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NEK1 loss-of-function mutation induces DNA damage accumulation in ALS patient-derived motoneurons

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Abstract

Mutations in genes coding for proteins involved in DNA damage response (DDR) and repair, such as C9orf72 and FUS (Fused in Sarcoma), are associated with neurodegenerative diseases and lead to amyotrophic lateral sclerosis (ALS). Heterozygous loss-of-function mutations in NEK1 (NIMA-related kinase 1) have also been recently found to cause ALS. NEK1 codes for a multifunctional protein, crucially involved in mitotic checkpoint control and DDR. To resolve pathological alterations associated with NEK1 mutation, we compared hiPSC-derived motoneurons carrying a NEK1 mutation with mutant C9orf72 and wild type neurons at basal level and after DNA damage induction. Motoneurons carrying a C9orf72 mutation exhibited cell specific signs of increased DNA damage. This phenotype was even more severe in NEK1c.2434A > T neurons that showed significantly increased DNA damage at basal level and impaired DDR after induction of DNA damage in a maturation-dependent manner. Our results provide first mechanistic insight in pathophysiological alterations induced by NEK1 mutations and point to a converging pathomechanism of different gene mutations causative for ALS. Therefore, our study contributes to the development of novel therapeutic strategies to reduce DNA damage accumulation in neurodegenerative diseases and ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the selective death of upper and lower motoneurons (MN), which leads to muscle denervation and eventually death by respiratory failure (Boillée et al., 2006). Although most ALS cases do not have a familial history (sporadic cases), specific mutations in different genes could be identified in about 10% of ALS patients (familial cases, i.e. fALS). In the last decades, several new genes have been identified that are associated with familial and sporadic ALS (Chen et al., 2013; Taylor et al., 2016). The complex pathogenic landscape and the functional heterogeneity of ALS genes are limiting factors for the development of effective ALS therapies. In fact, although eventually leading to the same disease phenotype, ALS-associated genetic mutations occur in genes that are involved in a plethora of different cellular pathways such as protein degradation, RNA processing, and DNA-damage repair (Lagier-Tourenne et al., 2010; Qiu et al., 2014; Vance et al., 2009; Wang et al., 2013).

Recently, whole genome sequencing of ALS patients and healthy individuals revealed an association of heterozygous mutations in the

1 These authors contributed equally to this work.
2 These authors contributed equally to this work.

Abbreviations: ALS, amyotrophic lateral sclerosis; ATM (serine/threonine kinase), BRCA1, Breast Cancer 1; CHK1, Checkpoint kinase 1; DDR, DNA damage response; DSB, double strand break; hiPSC, human induced pluripotent stem cells; HR, homologous recombination; NEK1, NIMA-related kinase 1; NHEJ, non-homologous end joining

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mitotic protein kinase never-in-mitosis A (NIMA-) related kinase 1 (NEK1) with ALS (Brenner et al., 2016; Cirulli et al., 2015). Thus, these studies highlighted NEK1 as a novel ALS gene. NEK1 is known to be involved in DNA damage response (DDR), since it specifically localizes to DNA damage foci after γ-irradiation (Chen et al., 2008; Polci et al., 2004). Moreover, cells lacking NEK1 display severe alterations in cell cycle checkpoints control, since the protein is required for activation of the cell cycle kinases CHK1 and CHK2 (checkpoint kinase 1/2). Both kinases are indispensable for proper arrest at G1/S, S- or G2/M-phase and efficient DNA repair after DNA damage (Chen et al., 2008). Consequently, NEK1-deficient cells suffer from unrepaired DNA damage, finally leading to chromosomal breaks and genomic instability (Chen et al., 2011).

Amongst others, ALS-related mutations have also been shown to affect different genes specifically involved in the maintenance of genomic stability and DNA repair mechanisms. For instance, mutations in Fused-in-Sarcoma (FUS) (Qiu et al., 2014; Wang et al., 2013) and C9orf72 (Lopez-Gonzalez et al., 2016) are associated with ALS and it has already been shown that they are causative of increased DNA damage. However, it is not known whether an ALS related NEK1 mutation causes increased DNA damage and impaired DDR after induction of DNA strand breaks. Since NEK1 is a key element in early DNA damage repair pathways (Chen et al., 2008, 2011; Polci et al., 2004) and altered DDR has already been associated with fatal neurodegeneration and ALS progression, we analyzed and characterized iPSC-derived MN derived from a newly generated NEK1c.2434A > T cell line. Based on this, we especially focused on the question whether the NEK1 mutation is indeed leading to haploinsufficiency causing alterations in DDR as already been demonstrated for C9orf72 (Lopez-Gonzalez et al., 2016). The analysis aims to clarify if mutations in different ALS related genes could lead to the same ALS associated motoneuronal pathology eventually leading to cell death.

2. Material and methods

2.1. Cultivation of human keratinocytes

Cultivation of keratinocytes from plucked human hair and cultivation CD-1 mouse embryonic fibroblasts (MEF) (Stemcell Technologies) was performed as already described (Aasen et al., 2008; Takahashi and Yamanaka, 2006). Briefly, keratinocytes obtained from the out-growth of several hair roots were cultured in Epilife medium with HKGS supplement (Invitrogen) on collagen IV-coated (20 μg/ml; Sigma-Aldrich) dishes. MEFs were cultivated according to manufacturer’s protocol.

