Neurobiology of Disease 94 (2016) 245–258

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Disruption of snRNP biogenesis factors Tgs1 and pICln induces phenotypes that mirror aspects of SMN-Gemins complex perturbation in *Drosophila*, providing new insights into spinal muscular atrophy

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ARTICLE INFO

Article history:
Received 26 October 2015
Revised 20 June 2016
Accepted 27 June 2016
Available online 4 July 2016

Keywords:
SMN complex
snRNP assembly
Unrip
Strap
wmd
pICln
trimethylguanosine synthase 1
Tgs1
Gemin2
Gemin3
spinal muscular atrophy
motor neuron disease

ABSTRACT

The neuromuscular disorder, spinal muscular atrophy (SMA), results from insufficient levels of the survival motor neuron (SMN) protein. Together with Gemins 2–8 and Unrip, SMN forms the large macromolecular SMN-Gemins complex, which is known to be indispensable for chaperoning the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs). It remains unclear whether disruption of this function is responsible for the selective neuromuscular degeneration in SMA. In the present study, we first show that loss of wmd, the *Drosophila* Unrip orthologue, has a negative impact on the motor system. However, due to lack of a functional relationship between wmd/Unrip and Gemin3, it is likely that Unrip joined the SMN-Gemins complex only recently in evolution. Second, we uncover that disruption of either Tgs1 or pICln, two cardinal players in snRNP biogenesis, results in viability and motor phenotypes that closely resemble those previously uncovered on loss of the constituent members of the SMN-Gemins complex. Interestingly, overexpression of both factors leads to motor dysfunction in *Drosophila*, a situation analogous to that of Gemin2. Toxicity is conserved in the yeast *S. pombe* where pICln overexpression induces a surplus of Sm proteins in the cytoplasm, indicating that a block in snRNP biogenesis is partly responsible for this phenotype. Importantly, we show a strong functional relationship and a physical interaction between Gemin3 and either Tgs1 or pICln. We propose that snRNP biogenesis is the pathway connecting the SMN-Gemins complex to a functional neuromuscular system, and its disturbance most likely leads to the motor dysfunction that is typical in SMA.

1. Introduction

The neuromuscular disorder, spinal muscular atrophy (SMA), is the leading genetic cause of infant death. Hallmark features include loss of spinal motor neurons as well as atrophy of the proximal limb and intercostal muscles. Available therapies are, at best, palliative. In the majority of cases, SMA is the result of insufficient levels of the ubiquitously-expressed survival motor neuron (SMN) protein (Burghes and Beattie, 2009; Monani and De Vivo, 2014). Together with Gemins 2–8 and Unrip, SMN forms the large macromolecular SMN-Gemins complex that is indispensable for chaperoning a key step in the biogenesis of small nuclear ribonucleoproteins (snRNPs), the core constituents of the spliceosome (Cauchi, 2010). It has recently been proposed that this function forms part of a broader role by the SMN-Gemins complex in RNP exchange (So et al., 2016). Whether SMA results from a disruption in snRNP biogenesis and the consequential pre-mRNA missplicing of an ensemble of genes that are critical for the function of the neuromuscular system is still unclear (Burghes and Beattie, 2009; Li et al., 2014; Workman et al., 2012). Predominantly supported by the localisation of its constituent members in transport granules within neuronal processes, the SMN-Gemins complex has been implicated in the axonal trafficking of mRNAs and an alternative hypothesis proposing that disruption of this non-canonical function is responsible for SMA's signature features has gained traction in recent years (Burghes and Beattie, 2009; Fallini et al., 2012).

The production cycle of Sm-class snRNPs involves a cytoplasmic phase in which the SMN-Gemins complex collaborates with the protein arginine methyltransferase 5 (PRMT5) complex to regulate the coupling of a heptameric ring of Sm proteins with small nuclear RNAs (snRNAs) thereby generating the snRNP core structure. Key events in this process were unravelled through extensive biochemical and structural studies *in vitro*. In the early assembly phase, nascent Sm proteins are sequestered by the PRMT5 complex, which unites WD45/MEP50, PRMT5 and

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Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2016.06.015
0969-9961/

pICln. A bound Sm protein subset (B/B′, D1 and D3) is symmetrically dimethylated on designated arginine residues by PRMT5 and, possibly PRMT7, a modification thought to enhance their affinity for the SMN-Gemins complex (Fischer et al., 2011). The conclusion of this phase is marked by the formation of two distinct Sm protein sub-complexes each sharing pICln, which prevents premature RNA interactions (Chari et al., 2008; Grimm et al., 2013).

