that are toxic through dysfunction of at least two RNA-binding proteins. The muscleblind-like (MBNL) family of proteins, comprising MBNL1, MBNL2 and MBNL3, normally bind to YGC (Y stands for pyrimidine) RNA motifs and are diverted away from their normal RNA targets by the expanded CUG RNA repeats in *DMPK* transcripts (Miller et al., 2000; Mankodi et al., 2001; Fardaei et al., 2002; Ho et al., 2004; Lin et al., 2006; Wang et al., 2015). Furthermore, expanded CUG repeats also induce hyperphosphorylation and increase stabilization of CUG-binding protein 1 (CELF1; also named CUGBP1) (Kuyumcu-Martinez et al., 2007). As a result of disrupting the function of these proteins, various mis-regulations in RNA metabolism have been described in individuals with DM1, some of which are associated with specific symptoms of the disease (Mankodi et al., 2002; Savkur et al., 2001; Tang et al., 2012; Fugier et al., 2011; Freyermuth et al., 2016).

Reduced MBNL function appears to be a key pathogenic event underlying the skeletal muscle alterations observed in DM1 (Kanadia et al., 2003; Nakamori et al., 2013; Wagner et al., 2016). Indeed, overexpression of MBNL1 rescues DM1-like skeletal muscle alterations in a mouse model of DM1 (Kanadia et al., 2006). Furthermore, compounds or strategies aimed at reducing MBNL1 binding to expanded CUG repeats alleviate skeletal muscle dysfunctions in cell and animal models of DM1 (Warf et al., 2009; García-López et al., 2011; Childs-Disney et al., 2013; Cerro-Herreros et al., 2016). In contrast, the molecular causes of cardiac dysfunction in DM1 and the involvement of MBNL proteins in these defects are not yet fully understood. Notably, compound loss of Mbnl1 and Mbnl2 in mice generated cardinal features of DM1, including heart conduction dysfunction (Lee et al., 2013). However, elevation of CUGBP1 is an early event in the heart of a mouse model of DM1 (Wang et al., 2007), and heart-specific overexpression of CUGBP1 in mice induces functional and molecular alterations (Koshelev et al., 2010). Furthermore,

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inhibition of CUGBP1 hyperphosphorylation ameliorates the cardiac phenotype in a mouse model of DM1 (Wang et al., 2009). Importantly, both MBNL and CUGBP1 are master switches for normal heart development as they regulate a large subset of alternative splicing transitions that occur post-natally (Wang et al., 2015; Kalsotra et al., 2008). These data suggest that the pathogenic events underlying the cardiac dysfunctions in DM1 remain to be fully defined.

To study the importance of MBNL proteins in the heart dysfunction induced by expanded CUG repeats in *DMPK*, we used a *Drosophila* model expressing pure expanded CUG repeats (250 CUG repeats) in cardiomyocytes (Chakraborty et al., 2015). Importantly, cardiac dysfunction described in these flies is completely reversed by the sole overexpression of Mbl, the *Drosophila* orthologue of MBNL proteins. To further support the relevance of this protein in DM1 cardiac dysfunction, we searched *in vitro* for compounds that inhibit the interaction between MBNL1 and expanded CUG repeats. One of these compounds, daunorubicin hydrochloride, releases endogenous Mbl from CUG RNA foci by directly binding to CUG RNA, resulting in the correction of Mbl-dependent splicing alterations and subsequent recovery of heart dysfunction and survival in DM1 flies. These data highlight not only the relevance of MBNL depletion related to heart dysfunction in DM1, but also provide evidence that strategies aimed at releasing MBNL proteins from expanded CUG RNA repeats might also be useful to treat cardiac dysfunction in DM1.

RESULTS

Muscleblind is sufficient to rescue the cardiac dysfunctions and reduced survival caused by expanded CUG repeats

To understand the role of Mbl in cardiac dysfunction induced by expanded CUG repeat expression, we generated recombinant flies that simultaneously expressed 250 pure CUG repeats and either Mbl isoform C (MblC; the best evolutionary conserved Mbl isoform in *Drosophila*) or green fluorescent protein (GFP), as control. Cardiac parameters were analyzed using semi-automated optical heartbeat analysis (SOHA) software on 7-day-old female flies as described previously (Chakraborty et al., 2015; Ocorr et al., 2014; Cerro-Herreros et al., 2017). Heart dysfunctions, including systolic and diastolic dysfunction, arrhythmia and reduced contractility, in DM1 model flies expressing GFP were identical to DM1 flies (Fig. S1) and were similar to the previously reported alterations (Chakraborty et al., 2015), indicating that the expression of the GFP reporter is innocuous in heart. In contrast to GFP, expression of MblC corrects the cardiac parameters of DM1 flies: *Drosophila* expressing 250 pure CUG repeats and MblC were similar to control flies that do not express the repeats. Notably, MblC expression rescued heart period length [HP; defined as the diastolic interval (DI) plus systolic interval (SI)] (Fig. 1A-C), mainly by a 2-fold reduction of the SI (the contraction period). The variability in the heart periodicity, quantified as 'arrhythmia index' (AI), and the heart contractility, or percentage of fractional shortening (%FS), were also rescued in DM1 flies by MblC overexpression (Fig. 1D,E). Finally, survival, which was decreased in DM1 model flies, was also corrected by MblC, with median survival increasing from 22 to 32 days, and the lifespan increasing from 31 to 50 days (Fig. 1F). These results support an important role of Mbl loss of function in the heart dysfunctions induced by expression of expanded CUG repeats.

