Figures and figure supplements

The transcription factors TFE3 and TFEB amplify p53 dependent transcriptional programs in response to DNA damage

Owen A Brady et al
Figure 1. TFE3 and TFEB translocate to the nucleus in response to genotoxic stress. (A) Immunofluorescence images of WT MEFs treated with 100 μM etoposide for up to 16 hr. Scale bar = 20 μm. (B) Quantification of TFE3 localization from cells shown in A. Levels represent mean ± standard deviation.
with n = 3 experiments and > 200 cells counted per trial. Significance determined with Student’s t-test (*p < 0.05, ***p < 0.001). (C) Immunofluorescence images displaying TFE3 translocation in WT MEFs in response to different DNA damaging agents: etoposide, cisplatin, and UVC irradiation, and Scale bar = 10 μm. (D) Representative Western blot showing TFE3 and TFEB nuclear distribution by subcellular fractionation of WT MEFs exposed to etoposide for 10 hr. (E) Representative Western blot showing TFE3 de-phosphorylation at Ser321 and gel shift in TFEB in WT MEFs exposed to etoposide for up to 8 hr. EBSS for 2 h hours used as a positive control for de-phosphorylation of TFE3 and TFEB. (F) Representative Western blot showing TFE3 and TFEB de-phosphorylation in HeLa cells in response to UV-C light. (G) Representative Western blot showing mTORC1 inhibition due to S6K and 4EBP1 de-phosphorylation in response to increasing etoposide treatment time. EBSS for 2 hr used as a positive control for maximum mTORC1 inhibition. All the western blots are representative of three independent experiments.

DOI: https://doi.org/10.7554/eLife.40856.002
Figure 1—figure supplement 1. (A) Immunofluorescence images of ARPE-19 cells treated with 100 μM etoposide or 50 μM Cisplatin for 24 hr or 10 hr after UVC irradiation. Scale bar = 10 μm. (B) Immunofluorescence images of HeLa cells treated with 100 μM etoposide for 24 hr or 50 μM Cisplatin for 16 hr after UVC irradiation. Scale bar = 10 μm. (C) Immunofluorescence images of RAW 264.7 cells treated with 100 μM etoposide or 50 μM Cisplatin for 24 hr or 10 hr after UVC irradiation. Scale bar = 10 μm. (D) Western blot analysis of MEF control, UV, etoposide, cisplatin treated for 24 hr. (E) Western blot analysis of ARPE-19 control, UV, etoposide, cisplatin treated for 24 hr. (F) Western blot analysis of HeLa control, UV, etoposide, cisplatin treated for 24 hr. (G) Western blot analysis of Raw264.7 control, UV, etoposide, cisplatin treated for 24 hr. (H) Western blot analysis of Total lysate, Memb. + Cytosol, Nuclei control, etoposide treated for 4, 8, 16, 24 hr. (I) Western blot analysis of ARPE-19 control, etoposide 4, 8, 16, 24 hr, EBSS 2h.
Figure 1—figure supplement 1 continued

12 hr and 4 hr after UVC irradiation. Scale bar = 10 μm. (C) Immunofluorescence images of RAW 264.7 cells treated with 100 μM etoposide for 10 hr or 35 μM Cisplatin for 10 hr or 4 hr after UVC irradiation. Scale bar = 10 μm. (D) Representative Western blot showing TFE3 de-phosphorylation at Ser321 and gel shift in TFEB in WT MEFs exposed to 100 μM etoposide for 8 hr or 50 μM Cisplatin for 10 hr or 10 hr after UVC irradiation. (E) Representative Western blot showing TFE3 de-phosphorylation at Ser321 and gel shift in TFEB in ARPE19 cells exposed to 100 μM etoposide for 24 hr or 50 μM Cisplatin for 24 hr or 24 hr after UVC irradiation. (F) Representative Western blot showing TFE3 de-phosphorylation at Ser321 and gel shift in TFEB in HeLa cells exposed to 100 μM etoposide for 24 hr or 50 μM Cisplatin for 18 hr. (G) Representative Western blot showing TFE3 de-phosphorylation at Ser321 and gel shift in TFEB in RAW 264.7 cells exposed to 100 μM etoposide for 8 hr or 50 μM Cisplatin for 8 hr or 4 hr after UVC irradiation. (H) Representative Western blot showing TFE3 nuclear distribution by subcellular fractionation of WT MEFs exposed to 50 μM Cisplatin for 10 hr. (I) Western blot showing etoposide dependent S6K de-phosphorylation in ARPE19 cells. All the immunoblots are representative of three independent experiments.

