The Methyltransferases PRMT4/CARM1 and PRMT5 Control Differentially Myogenesis in Zebrafish

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Abstract

In vertebrates, skeletal myogenesis involves the sequential activation of myogenic factors to lead ultimately to the differentiation into slow and fast muscle fibers. How transcriptional co-regulators such as arginine methyltransferases PRMT4/CARM1 and PRMT5 control myogenesis in vivo remains poorly understood. Loss-of-function experiments using morpholinos against PRMT4/CARM1 and PRMT5 combined with in situ hybridization, quantitative polymerase chain reaction, as well as immunohistochemistry indicate a positive, but differential, role of these enzymes during myogenesis in vivo. While PRMT5 regulates myod, myf5 and myogenin expression and thereby slow and fast fiber formation, PRMT4/CARM1 regulates myogenin expression, fast fiber formation and does not affect slow fiber formation. However, our results show that PRMT4/CARM1 is required for proper slow myosin heavy chain localization. Altogether, our results reveal a combinatorial role of PRMT4/CARM1 and PRMT5 for proper myogenesis in zebrafish.

Introduction

The proper control of gene expression is essential for normal growth, development and differentiation. An early step in the process of gene activation consists in modifying histones and unraveling DNA sequences within promoter regions thereby facilitating the recruitment of specific and general transcription factors. Skeletal muscle differentiation involves cooperation between the muscle regulatory factors (MRFs) Myod, Myf5, Mrf4 and Myogenin [1], members of the Myocyte-enhancer factors 2 (Mef2) family and chromatin remodelling or modifying enzymes [2]. Numerous histone modifying enzymes have been implicated in the regulation of myogenic genes in cell culture including members of protein arginine methyltransferase (PRMT)-families [2]. Of particular interest are two members of the PRMT family, PRMT4/CARM1 (hereafter, called CARM1) and PRMT5 as independent studies performed in cell culture or on dissected skeletal tissues suggested that they are required for myogenesis [3,4,5]. CARM1 methylates histone H3 on specific arginine residues to regulate transcription [6,7], as well as non-histone proteins such as the transcriptional co-activators CBP/p300 [8,9,10,11]. Over the last few years, several studies have shown that CARM1 acts as transcriptional co-activator in cell proliferation and in a multitude of signaling pathways [12,13]. Similarly, PRMT5 methylates histones H3 and H4 as well as non-histone proteins but has been shown to regulate negatively cell proliferation [13,14]. These data suggest that CARM1 and PRMT5 play a central role in muscle differentiation in cell culture and most likely in vivo. However, the mechanisms by which these enzymes control myogenesis have not been addressed yet. Indeed, no other report has addressed the precise role(s) of CARM1 and PRMT5 in vivo during development and particularly in myogenesis. To gain insights into their function in vivo, we turned to zebrafish to analyze their shared and divergent roles in myogenesis.

In zebrafish, cells are committed to the myogenic fate through the activation and the cooperation of MRFs and Mef2 proteins [15]. Then, committed myoblasts differentiate into muscles fibers with distinct genes expression profiles and contraction speeds, the so-called slow- and fast-twitch fibers. In the zebrafish embryo, progenitors of the slow and fast muscle fibers can be identified on the basis of their morphology, their position within the segmental plate before somites formation and the expression of a specific set of genes [16]. It has been shown recently that myf5 and myod, but not myogenin, are required and cooperate to drive myoblast progenitor (also called adaxial cells) commitment to the slow fiber type [17] whereas fast fiber differentiation occurs during the migration of slow fibers and relies on the expression of myf2 and myod but also of myogenin [17,18]. Here, we investigated the involvement of CARM1 and PRMT5 in myogenesis in zebrafish embryos. We found that both CARM1 and PRMT5 control myogenin expression whereas PRMT5, controls the expression of the early genes myod and myf5. Accordingly, we demonstrated that these enzymes affect proliferation negatively and act in myogenic differentiation. We subsequently showed that both PRMT5 and CARM1 control fast muscle fiber formation whereas PRMT5 triggers slow muscle fiber formation. Finally, we found that if a decrease of CARM1 expression was not affecting the initial step of...
slow muscle fiber formation it lead to a defect in their localization. Hence, our results indicate that PRMT5 and CARM1 play a combinatorial role in zebrafish myogenesis.

