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Journal

Human Mutation, 43(4)

ISSN

1059-7794

Authors

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Publication Date

2022-04-01

DOI

10.1002/humu.24337

Peer reviewed

HHS Public Access

Author manuscript

Hum Mutat. Author manuscript; available in PMC 2023 April 01.

Published in final edited form as:

Hum Mutat. 2022 April ; 43(4): 471–476. doi:10.1002/humu.24337.

A novel nonsense variant in the NFE2L1 transcription factor in a patient with developmental delay, hypotonia, genital anomalies and failure to thrive

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Abstract

The NFE2L1 transcription factor (also known as Nrf1 for Nuclear factor erythroid 2-related factor-1) is a broadly expressed basic leucine zipper protein that performs a critical role in the cellular stress response pathway. Here, we identified a heterozygous nonsense mutation located in the last exon of the gene that terminates translation prematurely resulting in the production of a truncated peptide devoid of the carboxyl-terminal region containing the DNA-binding and leucine-zipper dimerization interface of the protein. Variant derivatives were well expressed in vitro, and they inhibited the transactivation function of wild-type proteins in luciferase reporter assays. Our studies suggest that this dominant-negative effect of truncated variants is through the formation of inactive heterodimers with wild-type proteins preventing the expression of its target genes. These findings suggest the potential role of diminished NFE2L1 function as an explanation for the developmental delay, hypotonia, hypospadias, bifid scrotum and failure to thrive observed in the patient.

Keywords

NFE2L1; transcription factor; dominant negative; nonsense variant; global developmental delay

The *NFE2L1* gene, also known as Nuclear factor erythroid 2 (NF-E2)-elated factor-1 (Nrf1), is located on chromosome 17q21.32 (Chan, Cheung, Moi, Chan, & Kan, 1995; Luna et al., 1995). Nrf1 is expressed in various tissues and the highest expression is observed in heart, muscle, kidney, brain, and adipose tissue (Chan, Han, & Kan, 1993). The gene belongs to

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CONFLICT OF INTERESTS

The content is solely the responsibility of the authors, and they have no conflicts of interest to disclose.

WEB RESOURCES

gnomAD: https://gnomAD.broadinsititute.org/

CADD: https://cadd.gs.washington.edu/

the cap'n'collar subfamily of basic-leucine zipper (bZIP) transcription factors that dimerize with other bZIP proteins such as MAFG, MAFK, and MAFF, to regulate gene transcription (Johnsen, Murphy, Prydz, & Kolsto, 1998; Johnsen et al., 1996; Katsuoka & Yamamoto, 2016; Marini et al., 2002; Marini et al., 1997). NFE2L1 proteins activate through the antioxidant response element (ARE), a regulatory element found in the promoters of cytoprotective and antioxidant genes, as well as controlling expression of proteasome genes under basal and activated conditions. *NFE2L1* encodes several protein isoforms (M. Kwong, Kan, & Chan, 1999; Lee, Ho, & Chan, 2013; Lee et al., 2011; Myhrstad et al., 2001; Radhakrishnan et al., 2010; Zhao et al., 2011). TCF11 and Nrf1a (also designated previously as Nrf1) are long isoforms of the gene. Nrf1a differs by an internal exon encoding a 30-amino acid peptide found in TCF11 (H. M. Kim, Han, & Chan, 2016; Luna et al., 1995). Shorter protein isoforms have also been described, but evidence supporting their expression and function is currently insufficient (E. K. Kwong, Kim, Penalosa, & Chan, 2012; M. Wang, Qiu, Ru, Song, & Zhang, 2019; W. Wang, Kwok, & Chan, 2007). Global knockout of NFE2L1 in mice leads to embryonic lethality indicating that it is an essential gene (Chan et al., 1998). Tissue-specific knockouts indicate that *NFE2L1* protects against neurodegeneration and development of steatohepatitis and liver neoplasia (Lee et al., 2011; Xu et al., 2005). Aside from its role in the cellular stress response, loss of function analysis suggests that *NFE2L1* also regulates osteoblast and odontoblast differentiation, and lipid metabolism (Hirotsu, Hataya, Katsuoka, & Yamamoto, 2012; J. Kim, Xing, Wergedal, Chan, & Mohan, 2010; Narayanan et al., 2004; Tsujita et al., 2014; Widenmaier et al., 2017).

