The following full text is a publisher's version.

For additional information about this publication click this link.
https://hdl.handle.net/2066/219190

Please be advised that this information was generated on 2021-02-07 and may be subject to change.
Xrp1 genetically interacts with the ALS-associated FUS orthologue caz and mediates its toxicity

Moushami Mallik1,2,3, Marica Catinozzi1,2,3, Clemens B. Hug4, Li Zhang1,2, Marina Wagner1,2, Julia Bussmann1,2, Jonas Bittern4, Sina Mersmann1,2, Christian Klämbt1, Hannes C.A. Drexler5, Martijn A. Huynen6, Juan M. Vaquerizas4, and Erik Storkebaum1,2,3

Cabeza (caz) is the single Drosophila melanogaster orthologue of the human FET proteins FUS, TAF15, and EWSR1, which have been implicated in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia. In this study, we identified Xrp1, a nuclear chromatin-binding protein, as a key modifier of caz mutant phenotypes. Xrp1 expression was strongly up-regulated in caz mutants, and Xrp1 heterozygosity rescued their motor defects and life span. Interestingly, selective neuronal Xrp1 knockdown was sufficient to rescue, and neuronal Xrp1 overexpression phenocopied caz mutant phenotypes. The caz/Xrp1 genetic interaction depended on the functionality of the AT-hook DNA-binding domain in Xrp1, and the majority of Xrp1-interacting proteins are involved in gene expression regulation. Consistently, caz mutants displayed gene expression dysregulation, which was mitigated by Xrp1 heterozygosity. Finally, Xrp1 knockdown substantially rescued the motor deficits and life span of flies expressing ALS mutant FUS in motor neurons, implicating gene expression dysregulation in ALS-FUS pathogenesis.

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disorder characterized by motor neuron loss, leading to progressive muscle weakness and ultimately complete paralysis and death (Taylor et al., 2016). Mutations in several genes encoding RNA-binding proteins (RBPs) cause familial ALS (FALS), including TDP-43 (Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008), FUS (Kwiatkowski et al., 2009; Vance et al., 2009), TAF15 (Couthouis et al., 2011), EWSR1 (Couthouis et al., 2012), hnRNPA1 and hnRNPA2B1 (Kim et al., 2013b), and matrin-3 (Johnson et al., 2014). Furthermore, TDP-43–positive inclusions are found in sporadic ALS patients (Neumann et al., 2006; Taylor et al., 2016), and inclusions containing either TDP-43 or FUS are a pathological hallmark in ~45% and ~10% of patients with frontotemporal dementia (FTD), respectively (Ling et al., 2013). These findings implicated defects in RNA biogenesis in ALS and FTD pathogenesis.

Of the ALS-associated RBPs, FUS, EWSR1, and TAF15 (FET) proteins are highly homologous proteins that constitute the FET family (Schwartz et al., 2015). The FET proteins are DNA-binding proteins and RBPs involved in gene expression regulation, including transcription, mRNA splicing, and mRNA subcellular localization (Schwartz et al., 2015). Heterozygous mutations in FUS account for ~5% of FALS (Ling et al., 2013), while mutations in TAF15 and EWSR1 are rare (Couthouis et al., 2011, 2012). Most ALS-associated mutations cluster in the nuclear localization signal of FUS, resulting in a shift from a predominantly nuclear to a more cytoplasmic localization, formation of cytoplasmic aggregates, and reduced nuclear FUS levels (Da Cruz and Cleveland, 2011). This suggests that loss of nuclear FUS function may contribute to ALS pathogenesis, although evidence from ALS-FUS mouse models indicates that ALS-FUS mutations also result in a novel toxic function that triggers motor neuron degeneration (Scekic-Zahirovic et al., 2016, 2017; Sharma et al., 2016). Moreover, in FTD with FUS pathology (FTLD-FUS), the three FET proteins are found in pathogenic inclusions, with reduced levels or complete loss of nuclear FET proteins in inclusion-bearing cells, indicating that loss of nuclear FET function may contribute to FTLD-FUS (Neumann et al., 2011; Davidson et al., 2013).

The Drosophila melanogaster gene cabeza (caz) encodes the single fly orthologue of the three human FET proteins. Accordingly, Caz is a predominantly nuclear RBP that contains the functional domains of the human FET proteins (Schwartz et al., 2015). When expressed in mammalian cells, Caz elicits down-regulation of FUS protein levels (Immanuel et al., 1995), while FUS expression in Drosophila rescues caz mutant phenotypes (Wang et al., 2011), indicating functional homology. We
previously generated caz mutant animals, which exhibit pupal lethality because adult flies fail to eclose due to motor deficits (Frickenhaus et al., 2015). In this study, we performed a genetic screen to gain insight into the molecular mechanisms underlying caz mutant phenotypes. Exhaustive screening of ~80% of the Drosophila genome identified Xrp1 as the only gene for which heterozygosity could rescue caz mutant phenotypes. Xrp1 encodes a protein containing an AT-hook DNA-binding domain often found in proteins involved in chromatin remodeling, transcriptional regulation, and DNA repair (Reeves, 2010). Xrp1 expression was increased in caz mutants, and neuron-selective knockdown of Xrp1 was sufficient to rescue caz mutant phenotypes. Importantly, the DNA-binding capacity of the AT-hook domain of Xrp1 was required to mediate caz mutant phenotypes, and caz mutants displayed substantial gene expression dysregulation, which was significantly mitigated by heterozygosity for Xrp1. Finally, Xrp1 knockdown in motor neurons rescued phenotypes induced by ALS mutant FUS expression, underscoring the potential relevance of our findings for human disease. Together, we propose that caz mutant phenotypes are mediated by up-regulation of Xrp1, leading to gene expression dysregulation and neuronal dysfunction.

Results
A genetic screen to identify suppressors of caz mutant phenotypes
We previously generated two independent caz null alleles: (1) caz2, an imprecise excision allele, and (2) cazKO, generated by homologous recombination (Frickenhaus et al., 2015). Caz mutants die during the pupal stage due to motor incapability resulting in pharate adults failing to eclose from the pupal case. This phenotype was used to perform a dominant suppressor screen whereby males carrying chromosomal deficiencies were crossed to caz2 heterozygous females. Since caz is on the X chromosome, this approach allowed us to screen for genes on the second and third chromosomes for which hemizygosity would rescue the pupal lethality of caz2 males (Fig. 1A). This screen yielded only a single deficiency that rescued caz2 pupal lethality, Df(3R)ED2 (Fig. 1B).

Fine mapping using smaller overlapping deficiencies reduced the number of candidate genes to 11 (Fig. 1C). As Df(3R)Exel6181 and Df(3R)Exel6182 are neighboring but nonoverlapping deficiencies that have a common break point in Xrp1, the fact that heterozygosity for either of these deficiencies rescued caz2 pupal lethality suggested that heterozygous loss of Xrp1 may mediate the rescue (Fig. 1B and C).

Heterozygosity for Xrp1 rescues caz mutant pupal lethality
Xrp1 is predicted to encode seven alternative transcripts (Fig. S1A), four of which can be translated into a 668-aa “long” isoform (Xrp1Long) and the remaining three into a 406-aa “short” isoform (Xrp1Short). We generated two Xrp1 deletion alleles: Df(3R)Xrp1Plus is a ~25-kb deletion of Xrp1, Mpc1, and part of CG42613 (Fig. 1D), whereas Xrp1Ex-long selectively deletes two thirds of Xrp1, expected to abolish expression of all Xrp1Long transcripts, but possibly leaving expression of a Xrp1Short transcript (Xrp1-RD) intact (Fig. 1E). In addition, we used in vivo homologous recombination to generate the Xrp1KO allele, in which the entire Xrp1 coding region is precisely deleted (Figs. 1F and S1C).