Results and Discussion

CARM1 and PRMT5 are dynamically expressed during development

Expression pattern of CARM1 and PRMT5 during zebrafish development was first analyzed by in situ hybridization. We found that both CARM1 and PRMT5 were robustly expressed at 2-cell stage indicating that they are maternal genes (Figure 1A). At gastrulation, CARM1 was strongly expressed in the dorsal shield, whereas PRMT5 was weakly present in this domain. Of note, both CARM1 and PRMT5 were expressed within the somites during early somitogenesis and enriched in the presomitic mesoderm (PSM, Figure 1A–1C, asterisk), a structure that will give rise to the somites [19]. That both genes are expressed in the PSM suggests that CARM1 and PRMT5 may contribute to the maintenance of somite progenitors in this area.

Interestingly, PRMT5 and CARM1 were expressed in the somites from 6- to 18-somite stage during segmentation (Figure 1A and 1B). Around 24 hours post fertilization (24 hpf), somitogenesis is completed and PRMT5 was preferentially expressed in the posterior trunk and in tail somites with a weaker expression in anterior trunk somites, whereas CARM1 was expressed all along the axis with a strong expression in the anterior neural structures (Figure 1A). To gain further insight into the expression of CARM1 and PRMT5 in the somites, we analyzed them in flat-mounted embryos and in the corresponding cross sections at 14-somite stage (14 ss). PRMT5 expression was not detected in the adaxial progenitor cells and was enriched in the medial part of the somite, whereas CARM1 was ubiquitously expressed in the somite (Figure 1C and 1D). That PRMT5 and CARM1 are expressed in different parts of the somites suggests that they may act differentially on myogenesis.

CARM1 and PRMT5 are essential for proper myogenesis

To examine the involvement of CARM1 and PRMT5 in myogenesis, we interfered with their translation using antisense morpholinos (Mo). Expression of endogenous CARM1 and PRMT5 was strongly reduced after injection of their respective morpholino, as confirmed by western blot (Figure 2A and 2B). Phenotypes of the morphants on the somites were analyzed at 14–16 ss and 24 hpf under polarized light. PRMT5 loss of function gave rise to embryos with long and flat somites, whereas CARM1 morphants showed smaller and rounder somites (Figure 2C and Table 1). Of note, the same morphant phenotypes were obtained after injection of another specific morpholino against CARM1 or PRMT5 (data not shown). These phenotypes were rescued at both stages when CARM1 or PRMT5 mRNAs were co-injected with their cognate morpholino (Table 1, Figure 2C, panels MoP5+P5, MOC1+C1 and Figure 2D, panels j and k). Furthermore, the phenotypes observed at 24 hpf were dependent on the dose of morpholinos injected (Table 1 and Figure 2D). Altogether, these data demonstrate the specificity of CARM1 and PRMT5 morpholinos and indicate that both CARM1 and PRMT5 are required for proper axis formation. The round shape of the somites at 14 ss in CARM1 morphant suggests that CARM1 could be involved in the elongation of muscle progenitor cells that are

Figure 1. PRMT5 and CARM1 are differentially expressed during zebrafish embryogenesis. (A–D) CARM1 and PRMT5 in situ hybridization at the indicated stages. (A) Animal view (av), lateral view (lv, dorsal to right for 6 hpf, anterior to left for 24 hpf) and dorsal views (dv, anterior to top) of whole-mount zebrafish in situ hybridization. (B) Dorsal view (anterior to top) of CARM1 and PRMT5 expression during somitogenesis. (C) Dorsal flat-mounts of 14 ss embryos stained for CARM1 and PRMT5. A magnified region is shown on the right. (D) Transverse sections (30 μm) of the magnified region in (C) showing undetectable PRMT5 expression in adaxial cells (arrow), whereas CARM1 is ubiquitously expressed in the somite. *, Presomitic Mesoderm (PSM); ss, somite stage. doi:10.1371/journal.pone.0025427.g001

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initially round to generate the myotome, a transition stage involved in somite boundary formation [20].