In this study, we report a nonsense variant (c.1855C>T) in the *NFE2L1* gene in a patient who presented at the age of 17 months with profound failure to thrive, developmental delay, hypotonia, hypospadias, and bifid scrotum. This variant creates a premature stop codon in the DNA-binding and protein dimerization domain of NFE2L1. When tested in vitro, truncated NFE2L1 proteins exhibited dominant-negative properties, suggesting that this novel variant is pathogenic and provides a potential explanation for the phenotype in this patient.

CS is a now 3-year-old boy who was born at 35-weeks gestation via C-section due to breech presentation. Pregnancy was complicated by polyhydramnios, and he was born with hydrops and a birth weight of 5 lbs 15 oz (2.4Kg). He first presented to genetics for clinical evaluation at 17 months of age due to severe failure to thrive and global developmental delay. At that time, he was not yet able to crawl or pull to stand. At the time of initial evaluation his weight was <1st percentile with a z-score of −3.64, length was at the 1st percentile and head circumference was in the 9th percentile. Physical exam was notable for amblyopia, keratosis pilaris, eczema, dolicocephaly, short columella, high forehead, hypertelorism, borderline low ears, thin upper lip, hypospadias and bifid scrotum, loose skin, and a sallow skin tone. Initial differential included Smith-Lemli-Opitz syndrome or other sterol disorders, but he did not have 2,3 syndactyly. He was initially admitted to the hospital due to concern for severe malnutrition. Initial evaluation included normal total cholesterol and 7-dehydrocholesterol. Prenatal genetic testing was reported to include a normal karyotype and chromosomal microarray. Given severe failure to thrive he required G-tube placement and bolus tube feedings for all his nutrition. He demonstrated poor gut motility with severe constipation and persistent vomiting which improved with pyloric botox

injections. At 3 years of age, his weight is now in the 6th percentile and his height is in the 15th percentile. At 3 years of age, he can now pull to stand and cruise and has taken a few independent steps. He has good receptive language and can communicate wants through noises and gestures, but he has profound speech delay with no spoken words or phrases.

Whole exome sequencing was performed and identified a heterozygous variant of uncertain significance at nucleotide c.1855C>T in *NFE2L1* (NM_003204.2) in the proband but not in the proband's mother. This variant is novel (not in any individuals) in gnomAD. The *NFE2L1* gene encodes several protein isoforms, and the nonsense variant is in the penultimate exon of the two long protein isoforms, termed TCF11 and Nrf1a. Hence, the two transcripts derived from the mutant *NFE2L1* allele, if translated, would produce truncated proteins of 619 (TCF11-Arg619Ter) or 589 (Nrf1a-Arg589Ter) amino acids, that are lacking the basic-leucine zipper domain crucial for protein dimerization and DNA-binding (Fig 1A). In addition, constraint analysis of *NFE2L1* indicates an extreme intolerance of loss-of-function (LoF), with pLI of 1.0, indicating that LoF *NFE2L1* mutations are likely causative of severe phenotype, and in silico predictions also suggest a deleterious effect with an elevated CADD score of 36 (Rentzsch, Witten, Cooper, Shendure, & Kircher, 2019).

