Differential Toxicity of Nuclear RNA Foci versus Dipeptide Repeat Proteins in a *Drosophila* Model of C9ORF72 FTD/ALS

**Highlights**
- Intronic 160R form abundant nuclear sense RNA foci in *Drosophila*
- Nuclear RNA foci cause little toxicity and minimal changes in RNA processing
- Cytoplasmic 36R-poly(A) produce >100-fold more poly(GP) than intronic 160R
- Modest toxicity of 160R at higher temperature correlates with increased DPRs

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**In Brief**
Tran et al. engineered a novel *Drosophila* model of FTD/ALS with C9ORF72 repeat expansion and revealed that dipeptide repeat proteins, but not nuclear G4C2 repeat RNA foci, are a major source of toxicity in vivo.

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Differential Toxicity of Nuclear RNA Foci versus Dipeptide Repeat Proteins in a *Drosophila* Model of C9ORF72 FTD/ALS

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SUMMARY

Dipeptide repeat (DPR) proteins are toxic in various models of FTD/ALS with GGGGCC (G4C2) repeat expansion. However, it is unclear whether nuclear G4C2 RNA foci also induce neurotoxicity. Here, we describe a *Drosophila* model expressing 160 G4C2 repeats (160R) flanked by human intronic and exonic sequences. Spliced intronic 160R formed nuclear G4C2 sense RNA foci in glia and neurons about ten times more abundantly than in human neurons; however, they had little effect on global RNA processing and neuronal survival. In contrast, highly toxic 36R in the context of poly(A)+ mRNA were exported to the cytoplasm, where DPR proteins were produced at >100-fold higher level than in 160R flies. Moreover, the modest toxicity of intronic 160R expressed at higher temperature correlated with increased DPR production, but not RNA foci. Thus, nuclear RNA foci are neutral intermediates or possibly neuroprotective through preventing G4C2 RNA export and subsequent DPR production.

INTRODUCTION

A GGGGCC (G4C2) repeat expansion in the noncoding region of *C9ORF72* is the most common mutation in both frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) (DeJesus-Hernandez et al., 2011; Renton et al., 2011). How G4C2 repeat expansion contributes to disease pathogenesis is largely unknown. One potential pathogenic mechanism is mediated by abnormal dipeptide repeat (DPR) proteins arising from repeat-associated non-AUG (RAN) translation (Cleary and Ranum, 2014). DPR proteins, synthesized from both sense and antisense repeat transcripts and adjacent intronic sequences, are found mostly in cytoplasmic inclusions in *C9ORF72* patients and a mouse model (Ash et al., 2013; Gendron et al., 2013; Mori et al., 2013, 2013b; Zu et al., 2013; Chew et al., 2015). When overexpressed in cultured cells or *Drosophila*, different DPR proteins have varying degrees of toxicity (Kwon et al., 2014; May et al., 2014; Mzielińska et al., 2014; Tao et al., 2015; Wen et al., 2014; Yang et al., 2015; Zhang et al., 2014; Zu et al., 2013).

Another potential pathogenic mechanism is RNA-mediated toxicity, in which the formation of RNA foci and sequestration of specific RNA-binding proteins is associated with misregulation of RNA processing in repeat-expansion diseases (Mohan et al., 2014). In the case of *C9ORF72* FTD/ALS, RNA foci are found mostly in the nucleus of multiple cell types, such as brain cells and fibroblasts from patients and neurons derived from induced pluripotent stem cells (iPSCs) (Almeida et al., 2013; DeJesus-Hernandez et al., 2011; Donnelly et al., 2013; Gendron et al., 2013; Lagier-Tourenne et al., 2013; May et al., 2014; Mzielińska et al., 2014; Sareen et al., 2013; Zu et al., 2013). However, little is known about the pathogenicity of these RNA foci.

To address this question, we generated a *Drosophila* model expressing in a cell-type-specific manner a *C9ORF72* minigene containing up to 160 G4C2 repeats (160R) flanked by human intronic and exonic sequences. Overexpressed intronic 160R formed abundant nuclear sense RNA foci in neurons and glial cells but had surprisingly little toxicity in vivo. In contrast, highly toxic 36 G4C2 repeats (36R) expressed in the context of a poly(A)+ mRNA (Mzielińska et al., 2014) were exported to the cytoplasm and translated into DPR proteins at a level >100-fold higher than that by intronic 160R. Moreover, intronic 160R expressed at a higher temperature induced a modest toxicity that correlated with increased DPR production, but not G4C2 sense RNA foci. Thus, low levels of DPR proteins, but not nuclear RNA foci, are a major source of toxicity in vivo.