All three Xrp1 mutant alleles were homozygous viable, and quantitative PCR (qPCR) using primers that selectively detect Xrp1Long revealed loss of Xrp1Long transcript in homozygous Df(3R)Xrp1Plus, Xrp1Ex-long, and Xrp1KO animals (Fig. 1G). qPCR using primers detecting all Xrp1 isoforms revealed loss of Xrp1 transcript in Df(3R)Xrp1Plus and Xrp1KO flies, whereas in Xrp1Ex-long flies, some residual Xrp1 transcript could be detected (~8% of WT levels), presumably reflecting expression of the short Xrp1-RD mRNA isoform (Fig. 1H).

Crossing males heterozygous for either Df(3R)Xrp1Plus, Xrp1Ex-long, or Xrp1KO to caz/FM7 or cazKO/FM7 females revealed that heterozygosity for Df(3R)Xrp1Plus or Xrp1KO rescued caz mutant pupal lethality to a similar extent as Xrp1 deficiencies and a genomic caz transgene, independent of the caz null allele used (Fig. 1I and J). Heterozygosity for Xrp1Ex-long only partially rescued, presumably due to residual Xrp1 expression (Fig. 1I and J). Furthermore, the mild rough eye phenotype previously reported in caz mutant adult escaper flies (Wang et al., 2011) was rescued by Xrp1 heterozygosity. While a genetic interaction was previously reported between caz and TBPH, the Drosophila homologue of TARDBP, encoding TDP-43 (Wang et al., 2011), Xrp1 heterozygosity could not rescue the adult eclosion defect of TBPH mutants (Fig. S2). Thus, loss of 50% of Xrp1 gene dosage is sufficient to rescue pupal lethality induced by loss of caz but not TBPH function.

Xrp1 heterozygosity rescues motor deficits and life span of caz mutant flies
As caz mutant flies display motor deficits (Wang et al., 2011; Frickenhaus et al., 2015), we evaluated motor performance of caz mutant Xrp1 heterozygous flies using an automated negative geotaxis climbing assay (Niehues et al., 2015). While we failed to obtain caz mutant adult escapers, Xrp1 heterozygous caz mutant flies managed to climb the wall of a test vial, although their climbing speed was still significantly reduced as compared with control animals (Fig. 2A). Analysis of larval locomotion revealed that caz mutant third instar larvae display a significantly reduced crawling speed, which was fully rescued by heterozygosity for Xrp1 (Fig. S3A).

Evaluation of life span revealed that Xrp1 heterozygosity resulted in a substantial rescue of life span as compared with pupal lethality of caz2 and cazKO flies, although the life span of rescued caz mutants was still shorter than control flies (Fig. 2B). Thus, heterozygosity for Xrp1 partially but substantially rescues caz mutant motor performance and life span.

Selective knockdown of Xrp1 in neurons rescues caz mutant phenotypes
Selective reintroduction of Caz in neurons was shown to rescue caz mutant phenotypes (Wang et al., 2011; Frickenhaus et al., 2015), while selective inactivation of caz in neurons resulted in severe motor deficits and reduced life span (Frickenhaus et al., 2015), indicating that loss of caz function in neurons is both necessary and sufficient to induce caz mutant phenotypes.
We therefore evaluated whether selective knockdown of Xrp1 in neurons was sufficient to rescue caz mutant phenotypes despite the fact that the FlyAtlas and modEncode databases report Xrp1 expression in all tissues throughout development and adult life. Two independent transgenic Xrp1-RNAi lines revealed that selective knockdown of Xrp1 in neurons (elav-GAL4) fully rescued caz mutant pupal lethality (Fig. 2C) and partially rescued caz mutant motor performance for cazKO even to a similar extent as neuronal Caz reintroduction (Fig. 2D). Importantly, selective knockdown of Xrp1 in motor neurons (D42-GAL4) was sufficient to fully rescue the reduced crawling speed of caz mutant larvae (Fig. S3B), indicating that the larval locomotion deficit is attributable to dysfunction of motor neurons. Finally, the median life span of caz mutant flies with neuronal Xrp1 knockdown ranged from 30% to 50% of the life span of their respective controls (Fig. 2, E and F), comparable with the life span of Xrp1 heterozygous caz mutants. To evaluate the level of knockdown induced by the two Xrp1-RNAi lines, qPCR revealed residual Xrp1 transcript levels in Xrp1 mutant lines relative to WT controls (100%) determined by qPCR using primers either selectively detecting Xrp1 sharply isoform (G) or detecting all Xrp1 transcripts (H). n = 10. ***, P < 0.0001; one-way ANOVA. Mean ± SEM. (I and J) Frequency of adult male offspring from the indicated crosses that is heterozygous for the indicated Xrp1 allele. n > 87 per genotype. *, P < 0.05; ***, P < 0.0001; χ² test.
Increased Xrp1 expression mediates caz mutant phenotypes

Our finding that reducing Xrp1 expression rescues caz mutant phenotypes raised the possibility that these phenotypes are caused by up-regulation of Xrp1 expression, with particularly deleterious effects in neurons. Consistent with this hypothesis, qPCR revealed that Xrp1 mRNA levels are increased three- to fourfold in both CNS and body wall of caz mutants (Figs. 3 A and S4, B and C). Remarkably, in caz mutant Xrp1 heterozygotes, Xrp1 transcript levels were not significantly different from controls (Fig. 3 A). Thus, phenotypic rescue of caz mutants by Xrp1 heterozygosity is associated with normalization of Xrp1 expression levels. Vice versa, ubiquitous caz overexpression from the adult stage onwards did not reduce Xrp1 mRNA levels (Fig. 3 B). To evaluate whether Xrp1 gene dosage modifies caz expression levels, qPCR and Western blotting was performed on Xrp1 mutants and transgenic flies ubiquitously overexpressing Xrp1Long or Xrp1Short. These analyses revealed that Caz levels are not significantly changed in Xrp1 mutants (Fig. 3, C, E, and F) or upon Xrp1 overexpression (Fig. 3 D). Thus, loss of caz increases Xrp1 expression, but Caz overexpression does not down-regulate Xrp1, and alteration of Xrp1 levels has no effect on caz expression.

Xrp1 is a nuclear protein that binds chromatin

The Xrp1 protein is predicted to contain two conserved DNA-binding domains in its C terminus: (1) an AT-hook motif consisting of nine amino acids centered on the invariant tripeptide glycine-arginine-proline (Reeves, 2010) and (2) a basic-region leucine zipper (bZIP) motif found in the bZIP family of transcription factors, which typically consists of a basic region of ∼20 aa that mediates sequence-specific DNA binding along with a leucine zipper, a sequence of 40–60 hydrophobic amino acids in which leucine occurs every seventh residue, which mediates dimerization (Vinson et al., 2002). Despite the fact that Xrp1 was reported to heterodimerize with the bZIP protein Irbp18 (Francis et al., 2016), coimmunoprecipitation experiments on extracts of otherwise WT flies. Neuronal overexpression of either Xrp1Long or Xrp1Short induced developmental lethality, with a fraction of adult escapers emerging (Figs. 3 G and S4 D). These adult escapers displayed substantial motor performance deficits (Figs. 3 H and S4 E) and a significantly shortened life span (Figs. 3 I and S4 F). Thus, neuronal Xrp1 overexpression phenocopies caz mutant phenotypes. Together with the findings that Xrp1 heterozygosity or Xrp1 neuronal knockdown rescue caz mutant phenotypes, these results indicate that increased neuronal Xrp1 levels mediate caz mutant phenotypes.
Drosophila S2 cells cotransfected with N-terminal HA-tagged and Flag-tagged variants of either Xrp1<sup>Long</sup> or Xrp1<sup>Short</sup> indicated that Xrp1 does not homodimerize (Fig. S4, G and H).