Although transcripts containing premature termination codons are known to be degraded via nonsense-mediated mRNA decay (NMD), this surveillance system is not able to distinguish nonsense mutations and natural stop codons that are in the penultimate exons of genes (Khajavi, Inoue, & Lupski, 2006). Hence, TCF11 and Nrf1a transcripts containing the premature termination codon would likely escape NMD and generate truncated proteins. To verify this, V5-tagged TCF11-Arg619Ter and Nrf1a-Arg589Ter expression vectors were constructed and transfected into HEK293 cells for western blotting. Wild-type TCF11-V5 and Nrf1-V5 generated expected size proteins of approximately 130 kDa with anti-V5 antibody (Fig. 1B top panel, lanes 2 and 4), while TCF11-Arg619Ter-V5 and Nrf1aArg589Ter-V5 generated proteins that were 20 kDa smaller than their wild-type counterparts (Fig. 1B top panel, lanes 3 and 5). As expected, all V5-tagged proteins were detected with anti-TCF11, an antibody that is targeted against a portion of the peptide sequence that is present in both wild-type and mutant TCF11 and Nrf1a proteins (Fig. 1B second panel, lanes 2–5). In contrast, V5-tagged wild-type proteins (Fig. 1B third panel, lanes 2 and 4), but not truncation mutants (Fig. 1B third panel, lanes 3 and 5), were detected using an antibody against the C-terminus of TCF11 and Nrf1a. As expected, the C-terminus antibody detected only the endogenous TCF11/Nrf1a in both non-transfected cells and in cells transfected with truncation mutants (Fig. 1B third panel, lanes 1, 3, and 5). Western blotting with alpha-Tubulin antibody indicated similar amounts of protein were loaded in each lane (Fig. 1B bottom panel). These results demonstrate that the nonsense variant identified in NFE2L1 does not impair protein expression.

To determine transacting function, both TCF11-Arg619Ter-V5 and Nrf1aArg589Ter-V5 were evaluated for their ability to activate luciferase reporter expression in *NFEL2L1* knockout HAP1 cells. Cells were transfected with a firefly luciferase reporter under the control of the PSMB6 promoter along with wild-type TCF11, Nrf1a, or truncated mutant constructs. Both wild-type TCF11 and Nrf1a induced luciferase activity in a dose-dependent

manner (Fig1C and D). In contrast, TCF11-Arg619Ter and Nrf1a-Arg589Ter, confer no transcriptional activation. These results suggest that the transactivation ability of TCF11 and Nrf1a is abolished by the nonsense mutation.

We next tested whether truncated derivatives can exert a negative effect on wild-type proteins. Transfection of knockout cells with wild-type TCF11-V5 led to a two-fold increase in luciferase activity compared to cells transfected with empty vector (Fig. 2A, top panel). When equal amounts of TCF11-Arg619Ter mutant were co-transfected with wild-type TCF11, reporter activity was reduced back to levels like cells transfected with empty vector alone, and luciferase expression was not further reduced by increasing the amount of TCF11-Arg619Ter indicating that ectopic expression of wild-type Nrf1a plasmid is responsible for reporter activation in transfected cells. Similarly, co-transfection of mutant Nrf1Arg589Ter also suppressed reporter activation by wild-type Nrf1a (Fig. 2A, bottom panel).

To extend these findings, we determined the effects of Nrf1aArg589Ter mutant on endogenous expression of known NFE2L1 target genes. NFE2L1 has been shown to be a central mediator in the regulation of proteasome genes. Hence, quantitative RT-PCR was performed to analyze the expression of proteasome genes in HAP1 cells transfected with wild type Nrf1a, Nrf1aArg589Ter or vector control. Expression of various proteasome genes was diminished by Nrf1aArg589Ter in HAP1 cells, whereas RPLP01, which is not a known target of NFE2L1 remained unchanged (Fig. 2B). In contrast, transfection of wild type Nrf1a showed activation of proteasome genes, suggesting that the transactivation ability of wild-type Nrf1 proteins was impaired in the presence of the Nrf1aArg589Ter mutant. These results demonstrate the dominant negative effect of the truncation mutant protein over its wild-type counterpart with respect to target gene activation.