DOI: https://doi.org/10.7554/eLife.40856.004
Figure 2. DNA damage-induced TFE3 and TFEB activation is a p53 and mTORC1 dependent process. (A) Representative Western blot showing p53-dependent inhibition of mTORC1 in response to etoposide treatment in WT and p53\(^{-/-}\) MEFs. EBSS for 2 hr was used as a positive control for mTORC1 phosphorylation.

Figure 2 continued on next page.
inhibition and was relatively unaffected by p53 status. (B) Quantification of Western blot data shown in A. Values represent mean ± standard deviation with n = 5. Significance determined with Two-way ANOVA with Sidak’s multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). (C) Immunofluorescence images displaying TFE3 translocation after 8 hr etoposide in WT MEFs compared to p53-/- MEFs. Scale bar = 20 μm. (D) Quantification of results from C. Levels represent mean percentage of cells localized in the nucleus, cytosol, or evenly distributed between both with n = 3 and > 80 cells counted per condition per trial. Significance determined with Student’s t-test (*p < 0.05). (E) Immunofluorescence images displaying TFEB translocation after 8 hr etoposide in WT and p53-/- MEFs infected with Ad-TFEB-FLAG. Scale bar = 20 μm. (F) Quantification of results from E. Levels represent mean percentage of cells localized in the nucleus, cytosol, or evenly distributed between both with n = 2 and > 80 cells counted per condition per trial. Significance determined with Student’s t-test (*p < 0.05). (G) Immunofluorescence images displaying TFE3 cellular distribution after 24 hr etoposide in ARPE-19 cells expressing active Rag heterodimers. Asterisks indicate transfected cells. Scale bar = 10 μm. (H) Quantification of results from G. Values represent mean ± standard deviation of the percentage of cells with nuclear TFE3 with n = 2 experiments and > 300 cells counted per trial (****p < 0.0001).

DOI: https://doi.org/10.7554/eLife.40856.005
Figure 2—figure supplement 1. (A) Representative Western blot showing TFEB and TFE3 gel shifts in response to etoposide in WT MEF, but not in p53⁻/⁻ MEF. All the immunoblots are representative of three independent experiments. (B) qPCR data showing relative induction of lysosomal-autophagy genes in response to starvation in WT and p53⁻/⁻ MEF. Data normalized to untreated cells and represents geometric means ± standard deviation and significance determined with Student’s t-test (*p < 0.05, **p < 0.01, ***p < 0.001).

DOI: https://doi.org/10.7554/eLife.40856.010
Figure 3. Differentially regulated genes in WT versus TFEB/TFE3 DKO MEFs undergoing DNA damage. qRT-PCR-based quantification of basal and etoposide induced mRNA levels of Rad9a, Chek2, Trp53inp1, Mdm2, Bbc3, Bax, Sesn1, Sesn2, Dram1, Tp53, Cdkn1a, Laptm5, Ctsd, Wrap53, Egfr and Foxo3.
Figure 3 continued

Foxo3 in WT vs TFEB/TFE3 DKO MEFs. All qRT-PCR data represented as geometric mean ± standard deviation and significance tested using Student's t-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, #p < 0.00001).