Identification of compounds reducing MBNL1 binding to CUG repeats

To confirm the relevance of MBNL loss of function in cardiac dysfunctions in DM1, we developed a real-time fluorescence

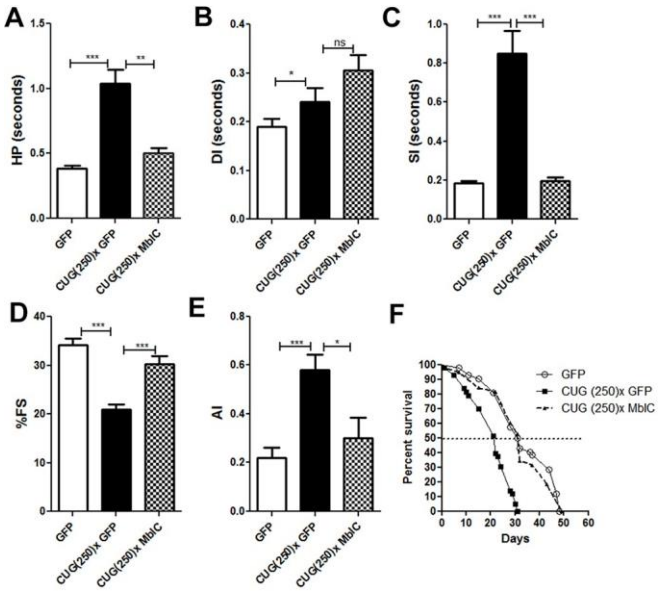

Fig. 1. Cardiac dysfunction and survival are rescued by MblC overexpression in DM1 model flies. In comparison to control flies expressing GFP, the heart period mean (HP; in A) increased in flies expressing expanded CUG repeats and GFP [CUG(250)× GFP] together. Increased HP is caused by extended diastolic and systolic intervals (DI and SI; in B and C, respectively). In contrast, simultaneous expression of CUG repeats and MblC [CUG(250)× MblC] achieved a reduction of HP length by a large decrease of SI. Heart contractility, measured as percentage of fractional shortening (%FS; in D), and arrhythmicity, measured as arrhythmia index (AI; in E), which were altered in DM1 flies, were also rescued by MblC overexpression (n=18 to 29). Median survival and lifespan were also rescued by MblC overexpression (F; $n\approx50$). ***P<0.001, **P<0.01, *P<0.05, ns, not significant (Student's t-test).

polarization/anisotropy assay to identify pharmacological compounds reducing MBNL1 binding to pathogenic CUG repeats. Fluorescence polarization measures the degree of polarization of a fluorophore, reflecting its rotational diffusion, a parameter that is inversely proportional to the molecule volume and thus reflects the molecular mass of the fluorescent-labelled molecule or complex. In short, fluorescence polarization can discriminate between a fluorescent-labelled RNA free in solution compared to the same RNA in complex with an RNA-binding protein. Consequently, a molecule that disrupts the binding between a fluorescent RNA and a protein can be identified by a reduction of the fluorescence polarization. In our assay, we used a chemically synthetized CUG repeat (26 repeats) RNA labelled with a tetramethylrhodamine and incubated with purified recombinant GST-MBNL1-HIS produced from *Escherichia coli*. An optimal shift of fluorescent polarization was observed with 50 nM of fluorescent CUG RNA incubated with 400 nM of recombinant MBNL1 protein. We screened in 96-well plates and at 10 µM final concentration ∼6500 compounds from two libraries, the Strasbourg Academic library, consisting of ∼5300 chemical or natural substances, and the Prestwick library, consisting of 1200 drugs approved by regulatory agencies. From this screen, we identified 76 molecules, of which 12 were verified by fluorescent polarization to inhibit binding of MBNL1 to CUG-repeat RNA *in vitro* (Fig. 2A). Next, we tested whether these compounds can reduce the sequestration of MBNL1 in human cultured cells. The colocalization of endogenous MBNL1 with foci of expanded CUG RNA was assessed by immunofluorescence coupled to RNA fluorescent *in situ* hybridisation (FISH) in primary cultures of myoblasts originated from a muscle biopsy of an individual with

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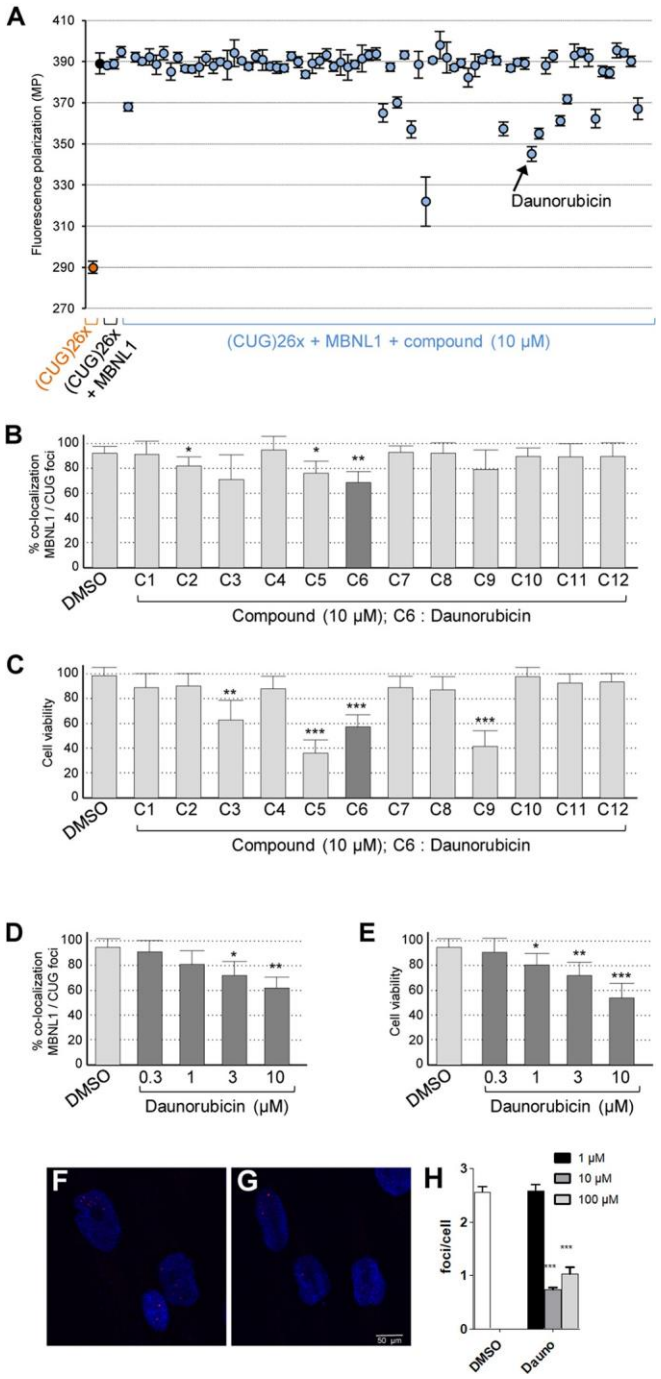