As Ensembl and NCBI Blastp searches failed to identify a human Xrp1 orthologue, we used HHpred (Zimmermann et al., 2018), the most sensitive homology detection tool, to identify human Xrp1 homologues. This yielded human homologues for the C-terminal ∼150 aa of Xrp1 that contain the conserved DNA-binding domains (Table S1). Although these human proteins all contain a bZIP domain, they do not appear to be Xrp1 orthologues, as reciprocal searches against Drosophila proteins using HHpred with the “best hits” from human, a reliable method to detect orthologues whose sequence homology is not apparent with pairwise searches (Szklarczyk et al., 2012), did not uncover Xrp1. Furthermore, none of the human Xrp1 homologues contained an AT-hook motif, and in fact, none of the bZIP proteins in the SMART database (Letunic and Bork, 2018) contained an AT-hook motif.

Consistent with the presence of two putative DNA-binding domains and its reported roles in protection against genotoxic stress and DNA repair (Brodsky et al., 2004; Akdemir et al., 2007; Francis et al., 2016), subcellular localization experiments revealed that Xrp1 is localized to the nucleus, where it colocalizes with Caz, both in Drosophila S2R+ cells and in motor neurons in vivo (Fig. 4, A–C). To evaluate whether Xrp1 binds chromatin, immunostaining for Xrp1 was performed on polytene chromosomes from larval salivary glands. Xrp1 was found to preferentially localize to euchromatic bands and to “puffs,” enlarged regions...
which indicate sites of active transcription (Fig. 4 D), suggesting a possible involvement of Xrp1 in regulation of gene expression. Furthermore, Xrp1 also localized to centromeric β-heterochromatin (Fig. 4 D). Caz prominently localized to puffs on polytene chromosomes (Fig. 4 E), and overall, Caz and Xrp1 displayed a distinct binding pattern with some overlap (e.g., arrowhead in Fig. 4, D and E).

**Xrp1-interacting proteins suggest a role in gene expression regulation**

To obtain a comprehensive overview of the molecular processes in which Xrp1 may be involved, we immunoprecipitated N-terminal Flag-tagged Xrp1 (either short or long isoform) from *Drosophila* S2 cells and identified interacting proteins by mass spectrometry (MS; Fig. 5 A). 106 Xrp1long-interacting proteins and 40 Xrp1short-interacting proteins were identified (Tables S2 and S3). The substantially higher number of Xrp1long-interacting proteins is likely attributable to its 262 additional N-terminal amino acids. Importantly, of the 40 Xrp1short-interacting proteins, 33 were also identified as Xrp1long-interacting proteins (Fig. 5 B). In addition, consistent with heterodimer formation between Xrp1 and Irbp18 (Francis et al., 2016), Irbp18 was identified as an Xrp1-interacting protein (Fig. 5 C and Table S2). Remarkably, out of a total of 112 Xrp1-interacting proteins, 62 (55.4%) are involved in gene expression regulation or DNA/RNA metabolism, including regulation of transcription (15), chromatin organization (12), DNA metabolism (9), DNA repair (5), RNA metabolism (14), and DNA-binding proteins (3) and RBPs (4; Fig. 5 C).
Following a similar experimental approach, we immuno-precipitated endogenous Caz from S2 cells and identified 88 Caz-interacting proteins (Table S4). Remarkably, 58 of these proteins (65.9%) were also identified as Xrp1-interacting proteins (Fig. 5 B), suggesting that Caz and Xrp1 are involved in similar molecular processes and/or commonly reside in protein complexes. The latter possibility is unlikely, as Caz did not coimmunoprecipitate with Xrp1 (Fig. S4 I).

The caz-Xrp1 genetic interaction depends on the functionality of the AT-hook domain of Xrp1

We next wanted to evaluate whether the rescue of caz mutant phenotypes by Xrp1 heterozygosity depends on the functionality of the AT-hook DNA-binding domain of Xrp1. We therefore evaluated whether a mild increase of Xrp1 levels in “rescued” Xrp1 heterozygous caz mutant flies would revert the rescue and result in pupal lethality, and if so, whether expression of Xrp1 with a subtle mutation in the AT-hook motif that precludes DNA binding would still revert the rescue.

As neuron-selective expression of Xrp1 from the standard pUAST transgenesis vector induces phenotypes by itself (Fig. 3, G–I; and Fig. S4, D–F), we used a modified pUAST vector with only three UAS sites, known to result in lower transgene expression levels (Fig. 6 A; Pfeiffer et al., 2010). The resulting 3×UAS-Xrp1 lines did not induce developmental lethality when selectively expressed in neurons (elav-GAL4; Fig. 6 C). Neuronal Xrp1 expression from 3×UAS transgenes was nevertheless able to revert the rescue of caz mutant pupal lethality by Xrp1 heterozygosity (Fig. 6, D and E). We therefore inactivated the Xrp1 AT-hook domain in the 3×UAS constructs by mutagenizing the RGR triplet of the KRK RGR PAK motif to AAA, known to abolish AT-hook–mediated DNA binding (Fig. 6 B; Metcalf and Wassarman, 2006; Turlure et al., 2006; Baker et al., 2013). The subtle AT-hook mutation altered the binding pattern of Xrp1 on polytene chromosomes (Fig. S3 C) but did not reduce the stability of the Xrp1 protein and in fact increased Xrp1 protein level (Fig. S1, D–F). In spite of this, AT-hook–mutant UAS-Xrp1 transgenes were no longer able to revert the rescue of caz mutant pupal lethality by Xrp1 heterozygosity (Fig. 6, D and E). These data demonstrate that the genetic interaction between caz and Xrp1 is dependent on the functionality of the AT-hook DNA-binding motif in Xrp1.

Gene expression dysregulation in caz mutants is rescued by Xrp1 heterozygosity

FUS is known to be involved in transcriptional regulation and miRNA splicing (Schwartz et al., 2012, 2015; Tan et al., 2012; Yang et al., 2016; Baker et al., 2016). As neuron-selective expression of Xrp1 from the standard pUAST transgenesis vector induces phenotypes by itself (Fig. 3, G–I; and Fig. S4, D–F), we used a modified pUAST vector with only three UAS sites, known to result in lower transgene expression levels (Fig. 6 A; Pfeiffer et al., 2010). The resulting 3×UAS-Xrp1 lines did not induce developmental lethality when selectively expressed in neurons (elav-GAL4; Fig. 6 C). Neuronal Xrp1 expression from 3×UAS transgenes was nevertheless able to revert the rescue of caz mutant pupal lethality by Xrp1 heterozygosity (Fig. 6, D and E). We therefore inactivated the Xrp1 AT-hook domain in the 3×UAS constructs by mutagenizing the RGR triplet of the KRK RGR PAK motif to AAA, known to abolish AT-hook–mediated DNA binding (Fig. 6 B; Metcalf and Wassarman, 2006; Turlure et al., 2006; Baker et al., 2013). The subtle AT-hook mutation altered the binding pattern of Xrp1 on polytene chromosomes (Fig. S3 C) but did not reduce the stability of the Xrp1 protein and in fact increased Xrp1 protein level (Fig. S1, D–F). In spite of this, AT-hook–mutant UAS-Xrp1 transgenes were no longer able to revert the rescue of caz mutant pupal lethality by Xrp1 heterozygosity (Fig. 6, D and E). These data demonstrate that the genetic interaction between caz and Xrp1 is dependent on the functionality of the AT-hook DNA-binding motif in Xrp1.