DOI: https://doi.org/10.7554/eLife.40856.012
Figure 3—figure supplement 1. qRT-PCR analysis of differentially expressed p53 upstream regulators and downstream effectors in WT versus TFEB/TFE3 DKO RAW264.7 cells. All data represented as geometric mean ± standard deviation and significance tested using Student’s t-test with n = 3 (*p < 0.05, **p < 0.01). DOI: https://doi.org/10.7554/eLife.40856.014
Figure 4. p53 induction in response to DNA damage is impaired in TFEB/TFE3 DKO RAW264.7 cells. (A) Representative Western blot showing p53 induction, p53 Ser15 phosphorylation, and Mdm2 levels in WT and TFE3/TFEB DKO RAW264.7 cells following etoposide treatment up to 8 hr. (B) Figure 4 continued on next page.
Quantification of p53 induction from data shown in A. Data represents mean relative p53 level ± standard deviation with n = 3. Significance tested with two-way ANOVA with Sidak’s multiple comparisons test (***p < 0.01, ****p < 0.0001). (C) Quantification of data shown in A. Total Mdm2 levels are significantly increased in TFEB/TFE3 DKO RAW264.7 cells compared to WT controls at 2- and 4 hr etoposide treatment. Data represents mean relative Mdm2 levels ± standard deviation with n=3. Significance tested with Student’s t-test (*p < 0.05, ***p < 0.001). (D) Representative Western blot of cycloheximide chase assay showing decreased p53 half-life in TFEB/TFE3 DKO RAW264.7 cells compared to WT controls. Cells were pre-treated with etoposide 2 hr to induce p53 expression and were chased in the presence of etoposide and cycloheximide. (E) Quantification of p53 levels from data shown in D. Data represents mean relative p53 level ± standard deviation with n = 3. Significance tested with Student’s t-test (*p < 0.05). (F) Representative Western blot showing rescue of p53 expression levels by treatment with nutlin-3 in TFEB/TFE3 DKO RAW264.7 cells after 8 hr etoposide treatment. (G) Quantification of p53 levels shown in F. Data represents mean relative p53 level ± standard deviation with n = 3. Significance tested using Student’s t-test (****p < 0.0001).

DOI: https://doi.org/10.7554/eLife.40856.016
Figure 5. Expression of constitutively active TFEB and TFE3 in HeLa cells increases total p53 protein levels and its extends half-life. (A) Representative Western blot showing elevated p53 protein levels in adenovirus infected HeLa cells expressing constitutively active mutants of TFEB and TFE3. Further Figure 5 continued on next page.
p53 protein level increases are seen with constitutively active TFEB and TFE3 after treatment with etoposide 8 hr. (B) Quantification of basal p53 protein level in HeLa cells expressing constitutively active TFEB and TFE3. Due to the high dynamic range and low detectability of basal endogenous p53 in control cells, values were normalized to intermediate expression samples, TFE3 S321A. Data represents mean relative p53 level ± standard deviation with n = 5 (*p < 0.05, ***p < ). (C) Immunofluorescence images of HeLa cells expressing constitutively active TFEB and TFE3 exhibit robust p53 accumulation in the nucleus compared to control cells. Scale bar = 20 μm. (D) Representative Western blot of cycloheximide chase assay showing increased stability of p53 in HeLa cells infected with control (Null) adenovirus or adenovirus expressing constitutively active TFEB and TFE3. (E) Quantification of cycloheximide chase assay shown in D. Values represent mean p53 protein levels ± standard deviation and each condition normalized relative to time 0 for that cell population with n = 4. Significance tested with two-way ANOVA with Dunnett’s multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

DOI: https://doi.org/10.7554/eLife.40856.021
Figure 5—figure supplement 1. (A) Western blot showing that TFEB-S211A expression, but not the NLS mutant, increases p53 protein levels in HeLa cells. (B) Immunofluorescence images of HeLa cells expressing either TFEB-S211A or TFEB-S211A/NLSmut. Only the nuclear localized TFEB-S211A
expressing cells show appreciable p53 nuclear accumulation. Transfected cells indicated with arrows and untransfected cells indicated with arrowheads. Scale bar = 20 μm. (C) qRT-PCR analysis of TFEB-S211A and TFEB-S321A expressing HeLa cells for expression of DNA damage response and p53-dependent genes. All qRT-PCR data represented as geometric mean ± standard deviation and significance tested using Student's t-test with n = 3 (^p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, #p < 0.00001).