Fig. 2. Validation screening of the 76 compounds tested at a concentration of 10 µM in DMSO to identify molecules reducing binding of MBNL1 to expanded-CUG-repeat RNA. (A) Fluorescence polarization of TRITC-labelled (CUG)26× RNA alone or in complex with GST-MBNL1-HIS recombinant protein is indicated by orange or blue dots, respectively. *N*=3 independent assays. (B) Percent of CUG RNA foci presenting a colocalization with endogenous MBNL1 in cultures of DM1 myoblasts upon drug treatment at 10 µM in DMSO for 24 h. *N*=3 independent cultures; 30 RNA foci were analyzed in each experiment. (C) Percent of living DM1 myoblasts upon drug treatment at 10 µM in DMSO for 24 h. (D) Percent of CUG RNA foci showing colocalization with endogenous MBNL1 in cultures of DM1 myoblasts upon daunorubicin treatment at 0.3, 1, 3 and 10 µM in DMSO for 24 h. *N*=4 independent cultures; at least 50 cells were analyzed each time. (E) Percent of living DM1 myoblasts upon daunorubicin treatment at 0.3, 1, 3 and 10 µM in DMSO for 24 h. (F-H) Foci detection in DM1 fibroblasts. Representative confocal images of FISH in DM1 fibroblasts treated with DMSO as control (F) or daunorubicin (G) showed reduced number of foci (red in F and G) with daunorubicin treatment. Nuclei were counterstained with DAPI (blue). (H) Quantification of foci confirmed a statistically significant difference at concentrations of daunorubicin higher than 1 µM. For all figure panels, error bars indicate s.e.m. Student's *t*-test: **P*<0.05; ***P*<0.01; ****P*<0.001.

DM1. Among the 12 compounds tested, three compounds − C2, C5 and C6 − decreased the sequestration of MBNL1 *in cellulo* (Fig. 2B). However, C5 was toxic and not investigated further (Fig. 2C). Subsequent studies in DM1 primary muscle cell cultures revealed that compound C6, daunorubicin hydrochloride, was achieving the most important reduction of MBNL1 colocalization with CUG RNA foci. Addition of 3-10 µM of daunorubicin reduced the colocalization of MBNL1 with CUG RNA foci by 20-30% in DM1 primary muscle cell cultures, but with some toxicity (Fig. 2D,E). Of note, daunorubicin treatment of control or DM1 primary muscle cell cultures did not modify protein expression of MBNL1, nor the mRNA expression of *DMPK* (Fig. S2A,B). We also quantified foci number in human DM1 fibroblasts (Arandel et al., 2017) at increasing concentrations of daunorubicin, and observed a significant reduction of foci number at concentrations higher than 1 µM (Fig. 2F-H). These data encouraged us to test whether daunorubicin could correct any phenotypic alterations in an animal model of DM1.

Daunorubicin rescues cardiac dysfunction and fly survival in DM1 model flies

Daunorubicin is a double-stranded DNA or RNA intercalant that belongs to the anthracyclines family. It has been used as curative or palliative treatment for several types of cancer for over 30 years (Hande, 1998; Gewirtz, 1999; Ellison et al., 1991; Kobylinska et al., 2016). To assess the potential of daunorubicin to rescue DM1 cardiac phenotypes, we fed DM1 model flies with the compound diluted in 1% DMSO to a final concentration of 1 µM in nutritive media. We had previously shown that, at the concentration used, DMSO does not alter the cardiac parameters in *Drosophila* (Chakraborty et al., 2015). For cardiac function assessment, 7-day-old flies fed with the compound were dissected to expose the beating heart in artificial aerated haemolymph, and videos were taken and analyzed by SOHA software (Chakraborty et al., 2015; Ocorr et al., 2014; Cerro-Herreros et al., 2017). In all analyses, flies that do not express CUG repeats but expressed GFP were used as controls. As compared to the DMSO-fed model flies, flies fed with daunorubicin showed significant improvement in HP length (Fig. 3A), with a significant 2-fold decrease in both the DI and SI (Fig. 3B,C). Furthermore, daunorubicin corrected AI (Fig. 3E), which decreased by almost 3-fold in treated flies, and rescued heart contractility, assessed by the calculation of % FS (Fig. 3D). Finally, DM1 model flies fed with DMSO had a median survival of only 28 days, while survival of DM1 flies fed with daunorubicin increased up to 40 days, which is close to the 47 days of median survival in control flies with no CTG repeats (Fig. 3F). As a control to discard a general effect of daunorubicin on flies' survival, we fed flies expressing GFP in cardiac muscle with daunorubicin and analyzed their survival curves. Daunorubicin had no effect on the survival of these control flies (Fig. S3A). In conclusion, daunorubicin improved all the cardiac parameters of DM1 flies, as well as their median survival, but did not affect control flies.