RESULTS

The Expanded G4C2 Repeat-Containing Intron Is Transcribed and Spliced in iPSC-Derived Human Neurons and Patient Brain Tissues

According to the latest information in the NCBI database, at least three splice variants of *C9ORF72* are expressed in human cells (Figure 1A). In variants 1 and 3 (V1 and V3), G4C2 repeats are located in the first intron. In contrast, transcription of V2,
whose expression level is much higher than V1 and V3 (see Figure S1 available online), starts downstream of G4C2 repeats (Figure 1A), which has been confirmed experimentally (Sareen et al., 2013). To quantify the effect of expanded G4C2 repeats on the transcription and splicing of the first intron of C9ORF72, we took advantage of a single-nucleotide polymorphism (SNP) in exon 3 (rs10757668) (Figure 1B), which is heterozygous (A/G) in 10%–15% of the general population, with the expanded G4C2 repeats associated with the G allele (DeJesus-Hernandez et al., 2011; Sareen et al., 2013). We performed allele-specific expression analysis of the junction between exon 1b and exon 3 in cells and brain tissues carrying the A/G SNP by pyrosequencing, a sequence-based detection technology that allows quantification of a sequence variation such as a SNP (Ronaghi, 2001). We found that the ratio between G- and A-containing V3 alleles was the same in iPSCs derived from three G4C2 repeat expansion carriers and three controls (Figure 1C).

A similar result was obtained from postmortem brain tissues (Figure 1D). If expanded G4C2 repeats blocked the full transcription or splicing of the first intron, one would expect that the ratio of unspliced RNA (as measured by the exon 1–intron 1 junction) to mature mRNA (as measured by the exon 1–exon 3 junction) would be higher. The ratio was identical in human neurons differentiated from four C9ORF72 and four control iPSC lines (Figure 1E) and in brain tissues from eight C9ORF72 patients and nine controls (Figure 1F), even though the levels of both intron 1-containing pre-mRNA and exon 1-containing mature mRNA were higher in iPSCs-derived C9ORF72 neurons (Figure S1B) and brain tissues (Figure S1C) than in their respective controls. Thus, the expanded G4C2 repeats do not meaningfully affect splicing of the first intron, and most if not all G4C2 sense RNAs in human neurons come from the spliced first intron of C9ORF72, consistent with our earlier finding that intron 1-containing C9ORF72 RNA could not be

Figure 1. Expanded G4C2 Repeats in C9ORF72 Are Fully Transcribed in Patient Cells and Tissues

(A) Schematic of the three known transcript variants of wild-type (top) and mutant (bottom) alleles of C9ORF72, named as in PubMed and the UCSC Genome Browser. The locations of primers used in (C) and (D) (green) and (E) and (F) (blue) are indicated.

(B) The G/A SNP in a control subject and in a carrier of expanded G4C2 repeats.

(C and D) Pyrosequencing analysis of the allele-specific expression of V3 in three C9ORF72 and three control iPSC lines (C) and in brain tissues of three patients and three controls (D).

(E and F) The relative ratio of total V1 and V3 pre-mRNAs (exon 1–intron 1 junction) to mature mRNAs (exon 1–exon 3 junction) in cortical neurons differentiated from four C9ORF72 and four control iPSC lines (E) and in brain tissues of eight C9ORF72 patients and nine controls (F). n.s., not statistically significant, by Student’s t test.

(G) RNA-seq analysis reveals a significantly lower V2 level in patient neurons than control neurons. The number of reads for V1 and V3 is too low for a meaningful analysis. ***p < 0.001, chi-square test.

See also Figure S1.
detected by northern blot (Almeida et al., 2013). In contrast, RNA-seq analysis revealed that mutant V2 allele (G allele) was expressed at a much lower level in patient iPSC-derived neurons than in controls (Figure 1G), consistent with several reports showing that the total level of V2 expressed from both alleles is lower in C9ORF72 patient samples (Almeida et al., 2013; Belzil et al., 2013; DeJesus-Hernandez et al., 2011; Donnelly et al., 2013; Sareen et al., 2013; Waite et al., 2014).