DOI: https://doi.org/10.7554/eLife.40856.024
Figure 6. TFEB and TFE3 are essential for etoposide-induced lysosomal membrane permeabilization in MEFs. (A) Immunofluorescence images showing LMP in MEFs. Red galectin-1 puncta appear co-localized or within the lumen of green Lamp1 positive lysosomes. No LMP is detected under basal conditions. (B) Graph showing the increase in Galectin-1/Lamp1+ puncta per cell over time following etoposide treatment. (C) Bar graph showing the comparison of Galectin-1/Lamp1+ puncta per cell between WT and DKO MEFs at 8h post etoposide treatment. (D) Bar graph showing the percentage of cells with permeabilized lysosomes, categorized by the number of Galectin-1/Lamp1+ puncta, in WT and DKO MEFs at 8h post etoposide treatment. **P < 0.0001. Figure 6 continued on next page.
conditions in either WT or TFEB/TFE3 DKO MEFs. Treatment with etoposide induces profound LMP in WT, but not TFEB/TFE3 DKO cells. No differences in LMP induction were detected in LLOMe treated cells, regardless of genotype. Scale bar = 20 μm, inset = 2 μm. (B) WT MEFs exhibit a time-dependent increase in LMP after etoposide treatment. Quantification of data shown in A of galectin-1+/Lamp1 + LMP puncta per WT MEF cell. Data represent mean number of puncta per cell ± standard deviation from randomly selected confocal images, with > 20 cells per counted for each time point over three separate experiments. (C) Quantification of total number of galectin-1+/Lamp1+ LMP puncta per cell in WT vs TFE3/TFEB DKO MEFs treated for 8 hr with etoposide. Distribution is representative of one of the three independent experiments performed and shows 29 randomly selected WT MEF cells and 51 randomly selected TFEB/TFE3 DKO MEF cells. Significance determined using Student's t-test (****p < 0.0001). (D) Quantification of overall galectin-1+/Lamp1+ LMP puncta distribution after 8 hr etoposide treatment in WT versus TFE3/TFEB DKO MEFs. Data were binned from three separate experiments with > 100 cells represented in each category and a minimum of 29 cells from each trial. DOI: https://doi.org/10.7554/eLife.40856.026
Figure 7. TFE3 and TFEB are necessary for proper execution of apoptosis in response to DNA damage in RAW264.7 cells. (A) Representative Western blot showing Caspase-3 cleavage in response to increasing time of etoposide treatment. (B) Quantification of data shown in A indicating defects in Caspase-3 cleavage in TFEB/TFE3 DKO RAW264.7 cells. Cleaved Caspase-3 levels are normalized to WT cells after 16 hr etoposide treatment with n = 3. Significance tested with Student’s t-test (#p < 0.00001). (C) Annexin V/7-AAD flow cytometry assay data showing an impaired progression through early (AnnexinV+/7-AAD-) and late (AnnexinV+/7-AAD+) apoptosis in TFEB/TFE3 DKO RAW264.7 cells after 16 and 24 hr etoposide treatment. (D) Quantification of data shown in C. Data taken from three independent experiments and significance tested using Student’s t-test (*p < 0.05, **p < 0.01, #p < 0.00001).