Daunorubicin redistributed Mbl from foci in *Drosophila* cardiomyocytes and rescued Mbl-dependent splicing defects

To observe the effect of daunorubicin on Mbl distribution, we performed FISH followed by immunofluorescence to detect CUG

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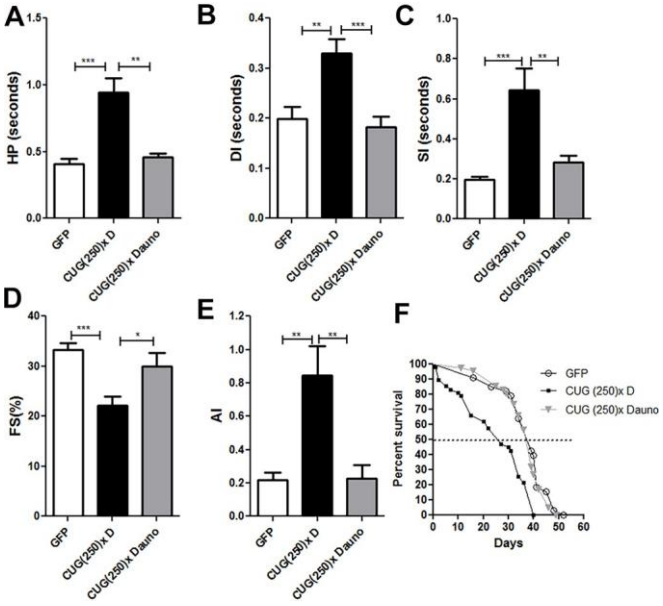

Fig. 3. All DM1 cardiac alterations were rescued by daunorubicin. (A-E) Alterations in HP, SI, DI, %FS and AI found in model flies fed with DMSO ('D') were significantly rescued to nearly normal conditions by treatment with daunorubicin hydrochloride (Dauno) (n=14 to 29). (F) Median survival was also rescued by daunorubicin treatment ($n\approx50$). ***P<0.001, **P<0.01, *P<0.05 (Student's t-test).

RNA foci and Mbl, respectively, in hearts of flies expressing expanded CUG repeats fed with 1 µM daunorubicin. As previously reported (Chakraborty et al., 2015), ribonuclear foci containing Mbl were present in the cardiac muscle nuclei of flies expressing CUG repeats. In cardiomyocytes of model flies fed with daunorubicin, we did not observe ribonuclear foci and Mbl was found homogeneously distributed in the nuclei (Fig. 4). As control, quantitative reverse transcription PCR (RT-qPCR) indicates that Mbl expression was not modified in DM1 flies treated with daunorubicin compared to DM1 flies fed or not with DMSO (Fig. 5A).

Next, we studied Mbl-dependent splicing events to test whether Mbl redistribution in the nuclei was enough to correct mis-splicing. Notably, daunorubicin corrects both *Serca* exon 13 (Fig. 5B) and *formin* (*Fhos*) exon 16′ (Fig. 5C,D) splicing events, which are Mbl-dependent events altered in heart of DM1 flies (Cerro-Herreros et al., 2017). These data show that daunorubicin induces Mbl redistribution in the nuclei enough to correct Mbl-dependent splicing events. In contrast, in flies expressing GFP in cardiomyocytes as controls, daunorubicin treatment did not alter the pattern of these splicing events, thus ruling out a general effect of daunorubicin on splicing regulation (Fig. S3B,C).

Daunorubicin interacts directly with CUG RNA repeats, modifying its stability

To test whether daunorubicin exerts its correcting effect by direct interaction with the CUG RNA, and competition with MBNL1, we performed electrophoretic mobility shift assay and analyzed the binding of recombinant GST-MBNL1 to internally labelled *in vitro* transcribed RNAs containing ten CUG repeats in the presence of increasing amounts of daunorubicin (Fig. 6A,B). According to these experiments, the quantity of daunorubicin needed to release half of MBNL bound to CUG 10× (*in vitro* IC50) would be 100 nM. We also used differential scanning fluorimetry (DSF) to monitor CUG-expanded RNA thermodynamics in the presence of growing concentrations of daunorubicin. The effect of a compound on RNA stability can be measured by DSF because RNA undergoes structural transitions from being double-stranded to single-stranded