A Drosophila Model of Intronic G\textsubscript{4}C\textsubscript{2} Repeat Expansion

To investigate the pathogenicity of intronic G\textsubscript{4}C\textsubscript{2} repeats, we designed and expressed an artificial human C9ORF72 minigene in Drosophila (Figure 2A). This minigene contains exon 1, part of the first intron with synthetic G\textsubscript{4}C\textsubscript{2} repeats of various lengths at the exact same location as in human cells, and exon 3 of human C9ORF72 (Figure 2A). DNA constructs containing uninterrupted 5R, 20R, 40R, 80R, and 160G\textsubscript{4}C\textsubscript{2} repeats (160R) were stable in E. coli (Figure 2B). C9ORF72 minigenes containing 5R, 80R, and 160R were then cloned into a transgenic fly vector (Figure 2A). The PhiC31 integrase-mediated site-specific integration system (Bateman et al., 2006) was used to ensure equal transcription of minigenes with different repeat length in flies. Southern blot analysis confirmed the repeat length of the transgenes (Figure S2A). G\textsubscript{4}C\textsubscript{2} repeats with five copies were not detected by Southern blot but were confirmed by PCR and sequencing analysis. The intronic 160R remained stable in transgenic flies over multiple generations.

To determine whether G\textsubscript{4}C\textsubscript{2} repeats of different length are expressed at the same level, we crossed 5R and 160R transgenic flies carrying the UAS-repeat sequences with Actin5C-Gal4 fly lines and did quantitative real-time PCR (qPCR) analyses. PCR analysis with primers located in exon 1 and exon 3 revealed only a single 120 bp band corresponding to the spliced form (Figure S2B). No difference was observed between 5R and 160R flies when the relative level of the exon 1–exon 3 junction was quantified with the same set of primers (Figure 2C), suggesting that intronic 160R did not affect transgene expression and splicing, as in human cells (Figure 1).

Flies Expressing Intronic 160R Do Not Have Obvious Neurodevelopmental Defects

C9ORF72 patients have age-dependent neurodegeneration, but no early neurodevelopmental defects have been observed. To determine whether flies expressing intronic 160R also develop normally—and are thus a more physiologically faithful model—we traced and quantified larval crawling behavior on an agarose plate (Nichols et al., 2012). In this assay, locomotor activity did not differ between larvae expressing 0, 5R, or 160R transgenic flies carrying the UAS-repeat sequences with Actin5C-Gal4 fly lines and did quantitative real-time PCR (qPCR) analyses. PCR analysis with primers located in exon 1 and exon 3 revealed only a single 120 bp band corresponding to the spliced form (Figure S2B). No difference was observed between 5R and 160R flies when the relative level of the exon 1–exon 3 junction was quantified with the same set of primers (Figure 2C), suggesting that intronic 160R did not affect transgene expression and splicing, as in human cells (Figure 1).

Figure 2. A Drosophila Model of FTD/ALS Expressing Expanded G\textsubscript{4}C\textsubscript{2} Repeats

(A) Schematic of human C9ORF72 minigenes containing 5R, 80R, or 160R, flanked by human intronic and exonic sequences. Green arrows: primer location used in (C).
(B) Construction of DNA plasmids containing 5R, 20R, 40R, 80R, or 160R. Plasmids were digested with EcoRI.
(C) Quantitative real-time PCR analysis of the relative levels of C9ORF72 minigene spliced RNAs.
(D) Effect of 160R on dendritic branching of dda neurons in third-instar larvae. No statistical difference was observed (n > 14 flies per genotype).
(E) Kaplan-Meier curves showing the lifespan of transgenic flies expressing 0, 5R, or 160R in glutamatergic neurons by OK371-Gal4. No statistical difference between 5R and 160R flies (log-rank test).
See also Figure S2.
(Figure S2D). To determine whether expanded G$_4$C$_2$ repeats affect the long-term viability of adult flies, we expressed intronic 5R or 160R in all cells by Actin5C-Gal4 (data not shown) or specifically in glutamatergic neurons with the OK371-Gal4 driver. When grown at 25°C, 160R flies did not show a change in their lifespan (Figure 2E).