PRMT5 and CARM1 regulate differentially myogenic factor expression

To further characterize the impact of CARM1- and PRMT5-loss of function, we first focused on their putative role on mesodermal markers expression at gastrulation. We found that although CARM1 and PRMT5 were expressed in the shield during gastrulation (Figure 1A), their knock down did not affect the expression of the organizer gene goosecoid/gsc (data not shown), or of the mesodermal gene spadetail/spt/tbx16 (Figure S1A), a key regulator of myod expression [21,22]. Hence, CARM1 and PRMT5 are not required in early mesoderm specification, from where the somites arise.

We then analyzed the impact of CARM1- or PRMT5-knock down on the expression of key regulators of zebrafish myogenesis,
myogenin (Figure S2). Strikingly, we observed that at 14 ss PRMT5 morphants exhibited a significant knock down had no effect on the expression of this gene (Figure 3). Accordingly, decrease of myod expression by either PRMT5 or CARM1 Mo diminished the expression of myogenin and mef2c at 18 ss (Figure 3B and Figure S2B). Of note, PRMT5 morphant were not due to the deregulation of the other enzyme.

Our study reveals that PRMT5 controls myogenesis at an earlier step by regulating myod and myf5 expression. Hence, it is likely that PRMT5 controls myogenesis via a two-tiers mechanism: first, our study shows that it up-regulates myod expression (Figure 3 and Figure S1B) and myf5 expression (Figure 3 and Figure S2B), second, as demonstrated in cell culture, it also cooperates with Myod or Myf5 to activate myogenin expression [5]. As PRMT5 morphant did not show any defect in spadetail expression (Figure S1A), a gene that controls the onset of myod expression [21,22], we propose that PRMT5 may activate directly myod and myf5 expression by being recruited onto their promoters as it has been demonstrated for myogenin in cell-based assays [4]. However, we cannot rule out that PRMT5 could also affect their expression indirectly or post-transcriptionally via its enzymatic activity.
Interestingly, our data reveal that CARM1 is required to specifically control the down-regulation of *myf5* expression in the mature somite (Figure 3). It has been shown that CARM1 methylates RNA-binding protein HuD thereby affecting the stability of HuD target mRNAs [23] and that CARM1 can act either positively or negatively on mRNA stability of a certain number of c-Fos target genes [24]. One can speculate that CARM1 could regulate *myf5* expression post-transcriptionally in a similar way. Our data showing that CARM1 regulates *myogenin* expression in vivo are in agreement with the original report showing that CARM1 activates *myogenin* expression in cell-based studies [3].

However, a recent study has reported a role of co-activator of Myogenin-dependent transcription for CARM1 but without affecting *myogenin* expression [5]. Of note, this study was performed in Myod-differentiated fibroblasts and C2C12 myoblasts. Here, our data indicate that in a whole organism, CARM1 regulates *myf5*, *myogenin* and *mef2c* expression. Hence, PRMT5 and CARM1 are not only co-activators of Myod- and Myogenin-dependent transcription respectively, but they also control the expression of these myogenic factors.

**PRMT5 and CARM1 control slow and fast myogenesis differentially**

To address whether PRMT5 and CARM1 could affect slow and/or fast myogenesis we analyzed the expression of *slow myosin heavy chain 1 (smyhc1)*, a slow fiber specific gene and of *fast myosin light chain (mlc2f)*, a fast fiber specific gene in PRMT5- and CARM1-depleted embryos as compared to control embryos. In PRMT5 morphants, *mlc2f* was dramatically reduced as compared to control morphants (Figure 4A) and *smyhc1* expression was completely lost in the lateral part of the somite (Figure 4C, arrow). CARM1 knock down restrained *smyhc1* expression medially (Figure 4C, red arrowhead) and *mlc2f* expression almost disappeared (Figure 4A). These data were confirmed by immunohistochemistry against fiber-specific myosin isoforms at two different stages (14 ss, Figure 4B and 4D; 18 ss, Figure S5). PRMT5 knock down lead to a strong decrease in slow muscle fiber formation and suppressed fast muscle fibers at both stages (Figure 4B and 4D and Figure S5).