**Gene expression dysregulation in caz mutants is rescued by Xrp1 heterozygosity**

FUS is known to be involved in transcriptional regulation and miRNA splicing (Schwartz et al., 2012, 2015; Tan et al., 2012; Yang et al., 2016; Baker et al., 2016). As neuron-selective expression of Xrp1 from the standard pUAST transgenesis vector induces phenotypes by itself (Fig. 3, G–I; and Fig. S4, D–F), we used a modified pUAST vector with only three UAS sites, known to result in lower transgene expression levels (Fig. 6 A; Pfeiffer et al., 2010). The resulting 3×UAS-Xrp1 lines did not induce developmental lethality when selectively expressed in neurons (elav-GAL4; Fig. 6 C). Neuronal Xrp1 expression from 3×UAS transgenes was nevertheless able to revert the rescue of caz mutant pupal lethality by Xrp1 heterozygosity (Fig. 6, D and E). We therefore inactivated the Xrp1 AT-hook domain in the 3×UAS constructs by mutagenizing the RGR triplet of the KRK RGR PAK motif to AAA, known to abolish AT-hook–mediated DNA binding (Fig. 6 B; Metcalf and Wassarman, 2006; Turlure et al., 2006; Baker et al., 2013). The subtle AT-hook mutation altered the binding pattern of Xrp1 on polytene chromosomes (Fig. S3 C) but did not reduce the stability of the Xrp1 protein and in fact increased Xrp1 protein level (Fig. S1, D–F). In spite of this, AT-hook–mutant UAS-Xrp1 transgenes were no longer able to revert the rescue of caz mutant pupal lethality by Xrp1 heterozygosity (Fig. 6, D and E). These data demonstrate that the genetic interaction between caz and Xrp1 is dependent on the functionality of the AT-hook DNA-binding motif in Xrp1.
et al., 2014), and knockdown or knockout of Fus in the mouse brain results in gene expression dysregulation (Ishigaki et al., 2012; Lagier-Tourenne et al., 2012; Scekic-Zahirovic et al., 2016). Furthermore, AT-hook proteins are often involved in gene expression regulation either as transcription factors or as chromatin architectural proteins (Reeves, 2010), and our results thus far indicate that Xrp1 is a nuclear chromatin-binding protein likely involved in gene expression regulation. We therefore hypothesized that loss of caz function may result in gene expression dysregulation, which could possibly be mitigated by heterozygosity for Xrp1.

To test this hypothesis, we used RNA sequencing (RNA-seq) to evaluate mRNA expression levels in third instar larval CNS of cazKO and cazKO Xrp1 heterozygous animals as well as Xrp1 heterozygous and WT animals as controls. Principal component analysis and clustering of the samples discriminated the four genotypes from each other, with a certain degree of overlap between Xrp1 heterozygous and WT samples (Fig. S5, A and B). Differential gene expression analysis between mRNA levels from WT and cazKO identified 1,641 up-regulated and 1,605 down-regulated genes in caz mutants (FRD-adjusted P value <0.05; Fig. 7, C and D). Most interestingly, in caz mutant Xrp1 heterozygous CNS, 315 up-regulated and 314 down-regulated genes were identified, with >90% of these displaying a less than twofold change (Fig. 7, C and D). Thus, heterozygosity for Xrp1 significantly mitigated gene expression dysregulation in caz mutant CNS. Principal component analysis confirmed the dramatic gene expression dysregulation in caz mutant CNS, which was significantly rescued in caz mutant Xrp1 heterozygous animals (Fig. S8 A).

Phenotypes induced by motor neuron–selective expression of ALS mutant Fus are substantially mitigated by Xrp1 knockdown

To evaluate the potential relevance of our findings for human ALS, we used a Drosophila ALS-FUS model. Selective expression of R518K mutant human FUS in motor neurons (D42-GAL4) yielded adult flies that developed progressive motor deficits and displayed a substantially shortened life span (Fig. 8, A–D). We therefore evaluated the effect of Xrp1 knockdown on motor behavior and life span of these flies. Coexpression of Xrp1-RNAi with transgenes encoding R518K mutant human FUS or a driver-only control yielded adult flies that developed progressive motor deficits and displayed a substantially shortened life span (Fig. 8, A–D). Therefore, the effect of Xrp1 knockdown on motor behavior and life span of these flies was significantly rescued in caz mutant Xrp1 heterozygous animals (Fig. S8 A).

Up- or down-regulation of a panel of 19 genes was validated by qPCR (Fig. S5 E), and gene ontology (GO) analysis showed that transcripts whose expression was altered in caz mutant animals were enriched for genes involved in processes such as axon and dendrite guidance, peripheral nervous system development, regulation of transcription and mRNA splicing, DNA repair, and mitotic spindle organization and assembly (Fig. 7 E). GO analysis for molecular function revealed that transcripts with altered expression in caz mutants were enriched for mRNAs encoding DNA- and chromatin-binding proteins as well as mRBPs, along with transcription factor activity, among others (Fig. S5 D). Overall, this is in line with the known functions of Caz and its mammalian FET protein orthologues (Schwartz et al., 2015) and strikingly similar to results from RNA-seq experiments in Fus−/− mice (Scekic-Zahirovic et al., 2016). In conclusion, our transcriptome analysis revealed that gene expression dysregulation in caz mutant CNS is substantially mitigated by Xrp1 heterozygosity.
displayed a severe climbing defect (reduction in speed by ~45%), which was rescued by knockdown of Xrp1 to a level that was not significantly different from D42-GAL4>UAS-Xrp1-RNAi control flies (Fig. 8 D). Thus, reduction of Xrp1 levels in motor neurons substantially rescues the motor deficits and shortened life span of a Drosophila ALS-FUS model.

To gain insight into the mechanism underlying this major phenotypic rescue, we evaluated the effect of FUS-R518K overexpression on caz and Xrp1 transcript levels. Ubiquitous overexpression of FUS-R518K in adult female flies moderately reduced caz transcript levels to ~60% of control levels (Fig. 8 E). Strikingly, Xrp1 transcript levels were about threefold increased upon FUS-R518K expression (Fig. 8 F). This substantial increase in Xrp1 expression cannot be attributed to the moderate reduction in caz levels because Xrp1 transcript levels were not altered in caz heterozygous females in spite of a ~50% reduction of caz transcript levels (Fig. 8, G and H). Thus, expression of ALS mutant FUS results in substantial up-regulation of Xrp1 expression independent of caz levels. The fact that Xrp1 knockdown substantially rescues phenotypes induced by ALS mutant human FUS indicates that these phenotypes are to a large extent mediated by increased Xrp1 expression.

**Discussion**

In this study, we identified Xrp1 as a genetic modifier of caz mutant phenotypes. Caz is the single Drosophila orthologue of the three human FET family proteins FUS, EWSR1, and TAF15 (Schwartz et al., 2015). Xrp1 expression was up-regulated by three- to fourfold in caz mutant animals, and heterozygosity for Xrp1 fully rescued the caz mutant eclosion defect and partially but substantially rescued adult motor performance and life span. Exhaustive genetic screening of the second and third chromosome, which together constitute ~80% of the fly genome, identified Xrp1 as the only gene for which reduction of gene dosage by 50% could rescue caz mutant pupal lethality, indicating that Xrp1 is a key modifier of caz mutant phenotypes. Interestingly, in spite of the previously reported ubiquitous expression of Xrp1 (Tsurui-Nishimura et al., 2013) and the fact that Xrp1 expression was not only increased in the CNS but also in the body wall and presumably other tissues of caz mutants, neuron-selective knockdown of Xrp1 was sufficient to rescue caz mutant phenotypes, and selective neuronal overexpression of Xrp1 in otherwise WT animals phenocopied caz mutant phenotypes. This is consistent with the previously reported key function of Caz in...
neurons (Wang et al., 2011; Frickenhaus et al., 2015). Together, our data indicate that caz mutant phenotypes are largely mediated by increased Xrp1 expression, with particularly deleterious effects in neurons (Fig. 9).