DOI: https://doi.org/10.7554/eLife.40856.029
**Figure 8.** Comparative gene expression of etoposide-treated WT and TFEB/TFE3-DKO RAW264.7 cells. (A) Principal component analysis of genes with q-value < 0.05 reveals distinct clustering of WT and TFEB/TFE3 DKO RAW 264.7 cells exposed to etoposide. (B) Volcano plot indicating distribution of genes regulated by the p53-DREAM pathway.
genes significantly down- and up-regulated in WT versus TFEB/TFE3 DKO RAW264.7 cells exposed to etoposide for 8 hr. Cutoffs indicate genes with q-value < 0.05. (C) Hierarchical cluster heat map showing expression of DREAM pathway genes in three independent samples of WT and TFEB/TFE3 DKO RAW264.7 cells following etoposide treatment for 8 hr. Each row shows the relative expression level of a single mRNA. Each column shows the expression level of a single sample. Up-regulated mRNAs are shown in red and down-regulated mRNAs are shown in green.

DOI: https://doi.org/10.7554/eLife.40856.032
Figure 8—figure supplement 1. (A) Enriched GO terms in the ‘Biological Process’ category of differentially expressed genes between etoposide-treated WT and TFEB/TFE3 DKO RAW264.7 cells. GO terms are ranked by q value.

Figure 8—figure supplement 1 continued on next page.
Figure 8—figure supplement 1 continued

value (< 0.05). (B) Heatmap showing unsupervised hierarchical clustering of the 50 most significantly (q value < 0.05) differentially expressed genes between WT and TFEB/TFE3 DKO RAW264.7 cells following 8 hr of etoposide treatment. Up-regulated genes are shown in red, down-regulated genes are shown in green. DOI: https://doi.org/10.7554/eLife.40856.033
Figure 9. TFEB and TFE3 promote expression of cell cycle regulators. (A) Profiles of Chip-seq analysis for TFE3 in CDK4 and CDK7 promoters in RAW264.7 cells under stress condition. (B) qPCR-based quantification of CDK4 and CDK7 mRNA levels in adenovirus infected HeLa cells expressing TFEB/3 DKO. Figure 9 continued on next page.
control (Null) or constitutively active mutants of TFEB and TFE3. Data represented as geometric mean ± standard deviation and significance tested using Student’s t-test with n = 3 (**p < 0.001, ****p < 0.0001). (C) Representative Western blot showing CDK4 and CDK7 levels in adenovirus infected HeLa cells expressing constitutively active mutants of TFEB and TFE3. Quantification of protein levels are shown on the right panels. Data represents mean relative CDK4 and CDK7 to GAPDH levels ± standard deviation. Significance tested using Student’s t-test with n = 3 (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). (D) Representative Western blot showing the expression of cell cycle regulators in response to etoposide treatment in WT versus TFEB/TFE3 DKO RAW264.7 cells. (E) Representative Western blot showing Mdm2 and p53 levels in WT and TFEB/TFE3 DKO RAW264.7 cells following etoposide treatment. EBSS, indicated as E, was used for 2 hr as a positive control for maximum mTORC1 inhibition. All the western blots are representative of three independent experiments. (F) Quantification of phospho-Rb/total-Rb ratios from data shown in D. Data represents mean relative phospho-Rb to total Rb level ± standard deviation with n = 3. Significance tested using Student’s t-test (*p < 0.05, **p < 0.01).

DOI: https://doi.org/10.7554/eLife.40856.034
Figure 10. Schematic representation of a novel p53-mTORC1-TFEB/TFE3 pathway activated by DNA damage stress. Following DNA damage stress, p53 rapidly promotes the transcription of numerous downstream targets involved in DNA repair, cell cycle arrest and apoptosis. Some p53 targets, such as the members of the sestrin family, cause a reduction in mTORC1 activity, thus leading to TFEB and TFE3 activation. This p53-dependent activation of TFEB and TFE3 results in enhanced p53 signaling.

DOI: https://doi.org/10.7554/eLife.40856.038