2.2. Generation of human iPS cells and characterization of pluripotency

Generation of human iPS cells by reprogramming human keratinocytes was performed as previously described (Stockmann et al., 2013). Lentiviral particles were produced as already published (Linta et al., 2012; Sommer et al., 2016; Stockmann et al., 2013; Warlich et al., 2016). Briefly, 3 – 10^6 cells were infected in Epilife medium containing 8 mg/ml polybrevin (Sigma-Aldrich). Infection was performed over 2 successive days. After expansion, iPS colonies were transferred onto Matrigel-coated (BD Biosciences) plates. Pluripotency tests were done using the StemLite Pluripotency Kit (Cell Signaling). In vitro differentiation necessary for germ-layer-specific verification was performed according to published protocols (Linta et al., 2012; Stockmann et al., 2013). Chromosome preparation for Karyotyping was carried out according to standard protocols (Linta et al., 2012) (supplemental data Fig. S1D).

2.3. Sequencing of NEK1c.2434A > T and CNTL2

To verify the presence of described mutation c.2434A > T within the NEK1 gene, affected area was sequenced for cell line NEK1c.2434A > T and CNTL2.

For that, total DNA was isolated using the QIAamp DNA Mini Kit (Qiagen) according to manufacturers’ protocol. A PCR using a specific primer pair (CTGATACCCGGGAAAATCTCA, TGTGAGAGGGAGGCCACCTCT) flanking the mutation site was performed to obtain a fragment, suitable for sequencing. After separation via Electrophoresis respective band (521 bp) was isolated using Gel Extraction Spin Kit 150 (Genomed) according to manufacturers’ protocol. All samples were sent and sequenced by mwg-biotech.

2.4. Cultivation of human iPSCs and motoneuronal differentiation

Cultivation of reprogrammed hiPSCs under feeder-free conditions as well the differentiation of hiPSCs into mature spinal MNs was performed as previously described (Hu and Zhang, 2009; Stockmann et al., 2013). Differentiated cells were cultured on 35 mm dishes (Ibidi) and kept in culture for up to 42 days.

2.5. Semi-quantitative real-time one-step RT-PCR

Semi-quantitative real-time one-step RT-PCR was performed as described (Kleger et al., 2010) using a Rotor-Gene Q System (Qiagen). 3 technical replicates of each cell line (n = 3) were used and results are shown relative to housekeeping gene HMBS. Validated primers pairs were purchased from Qiagen (Quantitect primer assay, Qiagen).

2.6. Fluorescence in situ hybridization (FISH)

To perform fluorescence in situ hybridization (FISH) cells were washed with phosphate buffer saline (PBS), fixed 4% Paraformaldehyde (PFA) and permeabilized with 0.2% Triton X-100 (Roche). The Cy3-labelled probe against hexanucleotide repeat GGGGCC was dissolved in 50% formamide in 2× saline-sodium-citrate (SSC) buffer plus 10% dextran sulphate (probe concentration 0.02 ng/μl). To hinder unspecific binding, 1 μg/μl of salmon sperm DNA was added. The probe was denatured for 5 min at 60°C, then kept for 3 min on ice. Next, the probe was added to the cells and incubated for 3-4 h at 42°C in a humid, dark chamber. After incubation, cells were washed with preheated solutions (42°C): three times 5 min with 50% formamide in 2× SSC, three times 5 min with 2× SSC and once for 5 min with 4× SSC plus 0.03% Tween 20 at RT. Then, cells were washed with PBS and aqua demin. Finally, cover slips were mounted with ProLong Gold Antifade reagent with Dapi (Invitrogen).

2.7. Southern blot

Southern Blot was performed according to a standard protocol described in previously published manuscripts (Hübers et al., 2014). Briefly, enzymatically digested DNA fragments were separated using a TRIS-Borat-EDTA agarose gel (0.9%), transferred onto Amersham Hybond NTM-XL (Fisher Scientific) and incubated with a 32P-labeled probe.
2.8. Western Blot

All Western Blots were carried out as previously described (Grabrucker et al., 2011) using 3 technical replicates of each cell line (n = 3). Briefly, total protein concentration of whole cell lysates was defined by Bradford Assay and equal protein amounts were separated through a polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, separated samples were transferred on a nitrocellulose membrane (GE Healthcare). Primary antibodies (NEK1, mouse, 1:500, santa cruz; Caspase-3, rabbit, 1:500, cell signaling; pBRCA1, rabbit, 1 μg/ml, abcam; BRCA1, rabbit, 1:500, abcam; Ku70, 1:1000, rabbit abcam; ATR; pATR, both rabbit, 1:1000, cell signaling) were detected using horseradish peroxidase- (HRP) conjugated secondary antibodies and the ECL detection Kit (Thermo Scientific). Primary Anti-NEK1 was detected using m-IgG HRP- (mouse, 1:200, chemcon), H2A.X phosphor S139 (mouse, 1:2000, abcam), Actinin (mouse, 1:200, chemcon), β-Catenin (mouse, 1:500, abcam) Tubulin-β-III (chicken, 1:1000, merck) and incubated for 12 h at 4 °C. Primary Antibodies were detected with fluorescence labelled secondary antibodies (all 1:500, Invitrogen): Alexa Fluor® 488, Alexa Fluor® 568, Alexa Fluor® 647 and mounted with ProLong Gold Antifade reagent. For quantification analysis, 3 technical replicates of each line were analyzed (n = 3).