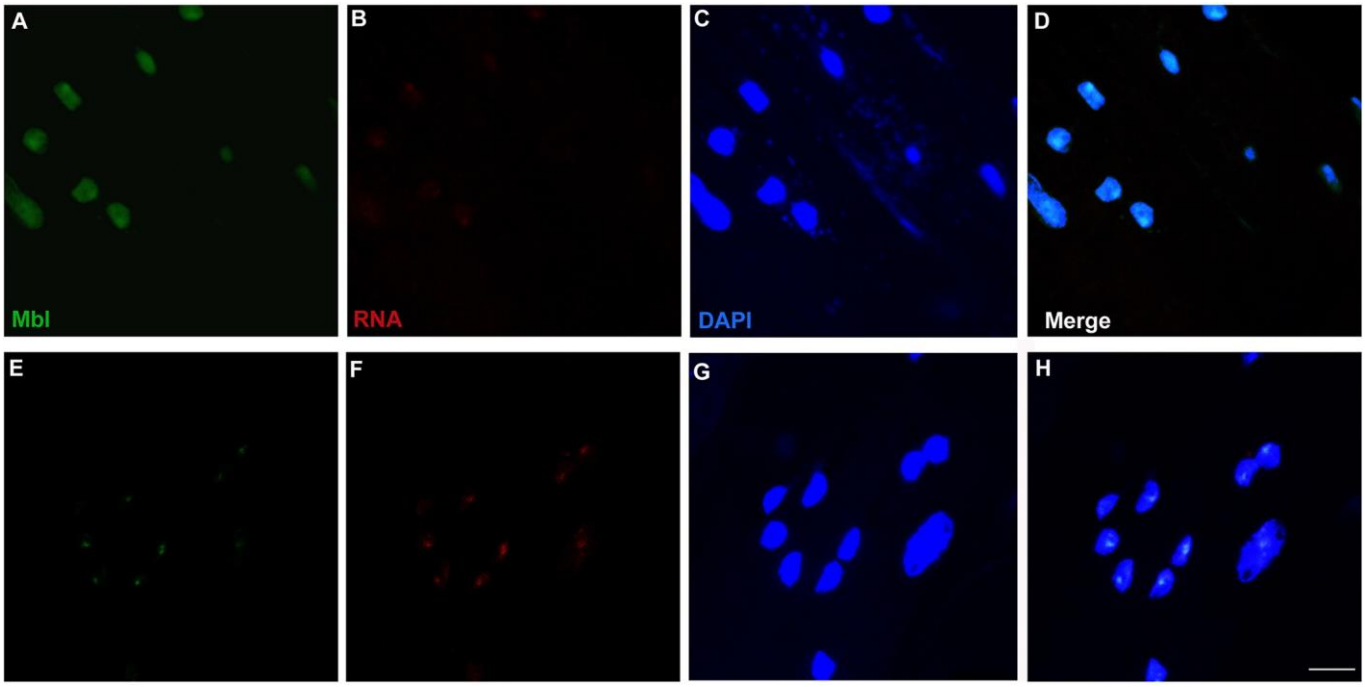

Fig. 4. Daunorubicin abrogates foci presence and redistributes Mbl in *Drosophila* cardiomyocytes. Representative confocal images of cardiomyocytes of *Drosophila* DM1 flies fed with daunorubicin (A-D) or DMSO (E-H). Acquisition settings were the same for images taken within the same channel. Double *in situ* hybridization and immunodetection of CUG RNA (red) and Mbl (green) showed that Mbl was dispersed in the nuclei of cardiomyocytes of flies fed with daunorubicin. Nuclei were counterstained with DAPI (blue). Scale bar: 10 µm.

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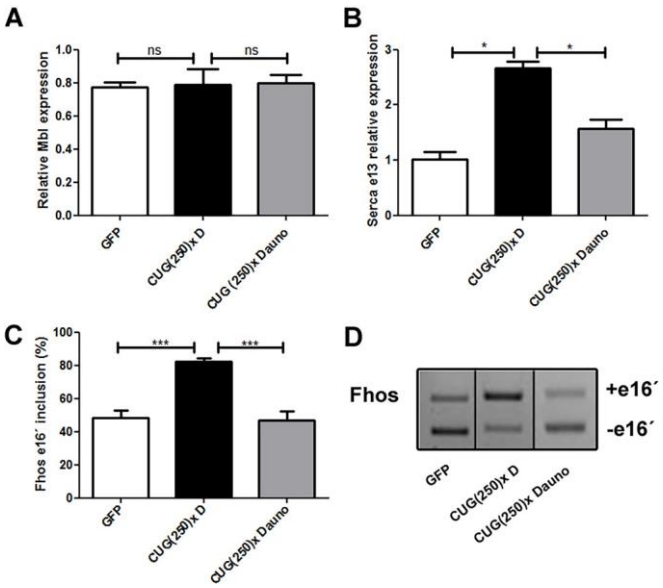

Fig. 5. In model flies fed with daunorubicin, Mbl transcript levels were not modified but Mbl-dependent splicing defects were restored. (A) RT-qPCR to assess Mbl expression levels relative to endogenous tubulin in cardiomyocytes showed that it was not modified by administration of daunorubicin. (B) RT-qPCR results of *Serca* exon 13 expression relative to *Rp49* confirmed a significant rescue of the expression of this exon in model flies fed with daunorubicin. (C) Semi-quantitative PCR to assess inclusion of *Fhos* exon 16′ in model flies fed with DMSO or daunorubicin. *Rp49* transcripts were detected as endogenous control. Quantification of percentage of exon inclusion (see D for a representative result) confirmed an improvement of *Fhos* mis-splicing in model flies fed with daunorubicin. **P*<0.05, ****P*<0.001 (Student's *t*-test).

upon thermal unfolding. These transitions are accompanied by increased availability of binding sites for the single-stranded-RNA-specific dye RiboGreen. In this type of assay, the initial fluorescence originates from binding of the dye to unstructured, single-stranded, binding-competent regions of the RNA. After an initial decrease, the fluorescence increases because of destabilization of compact RNA structures, thereby becoming competent to bind the dye and achieving a point of maximal fluorescence. Then, this process reverses due to the formation of secondary structures indicated by a decrease in the binding affinity of the dye until RNA is completely folded (Silvers et al., 2015). We represented the fluorescence intensity and the first derivatives of normalized fluorescence of RiboGreen with an RNA probe containing 12 repeats of CUG versus temperature in the presence of concentrations of daunorubicin ranging from 0.1 to 2 µM and observed an important decrease in the maximal fluorescence and a shift of the melting towards higher temperatures with growing concentrations of daunorubicin (Fig. 6C,D). These data are coherent with the known intercalation of daunorubicin with double-stranded RNA (Doskocil and Fric, 1973) and suggest that daunorubicin stabilizes a double-stranded hairpin conformation of the CUG RNA repeats, resulting in less available single-stranded binding sites for the RiboGreen dye. Extrapolating these results to the binding of MBNL1 to expanded CUG repeats, it is likely that daunorubicin, by stabilizing a double-stranded hairpin RNA structure, limits the accessibility of MBNL1 to free single-stranded YGC RNA motifs (Lambert et al., 2014; Delorimier et al., 2014; Park et al., 2017).