Xrp1 has previously been implicated in protection against genotoxic stress and DNA damage repair (Brodsky et al., 2004; Akdemir et al., 2007; Francis et al., 2016). Consistently, a number of Xrp1-interacting proteins are involved in DNA repair (Fig. 5 C), and GO analysis of our RNA-seq data revealed enrichment for genes involved in DNA repair among genes up-regulated in caz mutant CNS (Fig. 7 E). This study revealed a novel role for Xrp1 in gene expression regulation as a substantial number of Xrp1-interacting proteins are involved in regulation of transcription, chromatin organization, and RNA metabolism (Fig. 5 C). Interestingly, neuronal overexpression of the long Xrp1 isoform induced significantly stronger phenotypes as compared with the short isoform (Fig. 3, G–I; and Fig. S4, D–F), despite insertion of the transgenes in the same genomic site and similar expression levels (Fig. 3 B). This is likely attributable to the additional N-terminal 262 aa of the long isoform, allowing the binding of substantially more interacting proteins (Fig. 5 B and Table S2), which may result in more pronounced gene expression dysregulation and stronger phenotypes.

Consistent with a key role of Xrp1 in gene expression regulation, significant gene expression dysregulation was found in caz mutant CNS, which was substantially mitigated by normalizing Xrp1 levels in caz mutants (Fig. 7). Importantly, of the 3,246 differentially expressed genes in caz mutants, only 489 are still significantly up- or down-regulated caz mutant Xrp1 heterozygotes (Fig. 7 D). The 2,757 genes that are significantly changed in caz mutants but not in caz mutant Xrp1 heterozygotes are likely direct or indirect targets of Xrp1, and up- or down-regulation

Figure 8. Motor neuron–selective Xrp1 knockdown mitigates motor deficits and shortened life span induced by ALS mutant FUS expression. (A and B) Life span of control (driver only) flies and flies with motor neuron–selective (D42-GAL4) expression of human FUS-R518K, Xrp1-RNAi, or both transgenes. Data for male (A) and female (B) flies are shown. n > 75 per genotype. (C and D) Average climbing speed of adult female flies with motor neuron–selective (D42-GAL4) expression of human FUS-R518K, Xrp1-RNAi, or both transgenes versus driver-only controls. Flies were tested at 10 (C) and 16 (D) d of age. n > 100 per genotype. **, P < 0.01; ***, P < 0.005; Mann-Whitney test. (E and F) Transcript levels of caz (E) and Xrp1 (F) in heads of adult female flies 3 d after induction of ubiquitous FUS-R518K expression (tub-GAL4) versus driver-only controls. n = 9–11. ***, P < 0.0005; two-tailed unpaired t test. (G and H) Caz (G) and Xrp1 (H) transcript levels in the CNS of third instar female larvae heterozygous for caz+ or caz+107 versus WT controls. n = 11–13. ***, P < 0.001; one-way ANOVA. All graphs display mean ± SEM.
Mallik et al.

Xrp1 mediates caz mutant phenotypes

of these genes in caz mutants may contribute to neuronal dysfunction. The novel function of Xrp1 in gene expression regulation is likely dependent on the capacity of Xrp1 to bind DNA, presumably mediated by two predicted DNA-binding domains in its C terminus: an AT-hook motif and a bZIP motif. Whereas the functionality of the predicted bZIP motif remains to be investigated, the AT-hook motif of Xrp1 conforms with the consensus sequence, consisting of nine amino acids centered on the invariant tripeptide glycine-arginine-proline (Reeves, 2010). The DNA-binding capacity of the Xrp1 AT-hook motif is likely required to mediate gene expression regulation and dysregulation, as the introduction of a subtle mutation in this motif demonstrated that its functionality is essential to mediate caz mutant phenotypes. Based on our findings, we propose a working model in which caz mutant phenotypes are mediated by increased Xrp1 expression, leading to gene expression dysregulation and neuronal dysfunction (Fig. 9).

Extensive bioinformatic searches did not reveal a clear one-to-one Xrp1 orthologue in mammals. However, we believe that it is highly likely that Xrp1 has functional homologues in mammals. Candidate functional homologues include 27 human genes encoding proteins predicted to contain at least one AT-hook motif (Table S6), including the Rett syndrome gene MECP2 (Amir et al., 1999). Interestingly, DNA binding mediated by the MeCp2 AT-hook domains has been implicated in the pathogenesis of Rett syndrome (Baker et al., 2013), and FUS was reported to bind the MECP2 promotor and positively regulate MECP2 transcription (Tan et al., 2012). In addition, FUS also binds MECP2 mRNA (Lagier-Tourenne et al., 2012), indicating that MECP2 is both a transcriptional and mRNA target of FUS. Furthermore, brains from Fusu−/− mice or transgenic mice overexpressing ALS mutant FUS display up-regulation of Cbx2, Dot1l, Elf3, Prr12, and KMT2B (Scekic-Zahirovic et al., 2016; Shihashi et al., 2016), and 17 of the 27 AT-hook genes are reported FUS RNA targets (Table S6). En route toward identification of human functional homologues of Xrp1, it will be particularly important to gain detailed molecular insight into how Xrp1 regulates gene expression. A first step could be the identification of the genomic binding sites of Xrp1 and its putative target genes. Furthermore, since Xrp1 does not have other predicted functional domains apart from the AT-hook

Figure 9. Xrp1 is a key mediator of caz mutant phenotypes. (A) In WT animals, Caz controls Xrp1 levels, resulting in normal gene expression regulation. (B) Loss of caz function results in substantial up-regulation of Xrp1 expression, leading to gene expression dysregulation and neuronal dysfunction. (C) In caz mutant Xrp1 heterozygous animals, Xrp1 levels are normalized, resulting in rescue of gene expression dysregulation and neuronal dysfunction. (D) Expression of ALS mutant human FUS results in substantial up-regulation of Xrp1 expression, and motor neuron-selective expression induces neuronal dysfunction and reduced life span. (E) Simultaneous knockdown of Xrp1 in motor neurons expressing FUS-R518K rescues neuronal dysfunction and reduced life span.

Downloaded from http://rupress.org/jcb/article-pdf/217/11/3947/1376378/jcb_201802151.pdf by Radboud University user on 20 October 2020
and bZIP domains, it is tempting to speculate that Xrp1 regulates gene expression by recruiting other proteins that contain functional domains such as transactivation or histone-modifying domains to specific genomic sites. Several of the Xrp1-interacting proteins identified in this study contain such functional domains and have human orthologues (e.g., TAF9, ZMYM2, NFX1, HCF1/2, VRK1, RSF1, and BPTF).

Interestingly, gene expression dysregulation was previously implicated in ALS and FTD pathogenesis. For instance, a significant enrichment in de novo mutations in the chromatin regulatory pathway in sporadic ALS patients was reported (Chesi et al., 2013). Furthermore, involvement of the three FET proteins in regulation of transcription and mRNA splicing is well established (Schwartz et al., 2015), and in fact, several other RBPs that have been implicated in ALS and FTD pathogenesis are also known to play important roles in gene expression regulation (Ling et al., 2013). These include TDP-43, involved in regulation of transcription and mRNA splicing (Buratti and Baralle, 2010), which mislocalizes to cytoplasmic inclusions with nuclear clearance in >95% of ALS and 45% of FTD patients (Ling et al., 2013). Furthermore, the ALS-causing expanded hexanucleotide repeat in C9orf72 may be involved in the pathogenesis of human FUSopathies.

For the dominant suppressor screen, deficiencies covering the second and the third chromosome from the Bloomington Deficiency Kit were used as this kit provides maximal coverage (euchromatic coverage ≥ 97.5%) with a minimal number of deletions (Cook et al., 2012). Df/Balancer males were crossed to caz2/FM7 females to screen for the emergence of caz2/Y; Df/+ males in the offspring, which would indicate suppression of pupal lethality of caz2 males by hemizygosity for the deficiency. To narrow down the genomic region that is uncovered by Df(3R) ED2, the deficiency that mediated rescue of caz mutant pupal lethality, molecularly mapped smaller deficiency lines in this region were ordered from the BDSC. PCR genotyping of caz mutant males was used to exclude X chromosome nondisjunction in all experiments.