2.9. Immunocytochemistry

Immunofluorescence was performed as described in standard protocols (Stockmann et al., 2013). Cells were fixed with 4% PFA/PBS and primary antibodies were directed against: ChAT (rabbit, 1:500, abcam), NEK1 (rabbit, 1:1000, abcam), NF-H (chicken, 1:50,000, Antibodies online), γH2A.X phosphor S139 (mouse, 1:2000, abcam), Actinin (mouse, 1:200, chemcon), β-Catenin (mouse, 1:500, abcam) Tubulin-β-III (chicken, 1:1000, merck) and incubated for 12 h at 4 °C. Primary Antibodies were detected with fluorescence labelled secondary antibodies (all 1:500, Invitrogen): Alexa Fluor® 488, Alexa Fluor® 568, Alexa Fluor® 647 and mounted with ProLong Gold Antifade reagent. For quantification analysis, 3 technical replicates of each line were analyzed (n = 3).

2.10. Induction of DNA damage by γ-irradiation

DNA damage was induced by γ-irradiation (0.5 Gy) in undifferentiated hiPSCs as well as in MNs (21 days, 42 days). hiPSCs cells were lifted via hESC-dispase digestion (Stemcell Technologies), seeded on hESC-qualified-matrigel coated 35 mm dishes (ibidi) and cultured for 3–5 days. Medium was changed 1 h before colonies were exposed to γ-irradiation. iPSCs were then cultured in mTeSR1 medium (Stemcell Technology) for 1 h/24 h after treatment and either fixed for immunocytochemical analysis or prepared for Western blot analysis. To analyze the effect of irradiation in MNs, neuronal spheres were plated on 35 mm dishes. Cells were kept in culture for 21/42 days with changing medium 2 times per week. Medium was changed 1 h before cultures were treated. After 1 h/24 h of irradiation, cells were either fixed in 4% PFA/PBS or prepared for Western blot analysis.

2.11. Comet assay

Comet Assay was performed according to a standard protocol (Speit and Rothfuss, 2012). After corresponding treatment (without/1 h/24 h after irradiation), cells were lifted via Accutase (Sigma Aldrich) for 3 min at 37 °C. Afterwards, to ensure single-cell suspension samples were filtered through 30 μm Pre-Separation filters (Miltenyi Biotec). 15,000 cells per sample were mixed with 120 μl low melting-point agarose (0.5% in PBS) and added to microscope slides which had been covered with a bottom layer of 1.5% agarose. Slides were lysed for at least 1 h at 4 °C (pH 10) and alkali denaturated for 25 min (pH > 13). Electrophoresis was performed at 0.86 V/cm for 25 min. Slides were coded and images of 100 randomly selected cells stained with ethidium bromide were analyzed from each slide. Values were calculated using image analysis (Comet Assay IV, Perceptive Instruments, Haverhill). DNA migration was calculated by analyzing “tail length” and “tail intensity (% tail DNA)” as a mean of three experiments (3 technical replicates for each cell line), which were independently performed under the same experimental conditions.

2.12. Caspase-3 and ROS assay

Caspase-3 activity in 42 days old MNs ± irradiation was done according to the manufacturer’s protocol using the colorimetric Caspase 3 Assay Kit (abcam). Identification of the amount of reactive oxygen species (ROS) was performed as described in the manual of OxiSelect Intracellular ROS Assay Kit (Green Fluorescence) (Cell Biolabs Inc.). For analysis, 3 technical replicates were used for each cell line.

2.13. Cell cycle phospho antibody array kit detection and analysis

The phospho antibody array kit (Full Moon Biosystems) was performed according to the manufacturer’s protocol using 3 technical replicates for each line (n = 3). Briefly, MNs were lysated, centrifuged and stored at –80 °C until analysis. 25 μl lysate containing 39 μg protein was mixed with 75 μl labeling buffer (provided by manufacturer) and biotinylated for 2 h at RT. Biotinylated samples were stored at –80 °C. Blocking and coupling was performed according to the manufacturer’s protocol. The detection of antibody arrays was performed in a fluorescent slide scanner (Genepix 4000B microarray scanner, Molecular Devices). The 16-bits images were analyzed using GenePix Pro 6.1 software. For quantification, 6 replicates for each antibody detection and values of three independent experiments were used. Values were calculated as relative to the average value of corresponding CNTRL.

2.14. Statistical analysis

Fluorescence images were captured with an upright AxioScope microscope provided with a CCD camera (16 bits; 1280 × 1024 pixels) using the software Axiosvision (Zeiss). For Quantification analysis (γH2A.X− cells), 4–9 representative images were taken randomly from respective conditions (± irradiation) and at least 200 cells were analyzed for each condition using the Image J Fiji Software (www.imagej.nih.gov) (Schindelin et al., 2012). Each experiment was repeated at least 3 times. Statistical analysis of all data was performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) software. Results are represented as mean values ± standard error of the mean (SEM). Significance is shown as *p ≤ .05, **p ≤ .01, and ***p ≤ .001. Statistically significant differences were determined according to the parameters either by two-way ANOVA with Bonferroni post-test, one-way ANOVA with Bonferroni post-test or unpaired t-test. Statistical analysis of DNA damage categorization was performed using two-way repeated measures ANOVA which accounted for the measurement triplets of our experiments. Counts were considered as percentages rather than absolute numbers. A Bonferroni adjustment was applied to account for the pairwise multiple comparisons of different cell lines.

2.15. Ethical statement

Experiments with human material were in accordance with the ethical committee of Ulm University (Nr.0148/2009; 265/12) and in compliance with guidelines of the Federal Government of Germany (Nr. O.103).
### Key resources table

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3. Results and discussion

3.1. Generation of NEK1/C9orf72-mutant hiPSCs and motoneuron differentiation

To gain a deeper insight into the pathomechanisms triggered by NEK1 loss-of-function mutations in ALS, we analyzed human iPSC cells and differentiated motoneurons (MNs) derived from a patient carrying NEK1c.2434A > T mutation (Brenner et al., 2016).