**Overexpressed Intronic 160R Form Numerous Nuclear Sense RNA Foci with Little Effect on rRNA Biogenesis and mRNA Processing**

Sense and antisense RNA foci are a key pathological hallmark of C9ORF72 FTD/ALS. In iPSC-derived human neurons, most RNA foci are nuclear although a few are cytoplasmic (Almeida et al., 2013). In 160R flies, no antisense foci were observed; however, on average, 42 sense RNA foci were detected in the nucleus of glutamatergic neurons and 49 in glia expressing intronic 160R; no foci were observed in control flies and flies expressing 5R (Figures 3A and 3B). The average number of RNA foci per cell was more than ten times higher than that of sense RNA foci in iPSC-derived human neurons (Almeida et al., 2013), probably reflecting overexpression of G$_4$C$_2$ repeats by the UAS-Gal4 system.

These foci were not detected by a DNA probe targeting CUG or CCUG repeats (data not shown) and are made of RNA, as evidenced by their sensitivity to RNase but not DNase treatment (Figure S3A). In these cells, one large focus and numerous smaller foci were invariably present (Figures 3A and 3B).
small foci were similar to those in human fibroblasts or iPSC-derived neurons (Almeida et al., 2013) but distinct from G_{4}C_{2} repeats in muscle cells or salivary gland cells (Figure 3C). Salivary gland cells are much larger than neurons and have polytene chromosomes. Thus, expression of intronic 160R was much higher in this cell type; yet, very little was exported to the cytoplasm (Figure S3B). These large “foci” were always present on chromatin and seemed to be located at the site of transgene transcription. Indeed, they were成本 with an antibody against activated Pol-II (Figure 3B). Moreover, when two copies of intronic 160R were expressed from two different chromosomes, two large dots were invariably observed in each cell (Figure 3D). Thus, signals detected at the site of transcription in transgenic animal models should be distinguished from the actual RNA foci observed in patient cells.

RNA foci formed by intronic 160R did not localize to the nucleolus or grossly alter nucleolar morphology (Figure 3F). Indeed, 160R RNAs do not seem to directly impair nucleolar function. Instead, the level of different rRNA species did not differ between controls and flies overexpressing 5R or 160R (Figure S3C). To determine whether the numerous nuclear RNA foci alter mRNA processing in flies, we sequenced the transcriptomes of male fly heads in which a 5R or 160R transgene was ubiquitously expressed by Actin5C-Gal4. To reduce false positives caused by the difference in genetic backgrounds and other factors, we used flies bearing these transgenes on either the second or the third chromosome and obtained an average of 32 million non-redundant uniquely mapped reads per library (Table S1).

Gene expression in flies expressing 5R and 160R was highly correlated (Figures S3E and S3F). Because the number of RNA foci was comparable in flies expressing 160R from either the second or the third chromosome (Figure S3D), we analyzed differentially expressed genes (DEGs) from both groups and found 50 common DEGs; 45 of them showed differential expression in the same direction (Figure 3E; Table S2). Gene ontology analysis suggested that proteins with oxidoreductase activities were enriched among these 45 genes (Table S3). Only 7 of these 45 genes showed a change of >2-fold. Moreover, we found only 17 exon-skipping events common to flies expressing 160R from either the second or the third chromosome (Table S4). For example, in the sun gene, the percent spliced in (PSI) was 16.5% in 5R flies and 22.3% in 160R flies (Figure S3G). Such a small number of DEGs and exon-skipping changes argues against the hypothesis that those RNA foci are capable of sequestering sufficient RNA binding proteins to exert a global effect on RNA processing in our fly model.