Acceptance by the SMN-Gemins complex of the pre-organised Sm proteins from the pICln-D1/D2/F/E/G and pICln-B/D3 intermediates in parallel with the simultaneous dismissal of pICln propels the reaction into the late assembly phase (Chari et al., 2008; Grimm et al., 2013). Gemin2 handles the majority of Sm proteins by wrapping itself around the crescent-shaped Sm D1/D2/F/E/G pentamer and blocking RNA binding capacity until delivery of nuclear-exported snRNAs by Gemin5 (Battle et al., 2006; Grimm et al., 2013; Lau et al., 2009; Yong et al., 2010; Zhang et al., 2011). Unrip is required for Sm ring closure, during which the Sm B/D3 dimer replaces the Gemin6/Gemin7 dimer, a structurally-similar temporary surrogate around which the Sm pentamer might be initially arranged (Ma et al., 2005; Ogawa et al., 2009). The remodelling of RNA, and, eventually, RNP molecules during the assembly reaction is most probably fulfilled by DEAD-box RNA helicase Gemin3 (Charroux et al., 1999; Yan et al., 2003), although precise information in this regard is lacking. Prior to nuclear import, the 7-methylguanosine (m^7G) cap of assembled snRNPs is hypermethylated to a 2,2,7-trimethylguanosine (TMG) cap (Mouaikel et al., 2002), which along with the Sm ring acts as a nuclear-localisation signal (Fischer et al., 2011). The key player in this process is trimethylguanosine synthase 1 (Tgs1), which is thought to be recruited by the SMN-Gemins complex (Mouaikel et al., 2003).

The fruit fly *Drosophila melanogaster* is an attractive model system to study the *in vivo* function of human orthologues (Bellen and Yamamoto, 2015). To this end, we have previously shown that SMN and its Gemin associates participate in a common pathway that is essential for the correct function of the motor system (Borg and Cauchi, 2013; Borg et al., 2015). In contrast to the elaborate nine-membered human version, *Drosophila* is thought to possess a simpler SMN-Gemins complex that includes SMN, Gemin2, Gemin3 and Gemin5 as its constituent members (Cauchi et al., 2010). Bioinformatic analyses exclude the presence of additional orthologues with the exception of Unrip (also known as serine-threonine kinase receptor-associated protein or Strap) (Cauchi, 2010). In the present study, we first sought to investigate whether wmd, the *Drosophila* Unrip orthologue, has similar tissue-specific requirements as those reported for other members of the SMN-Gemins complex. Interestingly, we find that loss of wmd/Unrip function in the motor unit gives rise to viability and/or motor defects. However, we could detect neither a functional relationship nor a direct association between wmd/Unrip and Gemin3, hence, raising the possibility that wmd/Unrip works outside of the SMN-Gemins complex in *Drosophila* and was only added to the complex later in evolution.

We next asked whether the involvement of the SMN-Gemins complex in snRNP biogenesis is imperative for a functional neuromuscular system *in vivo*. To this end, we examined phenotypes resulting from the disruption of Tgs1 or pICln, two cardinal players in the snRNP biogenesis pathway, which have never been directly linked to axonal RNA metabolism. Intriguingly, we uncover that similar to SMN-Gemins complex members, both snRNP biogenesis factors are required for normal motor behaviour. Furthermore, overexpression of either Tgs1 or pICln in a pan-muscular pattern in wild-type flies has deleterious effects on adult viability and neuromuscular activity, a situation analogous to that reported for Gemin2 (Borg et al., 2015). Toxicity is conserved in the yeast *Schizosaccharomyces pombe (S. pombe)*, in which we find that the cytoplasmic retention of Sm proteins, likely indicating a block in snRNP biogenesis, is a contributing factor. Importantly, we show a strong genetic interaction and a physical association between Tgs1 or pICln and Gemin3. Our results provide convincing evidence favouring snRNP biogenesis as the pathway connecting the SMN-Gemins complex to optimal neuromuscular performance.