Interestingly, **PRMT5** was not detected in adaxial cells (Figure 1D) and our results suggest that this enzyme can control slow muscle fiber formation. Several possibilities can be proposed to explain this apparent paradox. First, even though PRMT5 was not detected in adaxial cells in our *in situ* hybridization experiments, it could be nevertheless very weakly expressed and thus active in these cells. Another possibility is that PRMT5 could act non-cell autonomously in adaxial cells. Finally, PRMT5 would not be required for the activation of *smyhc1* expression but for its maintenance. Indeed, if expression of *smyhc1* was weakly affected by PRMT5 knock down, its protein level was barely detectable in the same condition (Figure 4C and 4D). These data suggest that PRMT5 controls *smyhc1* expression post-transcriptionally. We can speculate that, either PRMT5 is specifically required for *smyhc1* translation, or PRMT5 is somehow involved in SMYHC1 stability. Interestingly, it has been shown recently that PRMT5 was required for the expression of myogenic microRNAs [25]. Hence, one possibility is that PRMT5 could control *smyhc1* translation by affecting the expression of these miRNAs. Further studies would be needed to elucidate whether and how PRMT5 controls SMYHC1 protein synthesis and/or stability.

![Figure 4. CARM1 and PRMT5 differentially control slow and fast myogenesis.](https://www.plosone.org/figure/4.4/carm1-and-prmt5-differentially-control-slow-and-fast-myogenesis-a-c.png)

(A, C) Dorsal flat-mounted 14 ss embryos (anterior to top) injected with the indicated Mo were analyzed by whole-mount *in situ* hybridization for (A) *mlc2f* and (C) *smyhc1* or (B, D) immunohistochemistry for (B) fast fibers, (D) slow fibers. In (C) a magnification of the area indicated by the black line is shown to the right of each panel. Arrow or white arrowhead, lateral expression; red arrowhead, medial expression; red bar, length of one somite. (B, D) Z-stack sections of the most anterior somites are shown.

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CARM1 morphants exhibited a lack of fast muscle fiber formation and a restriction of the slow fiber marker to the midline (Figure 4B and 4D and Figure S5). This pattern is reminiscent of embryos mutated in \(M\) or \(N\)-Cadherin that control slow muscle cell migration in the developing zebrafish myotome [26] and is in line with CARM1 morphant phenotype that generates round somites. We then went on to analyze slow fiber proper localization in CARM1 knock down. We found that slow muscle fiber localization was affected in CARM1 morphants (Figure 4D and Figure 5). More investigations will be needed to analyze whether CARM1 is involved in slow muscle fiber localization and regulates cadherin expression. Altogether, these results clearly show that PRMT5 is essential for slow and fast myogenesis, whereas CARM1 only controls fast fiber formation and is required for proper slow muscle fiber localization.

Having shown that CARM1 and PRMT5 control muscle cell fiber formation, we analyzed whether they also regulate cell proliferation in the somite as CARM1 and PRMT5 have been shown in cell culture to regulate cell proliferation, either positively [27] or negatively [14], respectively. Surprisingly, we found that at 14 ss, loss of either CARM1 or PRMT5 increased significantly the number of mitotic cells within the somite (Figure 6). This effect was rescued when CARM1 or PRMT5 mRNAs were co-injected with their cognate morpholino (Figure 6, MoP5+P5, MoC1+P5). Hence, these data indicate that both enzymes trigger cell cycle arrest and myogenic differentiation during development. We propose that CARM1 and PRMT5 could control the timing of muscle fiber formation by regulating the switch between proliferation and cell cycle exit.

**Figure 5.** CARM1 controls slow fiber localization. (A, B) One-cell stage embryos were injected with a CARM1 morpholino (MoC1) or a control morpholino (MoCt) and were analyzed at 24 hpf by whole-mount immunohistochemistry for slow fibers. (A) Lateral views, anterior to the left of 3D confocal reconstructions projected over four somites, (A’) single confocal scans at the dorsoventral level of the notochord and (A”) corresponding cross-section views. (B) Rostral cross-sections (50 μm) of 22 ss embryos injected with the indicated morpholino.

**Figure 6.** CARM1 and PRMT5 control cell cycle arrest during development. (A) Dorsal flat-mounted of 14 ss embryos (anterior top) injected with the indicated Mo or co-injected with the corresponding mRNA for rescue experiments were analyzed by immunohistochemistry for mitotic cells with an anti phospho-H3 (Ser10) antibody. (B) Representation of the number of mitotic cells per somite. Error bars represent standard deviations.