**Highly Toxic 36R-Poly(A) Are Exported to the Cytoplasm, and Intronic 160R Expressed at a Higher Temperature Induce Modest Toxicity and Increased DPR Production**

The finding that abundant nuclear G_{4}C_{2} RNA foci cause little toxicity was surprising, so we examined a fly model in which 36R are highly toxic (Mizielsinska et al., 2014). This model and another one expressing 30R (Xu et al., 2013) differ significantly from ours, where 160R are transcribed and spliced out as part of human intronic sequence. In those models, G_{4}C_{2} repeats are transcribed as part of a poly(A)^{+} mRNA. The steady-state level of 36R-poly(A) was about 2-fold higher than that of the spliced RNA in 160R flies (Figure S4B), which is likely due to their differential posttranscriptional regulation, since both transgenes were generated using the same site-specific integration system and expressed by the same Gal4 driver. Nonetheless, we examined 160R homozygous flies that expressed the spliced RNA at the same level as the 36R mRNA (Figure S4B) and found no visible eye neurodegenerative phenotype (Figure S4C).

In flies expressing 36R-poly(A) and grown at 25°C, most of the 36R-poly(A) RNA were exported to the cytoplasm of glial cells (Figure 4A) or motor neurons (data not shown), as expected for a poly(A)^{+} mRNA. Yet, in the nucleus of each cell, a bright large dot was still observed, which we believe is the site of the transgene transcription as demonstrated earlier (Figures 3B and 3D). In stark contrast to intronic 160R, ubiquitous expression of 36R-poly(A) by Actin5C-Gal4 resulted in a developmental lethal phenotype. Thus, to quantify DPR levels, we used GMR-Gal4 to express these transgenes in the eye and performed a previously described immunoassay for poly(GP) (Su et al., 2014). A similar quantitative assay is not available for poly(GR), which is highly toxic in flies (Mizielsinska et al., 2014; Yang et al., 2015). Therefore, we first performed immunostaining and detected poly(GR) in both neurons and glial cells expressing 36R-poly(A), but not in intronic 160R (Figures 4B and S4A). Similarly, the poly(GP) level was >100-fold higher in flies expressing 36R-poly(A) than in intronic 160R (Figure 4C). The high level of DPR proteins, especially poly(GR) produced from 36R-poly(A), correlates well with the eye-degenerative phenotype (Figure S4C) and the increased level of p62 (Figure S4D). Thus, the toxicity of expanded G_{4}C_{2} repeats is greatly influenced by their subcellular location.

In contrast to flies grown at 25°C (Figure 2E), intronic 160R expression at 29°C induced a modest toxicity as measured by the lifespan assay (Figure 4D). The number of RNA foci in both neurons and glial cells remained the same (Figure 4E). However, DPR production was 4-fold higher in 160R flies grown at 29°C than at 25°C (Figure 4F). These findings suggest that intronic 160R expressed at a higher temperature induces modest toxicity through increased DPR production, further supporting the notion that low levels of DPR proteins, but not nuclear sense RNA foci, are a major source of toxicity in our fly model.

**DISCUSSION**

In this study, we established a *Drosophila* model in which 160R were flanked by human intronic and exonic sequences, and transcribed and spliced in vivo. We first confirmed that expanded G_{4}C_{2} repeats, some of them containing more than 1,000 copies (Almeida et al., 2013), are transcribed and spliced as part of the first intron of C9ORF72 in human cells and patient brain tissues. This finding was not unexpected, as the highly evolved human transcription machinery contains multiple factors to perform this complex reaction in vivo (Shilatifard et al., 2003), in contrast to an observed high level of aborted transcription by bacteriophage T7 polymerase alone in an in vitro assay with short G_{4}C_{2} repeats (Haeusler et al., 2014). Some transcription elongation factors, such as Supt4h, facilitate transcription through long repeat sequences in human cells (Liu et al., 2012).

In general, most spliced introns stay in the nucleus, although some are exported to the cytoplasm (Hesselberth, 2013).
Indeed, intronic 160R formed abundant nuclear G₄C₂ RNA foci in both neurons and glia with only a few foci outside of the nucleus. The average number of these nuclear RNA foci was more than ten times higher than we observed in iPSC-derived neurons (Almeida et al., 2013). However, the transcriptome was largely unaffected, rRNA biogenesis was not significantly altered, and nucleolar morphology appeared to be normal. Thus, these foci by themselves do not elicit significant toxicity, at least in this experimental system. Our results comparing flies grown at 25°C versus 29°C also support the notion that nuclear G₄C₂ sense