To determine if truncated mutant derivatives could interact with wild-type proteins and thus interfere with their function, we examined their abilities to coimmunoprecipitate with one another following their ectopic expression in HEK293 cells. Cells co-transfected with HA-tagged wild-type Nrf1a and V5-tagged mutant Nrf1aArg589Ter were immunoprecipitated with anti-V5 antibody. Immunoblotting with anti-HA antibody showed that the mutant Nrf1aArg589Ter-V5 immunoprecipitate contained the wild-type Nrf1a-HA protein (Fig. 2C). Interaction with MAFG, a protein known to interact through the bZIP domain of Nrf1a, was monitored as a positive control, and as expected, specific precipitation of Myc-tagged MAFG occurred only in the presence of wild-type (Fig. 2D), but not mutant Nrf1aArg589Ter (Fig. 2E). These findings indicate that truncated mutants have the potential to compete with wild-type Nrf1a or TCF11 in forming complexes with other interacting proteins, thus disrupting the function of wild-type proteins.

Here we report a nonsense variant in *NFE2L1* (NM_003204.2, c.1855C>T) that was found on whole exome sequencing in a patient with syndromic global developmental delay and dysmorphic features, and to our knowledge, variants in the *NFE2L1* gene associated with any developmental delay and intellectual disability syndromes have not been reported. The nonsense variant is found in the last exon of the gene that is shared by two transcripts

(ENST00000362042.8 and ENST00000585291.5) encoding long protein isoforms of the *NFE2L1* gene, termed TCF11 and Nrf1a.

Of note, this patient was also found to have a low-level (13%) mosaic variant of uncertain significance in the *SPTBN1* gene. Cousin et al. reported heterozygous *SPTBN1* variants in a cohort of 29 patients with a neurodevelopmental syndrome showing significant phenotypic variability in neurologic and behavioral presentations in the cohort (Cousin et al., 2021). Our patient was included in the cohort and was the only patient with a mosaic variant. While the *SPTBN1* variant seen in our patient might contribute to the global developmental delay, other parts of the phenotype including failure to thrive, constipation and genitourinary abnormalities do not appear to be part of the phenotype.

TCF11 and Nrf1a function as heterodimers with small MAF proteins. While the truncated variants (Arg619Ter and Arg589Ter, respectively) appear to be stable proteins, the absence of the basic-leucine zipper domain cause by the truncation results in the loss of dimerization interface for Nrf1aArg589Ter to bind MAFG. Consistent with this is that both TCF11-Arg619Ter and Nrf1aArg589Ter failed to promote reporter gene activation. It remains to be determined how these truncated proteins impact on gene expression in vivo, but functional analysis in vitro indicates that both TCF11-Arg619Ter and Nrf1aArg589Ter exerts a dominant-negative effect over wild-type TCF11 and Nrf1a proteins, and despite its inability to bind MAFG, Nrf1aArg589Ter was found to interact with Nrf1a and TCF11 by co-immunoprecipitation. Thus, one possibility is that the truncated variants competitively dimerize and interfere, by sequestration of Nrf1a and TCF11 proteins or other cofactors in vivo, and the phenotype observed in the patient described here possibly involves dominant-negative inhibition of Nrf1a and TCF11 by truncated proteins.

Functionally, the *NFE2L1* gene has been linked primarily to cellular stress response pathways. For example, deletion of *NFE2L1* in neurons results in proteasome dysfunction, accumulation of ubiquitinated proteins, cell death and neurodegeneration. Knockout mouse models have also demonstrated a potential role for *NFE2L1* in metabolic processes. Liver-specific deletion of *NFE2L1* caused a spectrum of defects, including hepatic lipid accumulation. This is likely due to a role for *NFE2L1* in the regulation of fatty acid metabolism and lipoproteins receptor expression, as well as protecting against hepatic cholesterol accumulation. Although the patient was considered to have features that are characteristic of Smith-Lemli-Opitz (SLO) syndrome including developmental delay, facial dysmorphia, failure to thrive and genital anomalies, plasma levels of 7-dehydrocholesterol and cholesterol were normal however, which are not consistent with SLO. Currently, it is not known whether the genetic disorder in this patient also involves the liver as seen in *NFE2L1* knockout mice. Further detailed research on development, cellular stress response and metabolic changes in model systems including induced pluripotent stem cells, and knock-in mice should lead to a better understanding of the pathogenicity of the *NFE2L1* variant described here.