Given the ability of daunorubicin to intercalate within the DNA and thus to alter transcription and replication, we also quantified the expression level and length of expanded CTG repeats. PCR amplification revealed that the DNA region including the CTG repeats had the same length (1178 bp) in DM1 flies fed with either DMSO or daunorubicin (Fig. 6E). Furthermore, quantitative RT-qPCR indicated no changes of CTG transgene expression in DM1 flies fed with DMSO compared to daunorubicin treatment (Fig. 6F). These data support a mechanism of action based on daunorubicin's direct interaction with CUG RNA decreasing MBNL accessibility to the repeats, rather than a modification of the mutant DNA expression or length.

DISCUSSION

We previously reported alterations of different heart functions, including heart rate, rhythmicity, SI, DI and %FS in flies expressing pure expanded CUG repeats (Chakraborty et al., 2015). The relevance of our model of heart dysfunction in DM1 was supported by the similarities between the cardiac phenotype in DM1 model flies and those documented in DM1 patients. Here, we show that the sole expression of Mbl can rescue all these cardiac parameters to their normal values, except the DI, which was not altered by MblC overexpression. These results support the involvement of Mbl in the cardiac dysfunction in DM1 and the therapeutic potential of its overexpression. Our data are consistent with the compound loss of Mbnl1 and Mbnl2 that evokes DM1-like cardiac phenotypes in mice (Lee et al., 2013). Furthermore, we identified pharmacological compounds reducing MBNL1 sequestration by expanded CUG repeats and found that one of these drugs, daunorubicin hydrochloride, achieved a complete rescue of cardiac parameters, including survival, arrhythmia, %FS and HP, with an increase of both DI and SI in DM1 flies. The fact that DI was suppressed by daunorubicin and not by MblC suggested that either other Mbl isoforms are required for a complete rescue of Mbl function in heart or that daunorubicin affects other Mbl-independent components, which might be involved in DI duration in DM1. Potential Mbl-independent activity of daunorubicin might be related to its ability to cause DNA damage. Daunorubicin belongs to the anthracyclines family, which has been used in curative and palliative treatment of several types of cancer (Hande, 1998; Gewirtz, 1999). Although anthracyclines produce a wide range of biological reactions, their primary mechanism of tumour cytotoxicity has been ascribed to the inhibition of the topoisomerase II enzyme (Nielsen et al., 1996; Arcamone et al., 1997). Thus, anthracycline drugs may cause DNA damage such as fragmentation and single-strand breaks, which can influence microsatellite expanded repeat instability. Notably, treatment of cells with 1, 2 and 5 mg/ml (1.8 µM, 3.54 µM and 8.86 µM, respectively) of doxorubicin, a 14-hydroxylated version of daunorubicin, causes some reduction of CTG repeat length (Hashem et al., 2004). However, we assessed CTG repeat length in 1 µM daunorubicin-treated flies and found no alterations. Of interest, the normal dosage used for daunorubicin hydrochloride in adults under 60 years of age is 45 mg/m^2 once a day on days 1, 2 and 3 for the first course. This dosage would involve a concentration of 2 µM daunorubicin. DM1 flies fed with 1 µM daunorubicin throughout their lifespan have improved survival in comparison to DM1 flies fed with DMSO, suggesting that the cumulative daunorubicin doses achieved were not overtly toxic at this concentration.

Daunorubicin also interacts with double-stranded RNA (Doskocil and Fric, 1973). Our results indicate that daunorubicin directly interacts with expanded CUG RNA repeats and impairs binding of MBNL1 to these repeats, thus promoting MBNL1 release from CUG RNA foci in DM1 cells, as well as in DM1 *Drosophila* cardiomyocytes. Importantly, this reduced depletion of Mbl is enough to revert Mbl-dependent splicing events in DM1

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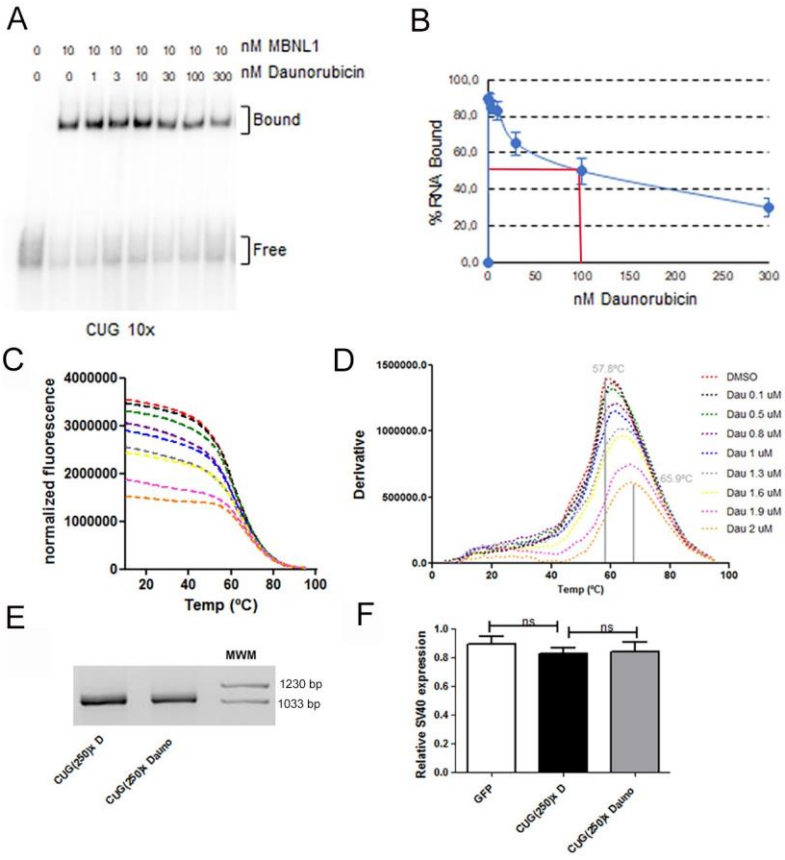