2. Results

2.1. wmd, the Drosophila orthologue of Unrip, is an essential gene

The *Drosophila* orthologue of WD-repeat protein Unrip has a high degree of homology to its vertebrate counterparts including human (55% identity; 80% similarity) throughout the entire length of the protein (Supplementary information, Fig. S1). Based on the improper lamination of dorsal and ventral wing surfaces observed in homozygotes with a *P*-element insertional mutation, Dworkin and Gibson (2006) named the gene *wing morphogenesis defect* or *wmd*. Knockdown of wmd/Unrip through the expression of an RNAi transgene (*wmd-IR^{STARK}*) targeting the 5′ untranslated region (UTR) shows that a ubiquitous reduction of wmd function leads to lethality. This outcome is obvious either when the high-expressing *α-Tub*-GAL4 driver is used or, in case of the low-expressing *da*-GAL4 driver, at culture temperatures that permit maximal GAL4 activity (29 °C) (Fig. 1A). As evidence of the specificity of the RNAi-based knockdown, we first demonstrate that escapers, in which RNAi is driven by *da*-GAL4 at lower culture temperatures (25 °C), have wing defects that are similar to those of flies that are transheterozygous for *wmd^{Matt}*, a 5′ UTR *P*-element insert (Supplementary information, Fig. S2), and a chromosomal deficiency (*Df[2R]BSC661*) that covers *wmd* amongst other genes (Fig. 1B–D). Secondly, expression of *wmd* mRNA was dramatically reduced in larvae with an RNAi-induced knockdown in all tissues (*da-GAL4 > wmd-IR-^{STARK}*) compared to controls (*da-GAL4/+*). No effect on the expression level of the housekeeping control *tat-binding protein-1* (*tbp-1*) was observed in larvae of either genotype (Fig. 1E).

2.2. wmd/Unrip is required for normal motor behaviour

We next asked whether wmd is required in the motor system. We found this to be the case. Hence, the lethality or reduced adult viability attributed to ubiquitous wmd knockdown can be recapitulated when knockdown is restricted to muscle tissues via the strong pan-muscular *how*-GAL4 and *C179*-GAL4 drivers at maximal GAL4 activity (29 °C). The phenotype is, however, more pronounced when RNAi driven by the pan-muscular *Mef2*-GAL4 driver, is enhanced by extra levels of Dicer-2 (Fig. 1A). Turning to the central nervous system (CNS), we show that pan-neuronal wmd reduction via the *elav*-GAL4 driver in combination with increased Dicer-2 levels results in lethality when flies are cultured at 29 °C. Since at lower temperatures flies with the same genotype (*elav-GAL4 > Dcr-2 + wmd-IR^{STARK}*) are adult viable, they were analysed for defects in motor function via a flight assay in which the height a fly falls in a cylinder determines its flight performance. Intriguingly, we show that flies exhibit an age-dependent progressive decline in flight performance. In this regard, starting at day 5 post-eclosion, a significant number of organisms are non-fliers, which do not stick to the walls of upper sectors, hence, dropping to lower sectors (Fig. 2A). Survival of the organisms throughout adulthood was also significantly affected (Fig. 2B). We have recently reported that muscle-restricted changes in the levels of SMN, Gemin2 or Gemin5 precipitate motor and viability defects associated with the Gemin3 hypomorph *Gem3^{BART}* (Borg et al., 2015). In this context, enquiring whether the same is also true for wmd, we found that in combination with Gem3-^{BART}, wmd knockdown in muscle had no effect on flight ability (Fig. 2A) or lifespan (data not shown) throughout adulthood. Despite the lack of a genetic interaction between *wmd/Unrip* and *Gemin3*, overall, these findings reveal a key requirement for wmd/Unrip in the motor system.

2.3. Depletion of Tgs1 in either muscle or neurons has a deleterious effect on adult viability

Tgs1 is a highly conserved enzyme that shows the highest homology at its C-terminus, the site of the methytransferase domain