In this study, we compared newly generated NEK1c.2434A > T (Fig. S2A) with two healthy controls: cell line CNTL1 was previously generated by our group (Higelin et al., 2016), while line CNTL2 was newly created from keratinocytes obtained from the daughter of the NEK1-ALS patient. This provided a control cell line, having a genetic background closely related to the mutant cell line. Since the correlation between NEK1 mutations and ALS has only recently been described, we included also a hiPSC line derived from an ALS patient carrying a repeat expansion of the hexanucleotide (GcG)1800 within C9orf72 gene (Fig. S2A). This mutation is the most frequent genetic cause of fALS (DeJesus-Hernandez et al., 2011) and previously published studies have shown that hiPSC derived neurons expressing ALS related C9orf72 mutation resemble pathological phenotypes (Lopez-Gonzalez et al., 2016). For these reasons, we included line C9orf72 as a “disease control” for pathologic phenotypes, in order to identify the alterations induced by mutation in NEK1 (Fig. 1A).

All newly generated cell lines fulfilled stem cell criteria by expressing typical pluripotency markers, having the ability to spontaneously differentiate into the three germ layers (ectoderm, endoderm and mesoderm) (Fig. S1A/B) and presenting normal karyotypes after reprogramming (Fig. S1C). Neuronal differentiation of iPSCs was achieved as
### Table A

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<td>Repeat expansion of GGGGCC (~1800)</td>
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<td>Spinal onset (paresis of dorsal interosseous muscle of left hand), progression to asymmetric amyotrophic tetraparesis with hyperreflexia, beginning pseudo-bulbar dysarthria, dysphagia, mild respiratory insufficiency (Brenner et al. 2016)</td>
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*Age at onset (age at keratinocytes preparation/biopsy, age at death)

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### Figure B

- Keratinocytes
- Human iPS cells
- Embryoid bodies
- Neuroepithelial rosettes
- Mature motoneurons

### Figure C

- iPS Cells
- CNTL1 + CNTL2
- NEK1

### Figure D

- Graph 1: % of CNTL1 with DAP
- Graph 2: % of NEK1 with DAP

### Figure E

- Graph 3: NPHS1 level in CNTL1

### Figure F

- Merge
- iPS Cells
- CNTL1 + CNTL2
- NEK1

### Figure G

- DNA sequence CNTL1: cta gat aca ttc tct aca act gaa
- DNA sequence NEK1: cta gat aca ttc tct aca act gaa
- AA sequence CNTL1: L D T S F T T E R
- AA sequence NEK1: L D T S F T T E R

### Figure H

- iPS Cells
- CNTL1
- NEK1
- β-actin
- NEK1 protein

### Figure I

- Merge
- iPS Cells
- CNTL1
- NEK1

(Caption on next page)
Fig. 1. Characterization of differentiated motoneurons derived from Controls and NEK1/C9orf72 patients.

(A) General description of donor cell lines, including gender, age of onset, genotype and clinical information.

(B) Time course of the motoneuronal differentiation protocol.

(C) Representative immunostainings showing ChAT+ neurons (green) from both controls and both ALS lines.

(D) Quantification of ChAT+ neurons in cultures of all generated cell lines. Observed differences between individual cell lines were not statistically significant (one-way ANOVA with Bonferroni post-hoc test, n = 3).

(E) mRNA expression level of neuronal marker neurofilament H (NF-H). Results are displayed relative to the housekeeping gene HMBS (n = 3).

(F) Fluorescent in situ hybridization (FISH) display nuclear RNA foci formation in keratinocytes from C9orf72 patient, not obvious in control. Southern Blot analysis revealed the presence of either wild type allele (green arrow) or the allele, harboring the typical repeat expansion (red arrow) in blood (lane 1), as well as in hiPSC sample (lane 2).

(G) Illustration, showing the DNA and amino acid (AA) sequence of NEK1c.2434A > T and CNTL, which show defined loss of function mutation c.2434A > T in case of ALS-patient, leading to a premature stop codon (highlighted by red asterisk).

(H) Western Blot analysis of whole cell lysates showed less NEK1 protein in NEK1c.2434A > T compared to both controls in iPSCs and 42 days old MNs. Statistical analysis revealed statistical differences between patient and CNTL only on iPSC level indicated by asterisk (CNTL2 vs NEK1c.2434A > T p ≤ 0.05). Statistically significant differences were determined by one-way ANOVA with Bonferroni post-hoc test to compare individual cell lines. Results were normalized to β-actin and displayed relative to untreated CNTL1 (n = 3). * p ≤ 0.05, ** p ≤ 0.001, ***p ≤ 0.001.

(I) NEK1 immunostainings showing the cellular distribution of the protein (green) in undifferentiated, highly proliferative iPSCs (left) and differentiated, post-mitotic MNs (right), positive for neurofilament H (NF-H). All scale bars: 10 μm.

previously described (Hu and Zhang, 2009). The protocol to induce specific motoneuronal differentiation includes embryoid bodies (EB) formation and manual selection of neuroepithelial rosettes. After final plating, 42 days were required to obtain mature MNs (Fig. 1B). In these cultures about 30% of all cells were chAT+ MNs (Fig. 1C/D). Of note, ALS-related mutations did not significantly affect the differentiation procedure. Differentiation efficiency was comparable between controls and mutant lines (no significant differences) and mRNA levels of neurofilament heavy chain (NF-H) were similar in all motoneuronal cultures (Fig. 1E).