Fig. 6. Daunorubicin binds directly to CUG RNA, stabilizes CUG RNA structure *in vitro* and does not modify the CUG-repeat expression level or length. (A) Gel-shift assays of 10 nM of purified bacterial recombinant GST-MBNL1 with 10 pM (2000 CPM) of uniformly [alphaP^{32}] internally labelled *in vitro* transcribed RNAs containing 10 CUG repeats in the presence of increasing amounts of daunorubicin. (B) Gel-shift quantification with IC50 represented by the red lines. (C) Normalized fluorescence intensity and (D) first derivatives of RiboGreen fluorescence versus temperature at different daunorubicin concentrations. Grey vertical bars mark the minimum (57.8°C) and maximum (65.9°C) melting temperatures. (E) Agarose gel showing CTG repeat length inserted in the genomic DNA of DM1 flies fed with either DMSO or daunorubicin. MWM, molecular weight marker. (F) Bar graph represents means±s.e.m. of SV40 expression (contained in the CTG transgene) relative to tubulin. According to E and F, the effect of daunorubicin is not mediated by a reduction in the CTG repeat length or expression level in model flies. ns, not significant according to Student's *t*-test.

flies. Overall, these results support a key role of Mbl protein sequestration in the cardiac dysfunctions observed in DM1.

Daunorubicin should be taken cautiously because of the genotoxic effects of this compound in long-term treatments. However, as proof of concept, our results suggest that inhibiting MBNL1 binding to expanded CUG repeats is a valid strategy to rescue cardiac phenotypes in DM1.

This study supports an important role for Mbl sequestration in the cardiac dysfunctions induced by expanded CUG repeats in DM. Furthermore, we uncovered a new FDA-approved compound with the ability of dissolving foci and competing MBNL1 away from CUG RNA foci. However, given the toxicity profile of daunorubicin, subsequent studies on murine models of the disease would be required to determine whether it could be considered as a suitable therapeutic approach for DM1.

MATERIALS AND METHODS

Drosophila strains

The cardiomyocyte-specific driver *GMH5-Gal4; UAS-GFP* was kindly provided by Dr Bodmer (Sanford Burham Institute, CA). Generation of CUG(250)× and UAS-MblC flies was previously described (Chakraborty et al., 2015; García-Casado et al., 2002). UAS-GFP strain was obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN).

Compound treatment

Daunorubicin hydrochloride (Sigma) was added to the standard fly food to a final concentration of 1 µM in 0.1% DMSO. The control group was fed with 0.1% DMSO in standard food. Flies were transferred every 3 days to new fresh food media, with or without compound, for the duration of their whole lifespan in survival curve experiments or during 7 days in the case of the group used for cardiac analysis and immunofluorescence.

Survival analyses

For survival analyses, a minimum of 50 flies were included per group and the survival curves were obtained at 29°C. Scoring of death was done as previously described (Chakraborty et al., 2015; Cerro-Herreros et al., 2017).

Cardiac physiological analysis

For the physiological analysis, female flies were collected just after eclosion and were maintained for 7 days at 29°C in compound and DMSO-supplemented food. For the heart beat recordings, semi-intact heart preparations were made as previously described (Magny et al., 2013; Cerro-Herreros et al., 2017; Chakraborty et al., 2015). A Leica microscope, connected to an ORCA Flash (Hamamatsu) high-speed digital camera was used to take 20 s recordings at a minimum speed of 150 frames/s. Different cardiac parameters were measured using SOHA software (Ocorr et al., 2014).

Alternative splicing quantification

For each biological replicate, total RNA was extracted using Qiazol (Qiagen) from at least 20 female hearts. One microgram of RNA was digested with DNase I (Invitrogen) and retro-transcribed with SuperScript II (Invitrogen) using random hexanucleotides. To analyze *Fhos* exon 16′ inclusion, 20 ng of cDNA was used in a standard semi-quantitative PCR reaction with GoTaq polymerase. *Serca* exon 13 expression, SV40 to measure repeats expression, and Mbl expression were checked with RT-qPCR as previously described (Cerro-Herreros et al., 2017, 2016).

Repeat number detection

To confirm the number of repeats in the flies fed with the compound or DMSO, 40 ng of genomic DNA was used as a template for the PCR amplification as described previously (Chakraborty et al., 2015). The region amplified by the primers includes long repeats (750 bp) and 428 bp of the CTG (250)× plasmid. PCR amplification was performed under the following conditions: 95°C for 2 min, followed by 30 cycles of 98°C for 20 s, 65°C for 30 s, 72°C for 1 min and final extension at 72°C for 5 min.

The PCR products were analyzed by electrophoresis at 110 V in 1.5% agarose gels.