The UAS-Xrp1-RNAi lines used in this study were P[TRiP, HMS500053]attP2 (34521; BDSC; UAS-Xrp1-RNAi-1) and P[GD9476]v33010 obtained from the Vienna Drosophila Resource Center (UAS-Xrp1-RNAi-2). The UAS-FUS-R518K transgenic line was generated by and obtained from Lanson et al. (2011).

**Materials and methods**

**Drosophila genetics**

Flies were housed in a temperature-controlled incubator with 12:12 h on/off light cycle at 25°C, and for some experiments, at 23°C (5×UAS-Xrp1 overexpression), in vials containing standard cornmeal medium. X chromosome–inserted elav-GALA (458; Bloomington Drosophila Stock Center [BDSC]) was used for panneuronal expression of UAS transgenes, OK371-GALA and D42-GALA were used for targeted expression in motor neurons, and tub-GALA4 was used for ubiquitous expression of UAS transgenes.

For the second and the third chromosome from the Bloomington Deficiency Kit were used as this kit provides maximal coverage (euchromatic coverage ≥ 97.5%) with a minimal number of deletions (Cook et al., 2012). Df/Balancer males were crossed to caz2/FM7 females to screen for the emergence of caz2/Y; Df/+ males in the offspring, which would indicate suppression of pupal lethality of caz2 males by hemizygosity for the deficiency. To narrow down the genomic region that is uncovered by Df(3R) ED2, the deficiency that mediated rescue of caz mutant pupal lethality, molecularly mapped smaller deficiency lines in this region were ordered from the BDSC. PCR genotyping of caz mutant males was used to exclude X chromosome nondisjunction in all experiments.

For generation of a clean Xrp1-null allele, in vivo homologous recombination was used to target the Xrp1 gene. Following a previously published strategy (Vilain et al., 2014), the presence of a Mi[MIC] transposon in the Xrp1 gene (Mi[MIC]Xrp1M10718) was exploited for site-specific insertion of a targeting construct (Figs. 1 and S1 C). For the construction of a targeting vector, a fosmid (FlyFos clone number FF017877 containing the extended Xrp1 genomic region was used to PCR amplify left and right homology arms using the primers Xrp1_LHA_FW, Xrp1_LHA_REV, Xrp1_RHA_FW, and Xrp1_RHA_REV (Table S7). To minimize the chance of introducing mutations during PCR amplification, Phusion high-fidelity DNA polymerase (New England Biolabs) was used with only 20 cycles of PCR amplification. The obtained PCR products were subcloned in a Zero Blunt TOPO PCR cloning vector (Invitrogen) and sequence verified. The presence of a HindIII site in primer Xrp1_LHA_FW and an EcoRI site in primer Xrp1_LHA_REV was subsequently used to clone the left homology arm into pABC (Choi et al., 2009). Next, the presence of an EcoRI site in Xrp1_RHA_FW and a KpnI site in Xrp1_RHA_REV was used to clone the right homology arm into the pABC vector that already contained the left homology arm.

The obtained targeting vector was sequence verified and injected into Mi[MIC]Xrp1M10718 embryos for site-specific integration of the targeting construct into the Mi[MIC] transposable element in the Xrp1 gene. Transgenic lines in which the transgenic construct was integrated into Mi[MIC]Xrp1M10718 in the correct orientation were identified. These lines were subsequently

---

**Journal of Cell Biology**

https://doi.org/10.1083/jcb.201802151
crossed to a transgenic line that expresses I-SceI under the control of a heat-inducible promoter. Given the presence of an I-SceI restriction site in primer Xrp1_LHA_FW, this will induce a double-strand break adjacent to the left homology arm of the targeting construct, allowing for precise removal of the Xrp1 and Mi(MIC) sequences left of the targeting construct through homologous recombination (Fig. S1C). Lines with successful homologous recombination were identified by PCR and sequencing of the obtained PCR fragments. Next, these lines were crossed to a transgenic line that expresses I-CreI under the control of a heat-inducible promoter. Given the presence of an I-CreI restriction site in primer Xrp1_RHA_REV, this will induce a double-strand break adjacent to the right homology arm of the targeting construct, allowing for precise removal of the Xrp1 and Mi(MIC) sequences right of the targeting construct through homologous recombination. Lines with successful homologous recombination were identified by PCR and sequencing of the obtained PCR fragments.

**Generation of UAS-Xrp1 transgenic lines**

For generation of UAS-Xrp1<sup>Long</sup> transgenic lines, RNA was extracted from WT flies and converted into cDNA, which was used as a template for PCR (primer sequences in Table S7) to amplify the transcript coding for the long Xrp1 isoform. Gold clone FI10013 containing the Xrp1<sup>Short</sup>cDNA was obtained from Kyoto Stock Center. Xrp1<sup>Long</sup> and Xrp1<sup>Short</sup> cDNAs were subsequently cloned into pUAST-attB using either NotI or EagI as well as XhoI restriction sites (Table S7). Site-directed PCR mutagenesis was used to generate AT-hook mutant versions of the long and short Xrp1 isoforms (mutagenesis primers are included in Table S7). WT and AT-hook mutant Xrp1 cDNAs were subsequently amplified by PCR using Phusion high-fidelity DNA polymerase (New England Biolabs) and primers containing XhoI and XbaI restriction sites (Table S7). The obtained PCR products were subcloned into the Zero Blunt TOPO PCR cloning vector (Invitrogen), and XhoI and XbaI were used to transfer the Xrp1 cDNAs to the pJFRC4 vector, which contains three UAS sites (Pfeiffer et al., 2010). UAS constructs were embryo injected following standard procedures. For each of the constructs, VK31 (on III) and VK37 (on II) genomic constructs were embryo injected following standard procedures. As neuronal expression of 5×UAS-Xrp1 transgenes in many cases resulted in developmental lethality with no adult escapers when genomic sequences on transgene expression. As neuronal expression of 5×UAS-Xrp1 transgenes were performed at 23°C. For assaying mobility, flies were collected within 24 h after eclosion and divided into groups of 10 individuals. Motor performance of 3-, 10-, or 16-d-old flies was evaluated as described earlier (Frickenhaus et al., 2015; Niehues et al., 2015), and average climbing speed (mm/s) was determined and compared between genotypes. As female D42-GAL4>FUS-R518K flies lived developed and scanned. Densitometric quantification of images was performed with ImageJ/FIJI (National Institutes of Health).

**Western blotting**

For Western blots, protein extracts were made by homogenization of third instar larval CNS in extraction buffer (50 mM Tris/HCl, pH 7.4, 150 mM KCl, 0.25 M sucrose, 5 mM MgCl<sub>2</sub>, and 0.5% Triton X-100). Lysates of Drosophila S2 cells transfected with plasmids encoding actin5C-GAL4 alone or cotransfected with plasmids encoding N-terminal HA-tagged Xrp1<sup>Short</sup> or Xrp1<sup>Long</sup> were prepared in cell lysis buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, and 0.5% NP-40 containing 1 U complete mini protease inhibitor cocktail [Roche]). Samples separated on 10% SDS-PAGE were electrotransferred onto polyvinylidenefluoride (EMD Millipore) for 45 min at 15 V. Blotted membranes were incubated overnight at 4°C with primary antibodies against Caz (mouse monoclonal 3F4; 1:30; Immanuel et al., 1995), HA epitope tag (mouse monoclonal HA.11; 1:1,000; Covance), and β-tubulin (mouse monoclonal E7; 1:700; Developmental Studies Hybridoma Bank). Immunoreactive proteins were visualized after incubation with anti-mouse IgG coupled to horseradish peroxidase (W402B; 1:2,500; Promega) for 1 h at RT. Blots were developed with enhanced chemiluminescence (GE Healthcare), and x-ray film images of chemiluminescence were developed and scanned. Densitometric quantification of images was performed with ImageJ/FIJI (National Institutes of Health).
ing actin5C-GAL4 alone or cotransfected with plasmids encoding N-terminal HA- or Flag-tagged Xrp1Short or Xrp1Long constructs, all under UAS control. 48 h after transfection, protein lysates of cells expressing HA- or Flag-tagged Xrp1 were either directly used or combined in a 1:1 ratio. 5% of protein extracts were used for Western blotting, while the remaining 95% were added to anti-HA agarose beads for 24 h at 4°C. Immunoprecipitation of HA-tagged proteins was performed using an Anti-HA Immunoprecipitation Kit (Sigma-Aldrich), according to the manufacturer’s protocol.