Figure 4. Increased DPR Production, but Not Nuclear RNA Foci, Contributes to Repeat Toxicity In Vivo
(A) RNA FISH in glial cells detects numerous nuclear RNA foci (red) formed by intronic 160R, while 36R-poly(A) RNA is exported to the cytoplasm. Green, nuclear membrane; blue, DAPI staining. Red arrows, transcription sites of transgenes. Scale bar, 5 μm.
(B) Poly(GR) (red) was detected mostly in the cytoplasm of neurons expressing 36R-poly(A), but not in cells expressing intronic 160R. Scale bar, 5 μm.
(C) One of the two experiments to measure poly(GP) in lysates from heads of flies raised at 25°C. Values are mean ± SEM. ***p < 0.0001 (one-way ANOVA). On average, the poly(GP) level is 122 times higher in 160R flies than in 36R flies.
(D) The lifespan of transgenic flies grown at 29°C expressing 0, 5R, or 160R in glutamatergic neurons. Statistical difference between 5R and 160R flies was assessed with the log-rank test (p < 0.001).
(E) Quantification of nuclear RNA foci in neurons and glia in flies grown at 25°C and 29°C. No statistical difference was observed (by unpaired nonparametric t test).
(F) Poly(GP) levels in lysates from heads of flies raised at 25°C and 29°C. Values are mean ± SEM. ***p < 0.0001 (one-way ANOVA).
See also Figure S4.
RNA foci are not a significant source of toxicity. Whether more abundant antisense foci are neurotoxic remains to be determined.

DPR proteins are mostly found in cytoplasmic inclusions of postmortem brain tissues of C9ORF72 patients and a mouse model (Ash et al., 2013; Gendron et al., 2013; Mori et al., 2013; Zuo et al., 2013; Chew et al., 2015). We speculate that they are produced from either intronic repeat RNAs and/or unspliced C9ORF72 pre-mRNAs that are transported to the cytoplasm. The poly(A) tail facilitates nuclear export and cytoplasmic translation of mature mRNAs (Weill et al., 2012). Indeed, we found that 36R expressed in the context of poly(A)+ mRNA were exported to the cytoplasm and produced DPRs at a level >100-fold higher than that in 160R flies (Figure 4C).

Many animal models fail to fully recapitulate human neurodegenerative diseases, because aging is a major risk factor for these disorders. During aging, a subset of nucleoporins is oxidatively damaged, resulting in increased nuclear permeability (D’Angelo et al., 2009). Thus, aging itself or environmental stress may cause increased leakage of expanded G4C2 repeats in an intron or an unspliced pre-mRNA into the cytoplasm, resulting in increased production of toxic DPRs in some vulnerable human neurons.

DPR proteins of various lengths may have different pathogenic mechanisms. We recently found that, in contrast to (GR)80 (Kwon et al., 2014), (GR)180 is located mostly in the cytoplasm and impairs the Notch signaling pathway, whereas (GA)240 recuits (GR)180 into cytoplasmic inclusions, thereby derepressing (GR)180 toxicity (Yang et al., 2015). Moreover, poly(GR) proteins of different length used in different studies can either directly or indirectly induce nucleolar stress (Kwon et al., 2014; Wen et al., 2014; Yang et al., 2015). Thus, much remains to be learned about the pathogenic mechanisms of DPR proteins of different lengths. Although the lifetime presence of sense RNA foci in the nucleus might still be detrimental, our results suggest that they could also be neutral intermediates or even neuroprotective by sequestering G4C2 repeats in the nucleus and therefore preventing cytoplasmic DPR production. Thus, inhibition of the toxicity of DPR proteins, especially nonaggregated forms, seems to represent a more promising therapeutic approach for C9ORF72 patients.

EXPERIMENTAL PROCEDURES

For details on all methods, please see Supplemental Information.

ACCESSION NUMBERS

RNA-seq data are available at the NCBI Gene Expression Omnibus database under accession number GSE72771.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.09.015.

AUTHOR CONTRIBUTIONS

H.T. and F.-B.G. conceived the project. H.T. performed all the fly experiments (with some help from U.C., Y.L., X.D., and J.A.N.). S.A. analyzed iPSCs and brain samples. J.M. and Z.W. performed bioinformatics analysis. T.F.G. and L.P. measured poly(GP) levels. H.T. and F.-B.G. wrote the manuscript with input from S.A., J.M., T.F.G., and Z.W. F.-B.G. supervised the project.

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