DM1 myoblast culture and staining

Primary human myoblast cells originating from muscle biopsies of genetically confirmed DM1 patients were maintained at 37°C with 10% CO_2 in skeletal muscle cell basal media with supplements (PromoCell, Heidelberg, Germany) and 10% fetal calf serum. For myoblast differentiation, cells were maintained for 4 days in DMEM with 2% fetal calf serum, the drug was added for 16 h and then assayed for CUG RNA foci and MBNL1 localization by classic RNA FISH immunofluorescence. Glass coverslips containing plated cells were fixed in PFA 4% for 15 min and washed two times with PBS. The coverslips or slides were incubated for 10 min in PBS plus 0.5% Triton X-100 and washed three times with PBS before pre-hybridization in 40% DMSO, 40% formamide, 10% BSA (10 mg/ml), 2× SCC for 30 min. The coverslips or slides were hybridized for 2 h in 40% formamide, 10% DMSO, 2× SCC, 2 mM vanadyl ribonucleoside, 60 µg/ml tRNA, 30 µg/ml BSA plus 0.75 µg CAG8x-Cy3 DNA oligonucleotide probe (Sigma). Following FISH, the coverslips or slide were washed twice successively in 2× SCC/50% formamide, in 2× SCC and in PBS. The coverslips or slides were incubated for 2 h with primary polyclonal antibody against MBNL1 (1/100 dilution, gift of Prof. Charles Thornton, University of Rochester Medical Center, Rochester, NY). Slides or coverslips were washed twice with PBS before incubation with a goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (1/500 dilution, #A32731, Fisher Scientific SA) for 60 min, incubated for 10 min in 2× SCC/DAPI (1/10,000 dilution) and rinsed twice in 2× SSC before mounting in Pro-Long media (Molecular Probes). Slides were examined using a fluorescence microscope (Leica).

Foci quantification in DM1 fibroblasts

Immortalized (hTERT) human DM1 (1300 CTG repeats) skin fibroblasts conditionally expressing MyoD were provided by D. Furling's laboratory in the Institute of Myologie, Paris. Fibroblast cells were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l glucose, 1% penicillin and streptomycin (P/S), and 10% fetal bovine serum (FBS; Sigma). Fibroblasts were aliquoted into 96-well plates (1.0×104 cells per well), incubated with daunorubicin or DMSO (48 h) and fixed in 4% PFA for 10 min at room temperature followed by several washes in 1× PBS. Fixed cells were incubated in pre-hybridization buffer (2× SSC, 30% deionized formamide) for 10 min at room temperature and hybridized with Cy3-$(CAG)_7$-Cy3-labelled probe diluted 1:500 in hybridization buffer [40% formamide, 2× SSC, 0.2% BSA, 10% dextran sulfate, 2 mM ribonucleoside-vanadyl complex, 10% tRNA (10 mg/ml) and 10% herring sperm] for 2 h at 37°C. After hybridization, cells were washed twice with pre-hybridization buffer for 15 min at 45°C, washed twice with 0.5× SSC for 5 min at 37°C, washed with 1× PBS for 15 min at room temperature, incubated with Hoechst 33342 (5 mg/ml) diluted 1:2000 in 1× PBS for 20 min at room temperature, and mounted with 20% Mowiol. Images were taken and analyzed using an IN Cell Analyzer 2200 Imaging System.

Immunofluorescence analysis in flies

For immunofluorescence analysis in *Drosophila* cardiomyocytes, 7-day-old dissected female fly hearts were fixed for 20 min in 4% paraformaldehyde and washed in PBT (PBS containing 0.3% Triton X-100) before staining. Mbl staining and FISH to detect ribonuclear CUG foci were performed as previously described (Llamusi et al., 2012). All the confocal images were taken with an Olympus FV1000 microscope.

Differential scanning fluorimetry (DSF)

DSF experiments were performed to understand the interaction of daunorubicin with the double-stranded CUG hairpin using DSF technique (Silvers et al., 2015). The experiment was performed using a StepOnePlus Real-Time PCR system (Life Technologies) with the melting curve software to measure the fluorescence intensity. A MicroAmp® Fast Optical 96-well plate (Life Technologies) was used with 50 µl of solution per well. The RiboGreen dye was used at a final concentration of 300 nM, whereas the synthetic double-stranded CUG RNA (12× CUG) was used at a final concentration of 600 nM. For each compound (in this case daunorubicin and DMSO), four technical replicates were performed and, for daunorubicin, eight different concentrations ranging from 0.1-2 µM were used. Sodium cacodylate buffer was used at pH 6.1, which is essential for experiments using RNA. During the DSF experiment, the temperature was increased from 4 to 95°C at an increment of 0.2°C with an equilibration time of 5 s at each temperature prior to measurement. The Excel sheets containing melting temperature, and normalized and derivative fluorescence data were exported into GraphPad Prism 5 software for further analysis and generation of graphics.

Electrophoretic mobility shift assay

10 pM (2000 CPM) of labelled CUG 10× RNA was incubated at 90°C for 5 min in binding buffer [BB; 0.75 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.0), 75 mM NaCl, 37.5 mM KCl, 5.25 mM DTT, 0.1 mg/ml BSA, 0.1 mg/ml Bulk tRNA] and allowed to cool to room temperature. After cooling, RNasin was added to a final concentration of 0.4 U/µl. GST-$MBNL1^{\Delta 101}$ was then added and the mixture was incubated on ice for 20 min. The solution mixture was loaded onto a non-denaturing 6.0% (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 40:1, w/w) containing 0.5× TBE [1× TBE is 90 mM Tris-base, 89 mM boric acid and 2 mM EDTA (pH 8.0)], which had been pre-electrophoresed at 110 V for 20 min at 4°C. The gel was electrophoresed at 110 V at 4°C for 3 h, then dried and exposed to a phosphorimager screen and imaged using a Typhoon 9410.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.C.-B., R.A., B.L.; Methodology: M.C., C.S., M.N., V.P., N.C.-B., R.A., B.L.; Validation: M.C., B.L.; Formal analysis: M.C., C.S., M.N., V.P.; Investigation: N.C.-B., R.A., B.L.; Data curation: M.C.; Writing - original draft: M.C., B.L.; Writing - review & editing: M.C., C.S., M.N., V.P., N.C.-B., R.A., B.L.; Supervision: N.C.-B., R.A., B.L.; Funding acquisition: N.C.-B., R.A., B.L.

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Supplementary information

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