To evaluate whether Caz and Xrp1 physically associate with each other, S2 cells expressing the actin5C-GAL4 plasmid alone (control) or in conjunction with the N-terminal HA-tagged Xrp1Short or Xrp1Long constructs were seeded at a density of 10^6 cells/ml in 1 ml Shields and Sang medium (Sigma-Aldrich) in 12-well plates 1 d before transfection. Transfection was performed using Fugene HD Transfection Reagent (Promega) according to manufacturer’s instructions. Cell lysates were prepared, cellular debris was pelleted by centrifugation at 12,000 g for 15 min and washed, and the protein-containing supernatant was incubated overnight at 4°C with 100 µl of either anti-Flag (10 µg; clone M2; F1804; Sigma-Aldrich) or anti-Caz (10 µg; 3F4; Immanuel et al., 1995) conjugated SureBeads Protein G Magnetic Beads (Bio-Rad Laboratories) according to the manufacturer’s instructions. Bound proteins were eluted by heating to 70°C for 10 min with 40 µl of 1× Laemmli buffer (Bio-Rad Laboratories).

Inputs, precipitates, and binding proteins were analyzed by SDS-PAGE and immunoblotting. The immunoblot analyses were performed using the following primary antibodies: anti-Flag (clone M2; F1804; 1:1,500; Sigma-Aldrich) or anti-Caz (10 µg; 3F4; Immanuel et al., 1995) conjugated SureBeads Protein G Magnetic Beads (Bio-Rad Laboratories) according to the manufacturer’s instructions. Bound proteins were eluted by heating to 70°C for 10 min with 40 µl of 1× Laemmli buffer (Bio-Rad Laboratories).

Liquid chromatography (LC)–tandem MS analysis

S2 cells were seeded at a density of 10^6 cells/ml in 3 ml Shields and Sang medium (Sigma-Aldrich) in six-well plates 1 d before transfection. Flag-tagged Xrp1Short or Xrp1Long plasmids were transfected, along with the actin5C-GAL4 construct. Three replicates were processed for each condition. After incubating the cells for 2 d with the transfection mix, cells were collected and lysed in cell lysis buffer (described above). Cellular debris was cleared by centrifugation at 12,000 g for 15 min. To pull down lysed in cell lysis buffer (described above). Cellular debris was pelleted by centrifugation at 12,000 g for 15 min and washed. The protein-containing supernatant was incubated overnight at 4°C with 100 µl of either anti-Flag (10 µg; clone M2; F1804; Sigma-Aldrich) or anti-Caz (10 µg; 3F4; Immanuel et al., 1995) conjugated SureBeads Protein G Magnetic Beads (Bio-Rad Laboratories) according to the manufacturer’s instructions. Bound proteins were eluted by heating to 70°C for 10 min with 40 µl of 1× Laemmli buffer (Bio-Rad Laboratories).

Inputs, precipitates, and binding proteins were analyzed by SDS-PAGE and immunoblotting. The immunoblot analyses were performed using the following primary antibodies: anti-Flag (clone M2; F1804; 1:1,500; Sigma-Aldrich) and anti-HA (Mono HA.11; 1:1,000; Covance).

MS data analysis

Raw MS files were processed using the MaxQuant computational platform (version 1.5.3.8; Cox and Mann, 2008). The Andromeda search engine integrated into MaxQuant was used for the identification of peptides and proteins by querying a concatenated forward and reverse UniProt Drosophila database (UP000000803_7227.fasta; release 2015-12), including common laboratory contaminants. The search for precursor and fragment ions was performed allowing an initial mass deviation of 20 ppm and 0.5 Da, respectively. Tryptsin with full enzyme specificity was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allowed. A maximum of two missed cleavages was selected, and only peptides with a minimum length of seven amino acids were allow
bution of measured values. A width of 0.3 SD and a downshift of 1.8 SD were used for this purpose. To identify proteins that displayed significant differences between the groups, ANOVA testing was performed (P < 0.05). Fold enrichment was calculated based on LFQ intensity values. The MS proteomics data have been deposited to the public PRIDE repository (Vizcaino et al., 2013) via the ProteomeXchange platform (http://proteomecentral.proteomexchange.org) with the dataset identifier PXD008417.

Immunocytochemistry and histochemistry

*Drosophila* S2R+ cells were seeded in a density of 4 × 10^5 cells/ml on Concanavalin A (Sigma-Aldrich)-treated circular microscope cover glasses (12 mm; VWR) in a 24-well cell culture dish. After 24 h at 25°C, cells were transfected with a mix containing the Fugene HD transfection reagent (Promega) and actinSC-GAL4 and UAS-HA::Xrp1<sup>short</sup> plasmids. 48 h later, cells were fixed in 4% PFA for 15 min followed by two 5-min washes in DPBS (1×; Gibco) at RT. After permeabilization with DPBS (1×) and 0.5% Triton X-100, cells were washed twice with DPBS. Cells were blocked for 1 h in 2% BSA and 10% goat serum in DPBS, followed by overnight incubation at 4°C with primary antibodies against Caz (mouse monoclonal clone 3F4; 1:30; Immanuel et al., 1995), lamin (mouse monoclonal ADL67.10; 1:100; Developmental Studies Hybridoma Bank), and HA (rabbit polyclonal; 1:50; Santa Cruz Biotechnology) diluted in 2% BSA and 10% goat serum in DPBS. After two washes in DPBS, secondary goat anti-mouse and anti-rabbit antibodies (Alexa Fluor 488 and 568; 1:500; Molecular Probes) were applied for 2 h at RT. The slides were counterstained with DAPI and mounted in Vectorshield antifade mounting medium (Vector Laboratories) for confocal microscopy. All images were acquired using ZEN 2010 software on a Zeiss LSM780 laser scanning confocal microscope using an EC Plan neofluar 1.3 NA 40× oil immersion objective.

RNA-seq and data analysis

For RNA-seq, total RNA was extracted from 15–20 brains dissected from wandering third instar male larvae using Nucleospin RNA (Macherey-Nagel) according to the manufacturer’s instructions. After performing quality control checks, the RNA was sent to the Max Planck Genome Center, where cDNA libraries were prepared using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) using standard procedures. The libraries were sequenced on an Illumina HiSeq 2500 instrument as 100-bp paired-end reads each according to the manufacturer’s standard protocols. Three biological replicates per genotype were sequenced, with an average of 8.6 million nonredundant read pairs uniquely mapped to the *Drosophila* genome.