The C9orf72 line maintained the (G4C2)1800 repeat expansion as shown by Southern Blot analysis, revealing the presence of the wild type gene (green arrow) and the mutated gene (red arrow) in blood and iPSC sample after reprogramming (lane2), although the repeats expansion was slightly shorter in iPSCs compared to the blood cells (Fig. 1F). Moreover, cells showed characteristic nuclear RNA foci highlighted by fluorescent in situ hybridization (FISH) with a labelled probe against the hexanucleotide repeat (G4C2) (Fig. 1F). These results confirmed the presence of the pathogenic G4C2 repeat expansion, thus excluding a possible repeat instability described for C9orf72 (Almeida et al., 2013). We also confirmed that the mutant NEK1 line conserved the heterozygous loss of function mutation 2434A > T (p.Arg812Ter) within the NEK1 gene, leading to a premature stop codon as indicated by red asterisk (Fig. S2A). As expected, the mutation was not detectable in the heterozygous loss of function mutation 2434A > T (p.Arg812Ter) within the NEK1 gene, leading to a premature stop codon as indicated by red asterisk (Fig. S2A). As expected, the mutation was not detectable in the heterozygous loss of function mutation 2434A > T cell line, which was derived from the patient’s daughter (Fig. 1G, Fig. S1D).

As the heterozygous mutation 2434A > T leads to a premature stop codon, the mutant cell line was characterized by reduced amount of NEK1 protein. Western Blot analysis of whole cell lysates from iPSC and MNs showed a reduction up to 50% of NEK1 protein in the mutant genotype to both controls at hiPSC and MN level (Fig. 1H). Our study confirmed previous findings that have indicated NEK1 haploinsufficiency as a pathogenic consequence of NEK1 mutation (Brenner et al., 2016). Since no previous work has described the cellular localization of NEK1 protein in ALS-related hiPSC, we sought to clarify its compartmental distribution in this model. In undifferentiated, highly proliferative iPSCs, NEK1 localization was mainly restricted to the nucleus, forming protein foci, probably reflecting different proliferation stages (Booth et al., 2014). Interestingly, differentiated, post-mitotic Chat+ MNs were devoid of intranuclear NEK1 but showed a cytoplasmic distribution, suggesting that in MNs NEK1 might also play a role in mitochondrial homeostasis control as already observed in immortalized cells (Chen et al., 2015) (Fig. 1I).

3.2. Increased DNA damage and impaired DNA damage response in ALS-derived motoneurons

Accumulation of DNA breaks and alterations of the DDR machinery have already been described in familial forms of ALS, more in particular associated with FUS and C9orf72 mutations (Higelin et al., 2016; Lopez-Gonzalez et al., 2016; Qiu et al., 2014; Wang et al., 2013). Since NEK1 has been shown to play a key role in DDR (Chen et al., 2008; 2011; Polci et al., 2004), we investigated whether ALS-related NEK1 mutations might trigger the accumulation of DNA breaks and alterations in DDR. To this end, we examined the accumulation of γH2A.X+ foci in iPSC (Fig. 2A) and MNs (Fig. 2B) at basal level and after inducing DNA strand breaks by irradiation. In iPSCs, the basal degree of nuclear γH2A.X foci was comparable amongst all cell lines. The number of cells having γH2A.X+ foci was drastically increased after irradiation (1 h after treatment), and returned to basal level after 24 h. No significant difference between mutant and control cells was detected, suggesting that DNA damage can be efficiently repaired by highly-mitotic cells despite ALS-related mutations. (Fig. 2C). In contrast, ALS patient-derived C9orf72 and NEK1c.2434A > T MNs showed elevated numbers of γH2A.X foci compared to CNTL. The increased number of cells having γH2A.X+ foci was detectable already at an early stage of neuronal development (21 days) (Fig. S2B) and this difference became even larger at a later stage of maturation (42 days). Moreover, NEK1c.2434A > T cells showed the strongest phenotype when compared to mutant C9orf72 MNs (C9orf72: 16% ± 1.5, NEK1c.2434A > T: 23% ± 1), suggesting an increased cell vulnerability in affected ALS MNs. When challenged with γ-irradiation, all genotypes responded by a strong increase of cells having γH2A.X+ foci after 1 h, with NEK1c.2434A > T MNs displaying the most prominent increase (89% ± 1.6). 24 h after irradiation, mutant and CNTL MNs presented reduced levels of cells having γH2A.X+ foci compared to the respective acute response (1 h after irradiation). However, in contrast to undifferentiated iPSCs, MNs failed to return to basal level, highlighting increased vulnerability of post-mitotic MNs. Of note, patient-derived cell lines C9orf72 and NEK1c.2434A > T showed less efficient DDR machinery, as the number of cells displaying γH2A.X+ foci were still increased compared to CNTL cells, with the mutant NEK1 cells showing again the strongest pathological phenotype (Fig. 2D), since C9orf72 at this stage was only significantly different to CNTRL but not CNTRL2.

Differences in the response of the two patient cell lines or the two CRNLs may account for maturation or aging of the cells as previously reported (Higelin et al., 2016), and/or human variability and epigenetic
Fig. 2. Motoneurons derived from ALS-patients show signs of increased DNA damage and impaired DNA damage repair after irradiation and during aging. (A,B) Immunostaining of DNA damage marker γH2A.X (green) in human (A) iPSCs and (B) 42 days old MNs from CNTL1, CNTL2, C9orf72 and NEK1c.2434A > T. Cells were stained either without irradiation or 1 h/24 h after irradiation. All scale bars: 10 μm.