In conclusion, we report the identification of a novel, potentially pathogenic variant in the *NFE2L1* gene in a patient case with syndromic intellectual disability and dysmorphic features.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank the patient's family for their participation in this study. This study was supported by UCI Department of Pathology funds and NIH/NIEHS grant ES029481 to JYC.

DATA AVAILABILITY STATEMENT

Data describing the identified variants have been submitted to ClinVar Database (https://www.ncbi.nlm.nih.gov/clinvar/).

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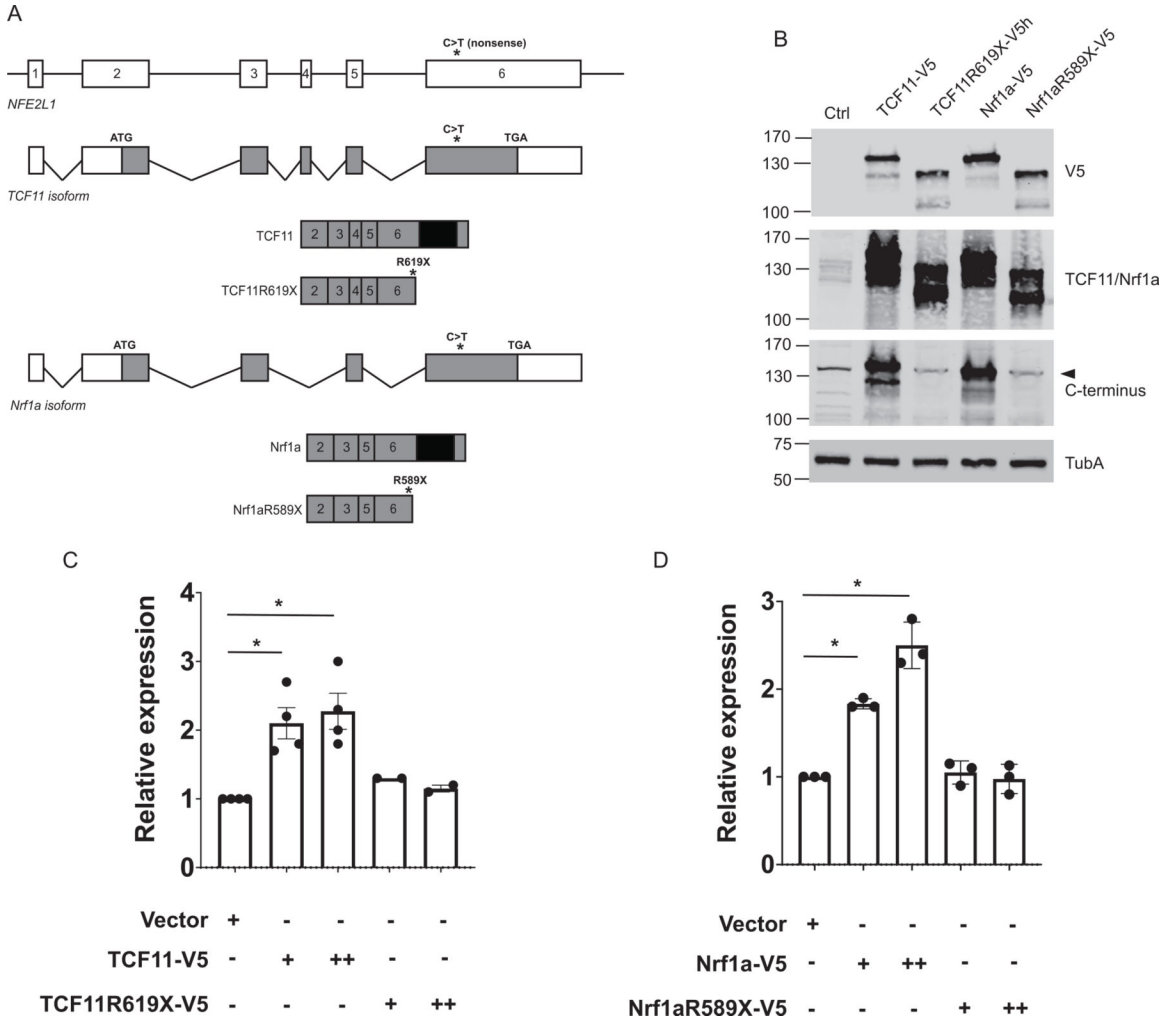