***, \(P < 0.0001\) extremely statistically significant; ns, not statistically significant, using a t-test.

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Here, we have established a combined action of CARM1 and PRMT5 to control MRF and myf2c expression as well as slow and fast myogenesis in zebrafish. Recent studies have emphasized the role of methylation of non histone proteins such as transcriptional co-regulators, transcription elongation factors and RNA binding proteins by CARM1 and PRMT5 [28]. It is tempting to speculate that through their enzymatic activity on specific substrates, these enzymes could regulate the transcriptional activity, protein-protein interactions, protein stability and/or protein sub-cellular localization of key factors involved in myogenesis. It was found that CARM1 interacts with Myogenin and Me2D, whereas PRMT5 can bind Myod, Myogenin and Me2D in vitro [5]. To further elucidate how these enzymes control myogenesis it would be of particular interest to analyze whether they methylate some myogenic factors. Indeed, methylation could modulate the interactions between MRFs or Me2 and specific partner(s), notably transcriptional co-activators/mediators or ATP-dependent chromatin remodeling enzymes to activate transcription on target genes.

In summary, this study demonstrates that CARM1 and PRMT5, two transcriptional co-regulators that have been shown respectively to act positively and negatively on proliferation in cell-based assays, are both inhibiting proliferation and are required for myogenic differentiation in a whole organism. However, they play distinct roles in myogenesis: PRMT5 regulates slow and fast fiber formation by controlling early myogenic genes (myod and myf2) as well as myogenin, whereas CARM1 is required for fast fiber formation by regulating myogenin expression. Interestingly, our data also show that if CARM1 has no impact on slow fiber formation it is required for their proper localization.

**Methods**

**Ethics Statement**

All embryos were handled according to relevant national and international guidelines. French Ministry of Agriculture approved the protocols in this study, with approval ID: B-31-555-10.

**Embryos, synthetic mRNAs, morpholinos and injections**

Zebrafish were raised according to standard procedures [29]. Injections were performed at one-cell stage with 250 pg of CARM1 RNA [6], 250 pg of PRMT5 RNA [30] and 6 ng of the indicated morpholinos (Gene-tools). Morpholino sequences were: PRMT5 5'-AGGCCCATCTGGTTAGGAGCAGAAGATG-3'; CARM1 5'-GAAGAAACGACGGACCCG CATCTTCAGGAGACCT-3'; Control 5'-CCTCTTACCTCAGTTCACAATTTTATA-3'.

**Whole-mount in situ hybridization, immunostaining and image acquisition**

Whole-mount in situ hybridization and antibody staining were performed according to standard protocols. In situ hybridization was performed with digoxigenin-labeled probes transcribed from plasmids containing cDNA for sp7 [31]; myod and myogenin [22], myf5 [32], myf2c [33], smyhc1 [34] and mld2f [35]. zCARM1 and zPRMT5 anti-sense probes were obtained by sub-cloning their corresponding ESTs BC078292 and BC095362 respectively (Open Biosystems, Huntsville, AL, US), into pBluescript KS (Stratagene, La Jolla, CA, US) using EcoRI/HindIII (zCARM1) and XmaI/HindIII (zPRMT5) and were transcribed with T3 polymerase. For cross-sections, embryos were embedded in 3% polyvinyl alcohol in glycerol and mounted in Moviol and images were recorded on a microscope (NIKON Eclipse 80i) using a 20× plan Apo na 0.5 or a 40× plan Apo na 1 with the NIS-element AR 2.30 software; or on a confocal microscope (TCS SP5, Leica Microsystems) with a 20× plan Apo na 0.7 objective (zoom ×4) using the scanner resonant mode. Confocal images are stacks of the anterior somites of 14 or 18 ss embryos as stated in figure legends. For whole embryos, imaging was performed using a sterescroscope (Leica MZ FL III) with the ACT-1C software.

**RNA extraction and reverse transcription**

Total RNAs were extracted from 25 embryos at 14 ss or 18 ss as stated in figure legends with the RNaseq mini kit (Qiagen, Valencia, CA, US), eluted in RNase free water and reverse-transcribed with the Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, US) according to the supplier’s instructions.