(C) Quantification of γH2A.X⁺ cells on hiPSC level (n = 3). All four lines responded nearly similar to irradiation 1 h and 24 h after irradiation as indicated by red asterisks (CNTL2 Ø vs CNTL2 1 h p ≤ .001, C9orf72 Ø vs C9orf72 1 h p ≤ .001, NEK1c.2434A > T Ø vs NEK1c.2434A > T 1 h p ≤ .001, vs NEK1c.2434A > T 1 h vs NEK1c.2434A > T 24 h p ≤ .01).

(D) Quantification of γH2A.X⁺ cells at a later time point of MN maturation (MNs 42 days) without irradiation or 1 h/24 h after irradiation (n = 3). All cell lines respond with a significant increase in γH2A.X⁺ cells 1 h after irradiation and a subsequent decrease 24 h after treatment as indicated by red asterisks (for all lines: Ø vs 1 h p < .001, 1 h vs 24 h p < .001). Patient-derived C9orf72 and NEK1c.2434A > T presented higher amounts of γH2A.X⁺ cells under control conditions without treatment (CNTL1 vs NEK1c.2434A > T p ≤ .001, CNTL2 vs NEK1c.2434A > T p ≤ .05, CNTL1 vs C9orf72 p ≤ .05). 1 h after irradiation NEK1c.2434A > T displayed higher degrees of damaged cells compared to all other lines (CNTL1 vs NEK1c.2434A > T p ≤ .001, CNTL2 vs NEK1c.2434A > T p ≤ .05, NEK1c.2434A > T vs C9orf72 p ≤ .01, CNTL1 vs CNTL2 p ≤ .01). After irradiation (24 h) both patient-derived cell lines presented a significant increased γH2A.X⁺ cell number compared to CNTLs (CNTL1 vs NEK1c.2434A > T p ≤ .001, CNTL2 vs NEK1c.2434A > T p ≤ .001, CNTL2 vs C9orf72 p ≤ .001, CNTL1 vs CNTL2 p ≤ .05). Statistically significant differences were determined by two-way ANOVA with Bonferroni post-test.

(E) Maturation-dependent increase γH2A.X⁺ cells in both controls, C9orf72 and NEK1c.2434A > T. Accumulation of DNA damage increased in all lines during maturation, but showed only significant differences for CNTL1 and NEK1c.2434A > T (CNTL1 21 days vs 42 days p ≤ .05, NEK1c.2434A > T 21 days vs 42 days p ≤ .01). Furthermore, the overall level of DNA damage was increased in patient-derived cell lines. Line NEK1c.2434A > T showed the largest response with respect to maturation. Unpaired t-test was performed to compare 21 days versus 42 days (n = 3).

(F) Classification of cells according to the number of nuclear γH2A.X⁺ foci (grey arrow) as correlation of the degree of DNA damage (red arrow) in the following categories: (I) no foci (II) low amount of damage (< 5 γH2A.X foci) (III) 5–10 γH2A.X foci (III) high amount of damage (≥ 10 γH2A.X foci). (G) Distribution of categorized DNA damage depending on the maturation state of neurons. Category III (high amount of damage) was only increased slightly in patient-derived cell lines in 21 days old MNs compared to CNTL1. 42 days old MNs, derived from patients showed increased amounts of cells, displaying high level of DNA damage compared to both controls (21 days: CNTL1 vs C9orf72 p ≤ .001, CNTL2 vs C9orf72 p ≤ .01, CNTL1 vs NEK1c.2434A > T p ≤ .001, CNTL2 vs NEK1c.2434A > T p ≤ .01). 42 days: CNTL1 vs NEK1c.2434A > T p ≤ .001, CNTL2 vs NEK1c.2434A > T p ≤ .01). Statistically significant differences between distribution patterns were determined by two-way ANOVA with Bonferroni post-test (n = 3). Green stars indicate significance against CNTL1, blue stars indicate significance against CNTL2. * p ≤ .05, **p ≤ .001, ***p ≤ .001.

To analyze whether an aging effect contributes to DNA damage accumulation, we compared mature 42 days old with young 21 days old MNs. An age-dependent increase of γH2A.X⁺ cells was visible in both controls and both patient-derived cell lines, but reached significance only in CNTL1 and NEK1c.2434A > T, with the NEK1 mutant showing the strongest increase (Fig. 2E). However since we detected that in γH2A.X⁺ cell the number of foci differed, the extent of age-dependent DNA damage accumulation was classified according to the number of nuclear γH2A.X⁺ foci within the nuclei in four categories: (I) no γH2A.X foci, (II) < 5 foci, (III) 5–10 foci and (IV) > 10 foci (Fig. 2F). Category III, presenting a high amount of DNA damage, was slightly increased in patient-derived cell lines and significant different to CNTL2 during early neuronal development. In 42 days old MNs, both patient-derived cells showed high level of DNA damage (Category III) with significant differences to both controls for line NEK1c.2434A > T (Fig. 2G). This supports the hypothesis of an affected DDR mechanism in ALS patient-derived MNs, which is in addition, altered during aging. This phenotype is stronger in the presence of an ALS-related NEK1 mutation suggesting that NEK1 mutated cells show a more activated DDR at baseline and after DNA damage stimuli (Chen et al., 2011) than mutated C9orf72.