Figure 1. The c.1855 C>T variant in *NFE2L1* results in protein truncation and loss of transacting function of TCF11 and Nrf1a.

(A) Schematic diagram of human NFE2L1, and transcripts encoding the TCF11 and Nrf1a isoforms. Exons are depicted as numbered boxes. Filled-in regions indicate translated portions of the transcripts, and the basic DNA-binding and leucine-zipper protein dimerization domain is shown in black. The position of the nonsense mutation is marked by an asterisk, and p.Arg619Ter and p.Arg589Ter truncated proteins encoded by the nonsense mutation are shown below their wild-type counterparts. **(B)** Western blotting analysis of V5-tagged wild-type and truncated TCF11a and Nrf1a proteins. HEK293 cells were transfected with 5ug TCF11-V5, Nrf1a-V5, TCF11Arg619Ter-V5, or Nrf1aArg589Ter-V5. After 48 hours, lysates were immunoblotted with anti-V5 antibody (top panel), anti-TCF11/Nrf1a (second panel), and an antibody against the C-terminus of TCF11a and Nrf1a (third panel). As a loading control, cell lysates were immunoblotted with anti-alpha tubulin antibody (bottom panel). **(C)** Transactivation by wild-type and truncated TCF11 and Nrf1a. NFE2L1 knockout HAP1 cells were co-transfected with PSMB6-Luc reporter, along with increasing amounts of effector plasmids (left panel) TCF11-V5 or TCF11-Arg619Ter-V5; or (right panel) Nrf1a-V5 or Nrf1Arg589Ter-V5. Twenty-four hours after, luciferase activity was

measured using Dual-Glo assay. Relative activities are expressed as arbitrary units after normalizing firefly luciferase activity to protein concentration. Values represent the average of at least three independent experiments each containing 3 replicates, and error bars represent SD, *P<0.05. P values were calculated by Student's t-test.