For subcellular localization of Xrp1 in motor neurons, brains/CNS from wandering third instar larvae expressing OK371-GAL4>UAS-mCD8::GFP alone (control) or in conjunction with UAS-Xrp1<sup>short</sup> plasmids were dissected in PBS and fixed in 4% PFA for 30 min. Tissues were washed 3 × 10 min in PBS/0.2% Triton X-100 and blocked for 1 h at RT in 10% goat serum in PBS. Tissues were incubated with primary antibodies against Caz (mouse monoclonal clone 3F4; 1:30; Immanuel et al., 1995), lamin (mouse monoclonal ADL67.10; 1:100; Developmental Studies Hybridoma Bank), or HA (rabbit polyclonal; 1:50; Santa Cruz Biotechnology) overnight at 4°C with gentle agitation. Appropriate secondary antibodies conjugated either with Alexa Fluor 405, Alexa Fluor 488, or Alexa Fluor 568 (Molecular Probes) were used to detect the given primary antibody. All images were acquired using ZEN 2010 software on a Zeiss LSM700 laser scanning confocal microscope using an EC Plan neofluar 1.3 NA 40× oil-immersion objective.

**Squash preparation of polytene chromosomes from larval salivary glands**

For preparing polytene chromosome squashes, salivary glands of WT third instar larvae were dissected in PBS (1×) and transferred to 1% Triton X-100 for 30 s. Fixation was in 4% PFA (1 min) and in 45% acetic acid/4% PFA (2 min). The glands were then incubated in 45% acetic acid (1 min) and subsequently squashed in the same solution under a coverslip to get polytene spreads. After freezing the slides in liquid nitrogen, coverslips were flipped off with a sharp blade, and slides were stored in 90% ethanol. For immunostaining, squash preparations were rehydrated twice for 5 min in PBS (1×). Immunostaining was performed following the Dangli and Bautz (1983) procedure using rabbit polyclonal anti-Xrp1 (1:50; Francis et al., 2016) followed by an Alexa Fluor 568–conjugated secondary antibody (1:500; Molecular Probes). The preparation was counterstained with DAPI and mounted in Vectashield antifade mounting medium (Vector Laboratories) for confocal microscopy. All images were acquired using ZEN 2010 software on a Zeiss LSM780 laser scanning confocal microscope using an EC Plan neofluor 1.3 NA 40× oil immersion objective.

**Xrp1 mediates caz mutant phenotypes**

For preprocessing the reads before alignment, e.g., quality- or adapter-trimming, was not necessary. The *Drosophila* reference genome was downloaded from Flybase. Revision 6.04 of the genome assembly and gene annotation was used for all analyses. We aligned reads to the reference transcriptome using the TopHat pipeline (version 2.0.14; Kim et al., 2013a) with Bowtie2 (version 2.2.5; Langmead and Salzberg, 2012) and the flags b2-very-sensitive and library-type = fr-firststrand. The mapped reads were assigned to genes using the HTseq-count script from the HTseq package (Anders et al., 2015). We used the intersection-nonempty mode to exclude ambiguous gene assignments. Aligned pairs with a mapping quality <10 were excluded, and RNA genes were removed from the gene list for further analysis.

Differential gene expression analysis was performed using DeSeq2 (version 1.11; Love et al., 2014). All comparisons were performed in a pairwise manner, comparing samples of each genotype separately against the WT. We chose to disable filtering genes based on Cook’s distance for the whole analysis because we observed that the high biological variability of Xrp1 heterozygous animals lead to the exclusion of a large number of genes. Genes were called differentially expressed if the log<sub>2</sub> fold change differed significantly from 0 with a false discovery rate–adjusted P value of <0.05. Expression levels for each annotated protein-coding gene were determined by the number of mapped reads per kilobase of exon per million mapped reads (RPKM).

Principal component analysis and hierarchical clustering of the global expression profile was performed on a variance-stabilized transformation of the read counts per gene using methods provided by the DeSeq2 R-package. For clustering, the distances between samples were calculated using the Manhattan distance metric. Based on the distance matrix, hierarchical clustering by complete linkage was performed using standard R functions.
GO-term enrichment analysis was done using the topGO package for R (version 2.22; Alexea et al., 2006). We extracted the sets of up- and down-regulated genes for each comparison from the DeSeq2 analysis and used the “weight01” algorithm in topGO in combination with Fisher’s exact test to check for enrichment of specific GO terms in these gene sets.

Statistical analysis
χ² statistics were used to analyze offspring frequency data. For life span analysis, the log-rank test was used to test for statistical significance. Motor performance was analyzed using the Mann-Whitney U test to compare climbing speed of individual flies per genotype and per run. As all flies were tested in three independent runs, three P values were generated per genotype. These P values were combined using the Fisher’s combined probability test. To analyze larval locomotion data, Mann-Whitney rank-sum tests were performed with MatLab. One-way ANOVA with Bonferroni correction was used to analyze Caz and Xrp1 mRNA and protein levels as data displayed normal distribution and equal variance. All images were assembled in figure panels using the Adobe Illustrator CS5 software.

Online supplemental material
Fig. S1 shows generation and characterization of Xrp1 mutant and transgenic lines. Fig. S2 shows that heterozygosity for Xrp1 does not rescue the adult eclosion defect of TBP44 mutant flies. Fig. S3 shows larval locomotion phenotypes and binding of WT or AT-hook mutant Xrp1 to polytene chromosomes. Figs. S4 shows characterization of the caz-Xrp1 genetic interaction. Fig. S5 shows that heterozygosity for Xrp1 mitigates gene expression dysregulation in caz mutant CNS. Table S1 lists human homologues of Xrp1. Tables S2, S3, and S4 list Xrp1long, Xrp1short, and Caz-interacting proteins, respectively. Table S5 lists RNA-seq results. Table S6 lists human AT-hook genes. Table S7 lists oligonucleotide primers.

Acknowledgments
We thank B. McCabe (École Polytechnique Fédérale de Lausanne Brain Mind Institute, Lausanne, Switzerland), P. Verstreken (VIB–IU Leuven, Leuven, Belgium), F. Feigun (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy), and U. Pandey (University of Pittsburgh, Pittsburgh, PA) for fly lines, D.C. Rio (University of California, Berkeley, Berkeley, CA) for the Xrp1 antibody, S. Bogdan for technical advice on S2 cultures, M. Been for help with figure design, and F. Schmalbein, Y. Schmidt, and A. Nolte for excellent technical assistance. This work is supported by funding to E. Storkebaum from the state North Rhine Westphalia, the Max Planck Society, The Bruno and Ilse Frick Foundation for ALS Research, the Minna-James-Heineman-Stiftung, the French Muscular Dystrophy Association, the US Muscular Dystrophy Association, the EU Joint Programme–Neurodegenerative Disease Research (grants ZonMW 733051075 [TransNeuro] and ZonMW 733051073 [LocalNMD]), and a European Research Council consolidator grant (ERC-2017-COG 770244). M. Mallik was supported by a Humboldt Foundation research fellowship, C.B. Hug by a fellowship from the International Max Planck Research School, and J. Bussmann by a fellowship from the Cell Dynamics and Disease (CEDAD) graduate school. Stocks were obtained from the BDSC (National Institutes of Health grant P40OD018537).

The authors declare no competing financial interests.

Author contributions: M. Mallik and E. Storkebaum designed the research; M. Mallik, C. Catinozzi, M. Wagner, L. Zhang, J. Bussmann, J. Bittern, S. Mersmann, H.C.A. Drexler, and E. Storkebaum performed the research; C.B. Hug, M.A. Huynen, and J.M. Vaquerizas performed bioinformatic analysis; M. Mallik, C. Catinozzi, L. Zhang, and E. Storkebaum analyzed the data; all authors commented on the paper; M. Mallik, C.B. Hug, and E. Storkebaum made figures; M. Mallik and E. Storkebaum wrote the paper with contributions from C.B. Hug and J.M. Vaquerizas.

Submitted: 27 February 2018
Revised: 13 July 2018
Accepted: 14 August 2018

References
Xrp1 mediates caz mutant phenotypes.