To finally dissect the extent of DNA breaks accumulation in ALS MNs we performed comet assays (Fig. 3A) and quantified the tail length (Fig. 3B) and the percentage of tail intensity (Fig. 3C) generated by DNA damage. In this assay, control and patient-derived lines showed similar results before irradiation but a significant increase of DNA damage was seen in NEK1c.2434A > T MNs 24 h after irradiation. This highlights the increased vulnerability of the NEK1 mutant cells not only in comparison to controls, but also to ALS-related C9orf72 mutants, whose parameters were comparable to the healthy genotype. Conversely to our results increased levels of DNA damage were previously shown in a maturation dependent manner for C9orf72 MNs (Lopez-Gonzalez et al., 2016). For C9orf72 it has already been demonstrated that the production of depeptide repeats (DRP) compromises mitochondrial function and increases oxidative stress leading to DNA damage accumulation (Lopez-Gonzalez et al., 2016). This pathomechanism could not be verified in our analysis when we analyzed the degree of free radicals in C9orf72 and NEK1 mutants (Fig. 2C).

However, this divergence in the analysis of C9orf72 cell lines might be explained by the differentiation method and the maturation stages. Our oldest cultures were 6 weeks old as opposed to the study by Lopez and colleagues, in which significances in DNA damage in mutant C9orf72 was observed at 8 weeks in culture. Differences in NEK1 versus C9orf72 may reflect different pathomechanism and dysregulation of key cellular pathways (Gao et al., 2017).

Unrepaired DNA damage leads to chromosomal breaks and to the accumulation of genomic rearrangements (Khanna and Jackson, 2001) if key components of DDR pathways are affected. Finally, this might then accumulate and cause neurodegeneration and neuronal cell death. Our results indicate that mature MNs accumulate DNA damage, however NEK1c.2434A > T fail to repair induced DNA damage leading to an increased amount of DNA strand breaks visible 24 h after treatment. The fact that this phenotype is detectable by comet assay in NEK1c.2434A > T but not in C9orf72 further suggests that accumulated and unrepaired DNA damage is more clearly seen in NEK1 mutated cells, at least at that time point of analysis. In addition, in motoneuronal cultures, caspase-3 levels were also up-regulated only in NEK1c.2434A > T 24 h after irradiation (Fig. 3D). To confirm apoptotic behavior after DNA damage induction, we quantified the amount of caspase-3 activation, full length protein showed no differences amongst patient and control, however, analysis of active caspase-3 revealed an increase in cleaved caspase-3 (17 kDa/19 kDa) 24 h after irradiation in NEK1c.2434A > T, indicating increased vulnerability of patient-derived cells. As a consequence this finally leads to motoneuronal cell death in neurons carrying ALS-related NEK1 mutation c.2434A > T in response to DNA damage.

3.3. NEK1 mutated motoneurons are characterized by a misregulation of DNA damage response machinery

The main DDR pathways that are activated by DNA damage induced by irradiation are initiated by a complex phosphorylation cascade leading to recruitment and accumulation of various repair factors at DNA damage foci sites. DNA double-strand break (DSB) repair requires on the one hand the phosphorylation of γH2A.X by ATM serine/threonine kinase (ATM) and Ataxia telangiectasia and Rad3 related (ATR) (Burma et al., 2001). These factors subsequently recruit a large number of different mediators, regulating the activation and the localization of BRCA1 to DNA damage foci (Wu et al., 2009). In response, BRCA1 is phosphorylated (pBRCA1) and induces cell cycle checkpoint activation (Fabbro et al., 2004; Xu et al., 2001) and DNA repair (Scully et al., 2008).
Fig. 3. ALS related NEK1 mutation leads to increased accumulation of DNA breaks in mature motoneurons 24 h after irradiation.

(A) Representative images, showing that DNA strand breaks are more abundant in mature MNs of NEK1c.2434A > T 24 h after DNA damage induction as shown by comet assay.

(B, C) Quantification of tail length and percentage of tail intensity from control and patients in 42 days old motoneuronal cultures without and after irradiation. A significant increase in accumulated DNA damage was visible 24 h after treatment for NEK1c.2434A > T compared to other cell lines (Tail length: CNTL1 vs NEK1c.2434A > T, p ≤ .01, CNTL2 vs NEK1c.2434A > T, p ≤ .01, C9orf72 vs NEK1c.2434A > T, p ≤ .01, Tail intensity: CNTL1 vs NEK1c.2434A > T, p ≤ .001, CNTL2 vs NEK1c.2434A > T, p ≤ .01, C9orf72 vs NEK1c.2434A > T, p ≤ .01). Statistically significant differences were determined by two-way ANOVA with Bonferroni post-test (n = 3). * p ≤ .05, ** p ≤ .001, *** p ≤ .001.

(D) Caspase-3 activity of CNTL1, CNTL2, C9orf72 and NEK1c.2434A > T without and 1 h/24 h after irradiation showed that cultures derived from NEK1c.2434A > T showed higher Caspase-3 activity 24 h after irradiation compared to all other cell lines. This trend showed only significant differences compared to CNTL2 (CNTL2 vs NEK1c.2434A > T, p ≤ .05). Statistically significant differences were determined by two-way ANOVA with Bonferroni post-test (n = 3). * p ≤ .05, ** p ≤ .001, *** p ≤ .001.