**Quantitative PCR**

Q-PCR analyzes were performed on an i-Cycler device (Biorad, Hercules, CA, US) with the SYBR greenJumpStart Taq Ready mix (Sigma Aldrich, St. Louis, MO, US), according to the manufacturer’s instructions. All experiments included a standard curve. Samples were analyzed in triplicates and the mean and standard deviation were calculated. Samples were normalized to the number of EF1 mRNA copies. Primer sequences were the following: myod fw 5'-GCCAAAAGTAGGAGATTCTGTA-3'; rev 5'-GCCCATAAATTCATCATGTC-3'; myf5 fw 5'-GAGAGCATGGTGTGGACTGC-3'; rev 5'-GAATCCTTCTGGTGGAGA-3'; myogenin fw 5'-AAACCATCTCCATGTCGACG-3'; rev 5'-GGGTTCATCAATGGTGCCTC-3'; Mef2c fw 5'-GGTGCTCCTGACGGGAACACATG-3'; rev 5'-GCCCACTATTCCTCCAGGTA-3'; EF1-alpha fw 5'-GATGCAGCCCCAGTGTCCTCAGA-3'; rev 5'-TGATGACCTGAGCGTTGAAG-3'.

**Western Blotting**

Whole cell extracts from 25 zebrafish embryos were classically prepared in 50 µl Laemmli sample buffer. 10 µl of each sample (5 embryos) were loaded per lane and subjected to SDS-PAGE. The following commercial antibodies were used: anti-CARM1 (US Biologicals, P9004-20C), anti-PRMT5 (Upstate, 07-405), anti-alpha-Tubulin (Sigma, T9026), anti-Rabbit IgG HRP conjugate (Promega, W1011) and anti-Mouse IgG HRP conjugate (Promega, W4021).

**Supporting Information**

**Figure S1 PRMT5 and CARM1 regulate myogenic factor expression.** (A-B) In situ hybridization of embryos with the indicated mRNA probe and injected with the indicated Mo (A) at the shield stage or (B) at 14 ss. (A) Animal view (dorsal to right). (B) Dorsal flat-mounted embryos stained for myod with a magnified region to the right. (TIF)

**Figure S2 PRMT5 and CARM1 regulate specifically myogenic factors expression at 14-somite stage (14 ss).** Whole-mount in situ hybridization of embryos injected at one-cell stage with the indicated morpholino (Mo). Experiments were done twice with n = 20 for each condition. Embryos were collected at 14 ss and were analyzed for myogenic factors expression by (A)
in situ hybridization, lateral view, anterior to the left or by (B) real time PCR with standard deviations relative to a control morpholino. Q-PCR procedures are detailed in the methods section. (A) Asterisk, anterior expression of myf5 in CARM1 morphant; arrow, down regulation of myogenin expression in the posterior somites in PRMT5 morphant. (B) Error bars represent standard deviations. *, P<0.01; **, P<0.001; ***, P<0.0001; ns, not statistically significant.

(TIF)

Figure S3 Knock down of CARM1 or PRMT5 does not affect their mutual expression. One-cell stage embryos injected with either a control morpholino (MoCt), or a morpholino against PRMT5 (MoP5) or against CARM1 (MoC1) (left panels). Mos were co-injected with either PRMT5 mRNA (MoP5+P5) or CARM1 (MoC1+C1) (right panels). Embryos were collected at 14-somite stage and analyzed for CARMI and PRMT5 expression by in situ hybridization. Note that PRMT5 expression is strongly enhanced in MoP5-injected embryos (white asterisk), which can be rescued by the co-expression of PRMT5 mRNA (black asterisk).

(TIF)

Figure S4 PRMT5 and CARM1 mRNAs rescue specifically myogenin expression affected by their cognate morpholino(*). One-cell stage embryos were injected with either a morpholino control (MoCt), or a morpholino against PRMT5 (MoP5) or CARM1 (MoC1), alone or in combination with either PRMT5 or CARM1 mRNA. Embryos were collected at 14-somite stage and were analyzed for myogenin expression by in situ hybridization. Experiments were done twice (n = 22 for each condition).

(TIF)

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Author Contributions

Conceived and designed the experiments: LV JB. Performed the experiments: JB CD. Analyzed the data: LV JB. Contributed reagents/materials/analysis tools: LV JB CD. Wrote the paper: LV JB.
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