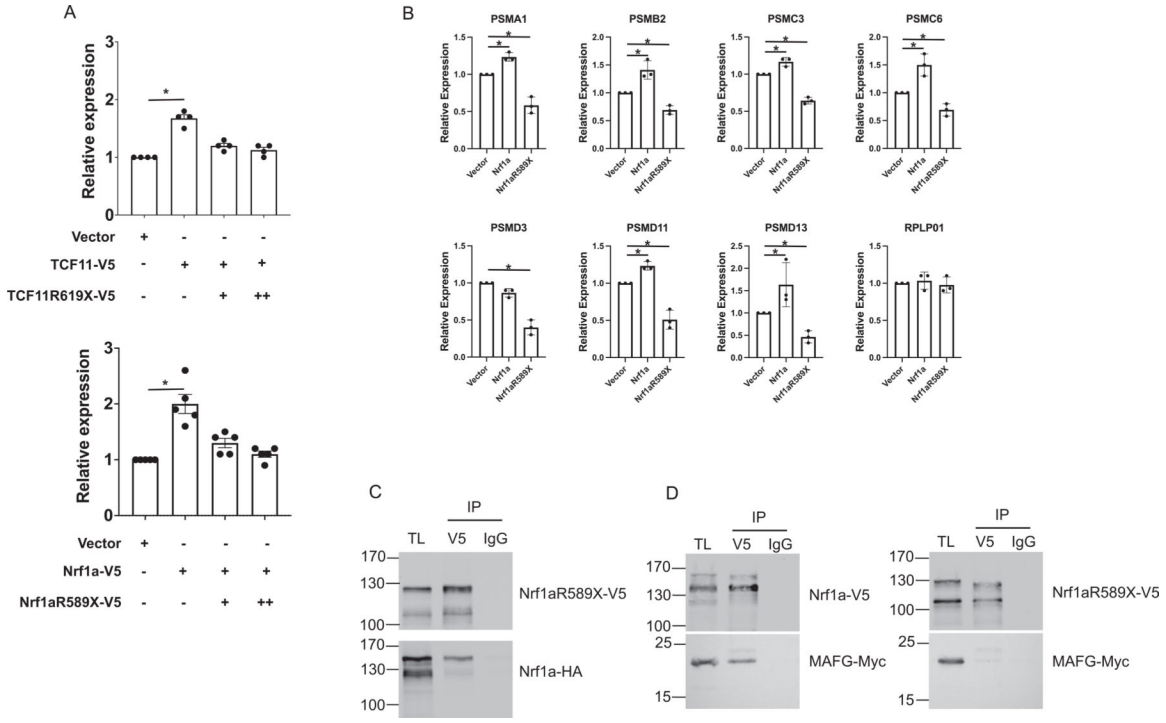

Figure 2. The c.1855C>T protein truncating variant of *NFE2L1* acts as a dominant negative mutation.

(A) Transactivation by TCF11 and Nrf1a is inhibited by truncation mutants. NFE2L1 knockout HAP1 cells were co-transfected with PSMB6-Luc, along with (top panel) TCF11-V5 and increasing amounts of TCF11Arg619Ter-V5 or (bottom panel) Nrf1a-V5 and increasing amounts of Nrf1aArg589Ter-V5. After 24 hours, a Dual-Glo assay was done to measure luciferase activity. Fold activation was calculated after normalizing firefly luciferase activity to protein concentration. Data represent the average of at least three independent experiments performed in triplicates. Error bars represent SD, and p values were calculated by Student's t-test. **(B)** Expression of endogenous Nrf1a-responsive genes is suppressed by Nrf1aArg589Ter. HAP1 cells were transfected with vector control, Nrf1a-V5 or Nrf1aArg589Ter-V5 expression vector. After 24 hours, total RNA was isolated, and analyzed for gene expression by RT-qPCR. Bar graphs depict expression of indicated genes relative to expression levels in vector transfected cells. P values were calculated by Student's t-test (n=3). Asterisk (*) indicates p value smaller than 0.05 ($p<0.05$). **(C)** Nrf1aArg589Ter associates with Nrf1a and TCF11. HEK293 cells were transfected with V5-tagged Nrf1aArg589Ter along with empty vector or HA-tagged Nrf1a. After 48 hours, cell lysates were prepared and subjected to immunoprecipitation with anti-V5 antibody followed by western blotting with indicated antibody. The presence of Nrf1a-HA and Nrf1aArg589Ter-V5 in cell extracts prior to immunoprecipitation was controlled using anti-V5 and anti-HA antibodies (see total lysate lanes). **(D)** Truncated Nrf1a does not bind MAFG. HEK293 cells were transfected with Myc-tagged MAFG along with V5-tagged Nrf1aArg589Ter or wild type Nrf1a. After 48 hours, cell lysates were prepared and subjected to immunoprecipitation with anti-V5 antibody followed by western blotting with indicated antibody. The presence of MAFG-Myc, Nrf1a-V5, and Nrf1aArg589Ter-V5 in cell

extracts prior to immunoprecipitation was controlled using anti-V5 and anti-Myc antibodies (see total lysate lanes).