(E) Western Blot analysis of all four lines 24 h after DNA damage induction showed an increase in caspase-3 cleavage to activated cl.casp-3 (19 kDa) and cl.casp-3 (17 kDa) in NEK1c.2434A > T. The amount of full length protein was not affected amongst patient and control. Values are shown relative to β-actin.
et al., 1999) via non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ, the main radiation-induced DSB repair mechanism, is also initiated through the recruitment of a Ku70/Ku80 heterodimer (Fell and Schild-Poulter, 2012). To determine DNA repair mechanism affected in ALS-patient-derived cells, we first quantified the amount of pBRCA1/BRCA1, KU70 and pATR/ATR in mature motoneuronal cultures before and after induction of DNA damage. Interestingly, the total amount of cellular BRCA1 was nearly identical in all cells (Fig. 4A), however mutated C9orf72 and NEK1c.2434A>T cell lines showed a clear trend towards increased amounts of pBRCA1 under physiological conditions compared to both controls. After induction of DNA damage, cells responded with a slight increase in pBRCA1 and this remained higher in patient-derived lines C9orf72 and NEK1c.2434A>T 24 h after irradiation (Fig. 4A). The amount of DNA damage marker...
Fig. 4. Increased phosphorylated BRCA1 in motoneurons derived from ALS-patients. (A-C) Western Blot Analysis of whole cell lysates from mature 42 days old MNs (n = 3) showing the protein amount of either (A) phosphorylated Breast Cancer 1 (pBRCA1/BRCA1), (B) Ku70 and (C) phosphorylated Ataxia telangiectasia and Rad3 related (pATR/ATR) in all four cell lines without treatment, 1 h and 24 h after irradiation. Results were normalized on β-actin and relative to untreated CNTL1. (B) Ku70 level was the same amongst all cell lines, but slightly decreased equally after irradiation for CNTL2, C9orf72 and NEK1c.2434A > T. (C) ATR level was nearly identical amongst all cell lines before and 1 h after irradiation, however, C9orf72 showed higher levels 24 h after treatment. Protein amount of phosphorylated ATR (pATR) slightly increased 1 h after irradiation for CNTL1, C9orf72 and NEK1c.2434A > T but showed no differences between patients and CNTL after DNA damage induction. (D) Heatmap showing phosphorylation levels of proteins involved during cell cycle control and DNA damage response in CNTL and NEK1c.2434A > T in 42 days old MNs (n = 3). Results were displayed relative to control. An overall downregulation of phosphorylated proteins was visible in NEK1 mutated cells. Statistical significant differences and alterations close to statistically significance were obtained by unpaired t-test to compare CNTL and NEK1c.2434A > T as indicated by red arrows.

Subsequently, to understand which mechanism or pathways are affected by mutated NEK1, we employed a specific phospho-protein arrays to screen for expression changes in cell cycle control and DDR proteins. In NEK1c.2434A > T, we could observe a differential regulation (mainly downregulation) of specific proteins, indicating an impairment in phosphorylation of some key regulatory elements (Fig. 4D). In addition to increased phosphorylation of BRCA1 and γH2A.X, which are early downstream events in the ATM-dependent signaling response and which require ATM-induced activation (Chen et al., 2011; Gatei et al., 2000), we saw in consensus with the literature an impaired phosphorylation of ChK2/1, p53 and smc1 re

In summary, our study provides good evidence that a NEK1 mutation associated with ALS leads to NEK1 haploinsufficiency in human MNs and to increased DNA damage leading to increased vulnerability and motoneuronal death. The cellular function of NEK1 is largely unknown, however, its role in early DDR as an essential regulatory kinase is well documented (Poleti et al., 2004) and NEK1−/− cells are characterized by unrepairued DNA damage and chromosomal breaks (Chen et al., 2008, 2011). In mutated NEK1c.2434A > T MNs γH2A.X was up-regulated and localized to DNA damage foci and phosphorylation of BRCA1 was present in MNs after irradiation. This indicates that reduced levels of NEK1 are still sufficient to activate the DDR pathway. When the system was challenged by an external induction of DNA damage, we clearly saw a less efficient DNA damage repair, leading to accumulation of DNA damage, increased DNA strand breaks and finally to cell death. The response to DNA damage is based on many cellular pathways activating a large number of different cellular proteins (Shiloh, 2003). In line with the notion that NEK1 is required for efficient ChK1/Chk2 activation (Chen et al., 2008) within ATM pathway, our data show that NEK1 deficiency triggers a complex misregulation of a complex phosphorylation pathway. This involves the reduced activation of further key elements, targets of ATM kinase activity (i.e. Chk1/2, p53). This results are in consensus with findings, showing a defective ATM-mediated DNA repair as pathological consequences of ALS associated C9orf72 expansions (Walker et al., 2017).

Since altered DNA repair mechanisms are potent driving forces for the progression of neurodegenerative diseases (Higelin et al., 2016; Lopez-Gonzalez et al., 2016; Qi et al., 2014; Walker et al., 2017; Wang et al., 2013), they should be taken into account when designing new therapeutic targets to tackle ALS related neurodegeneration.

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Author's contributions

TMB, MD, JH and AC designed and outlined the study. JH and AC carried out experiments and performed the data analysis. LLSS reprogrammed/characterized cell line C9orf72 and performed FISH experiments. SO performed and analyzed irradiation experiments on iPSC level for CNTL2 and NEK1. AKL contributed to data analysis and statistics. JB and RS performed and analyzed comet assay. GB analyzed karyotypes. PMA provided keratinocytes. JD, MD and TMB wrote the manuscript.
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Declaration of interest

None.

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