Cell–fate determination by ubiquitin–dependent regulation of translation

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Metazoan development depends on the accurate execution of differentiation programs that allow pluripotent stem cells to adopt specific fates. Differentiation requires changes to chromatin architecture and transcriptional networks, yet whether other regulatory events support cell-fate determination is less well understood. Here we identify the ubiquitin ligase CUL3 in complex with its vertebrate-specific substrate adaptor KBTBD8 (CUL3KBTBD8) as an essential regulator of human and Xenopus tropicalis neural crest specification. CUL3KBTBD8 monoubiquitylates NOLC1 and its paralogue TCOF1, the mutation of which underlies the neurocrisis syndrome. Ubiquitylation drives formation of a TCOF1–NOLC1 platform that connects RNA polymerase I with ribosome modification enzymes and remodels the transcriptional program of differentiating cells in favour of neural crest specification. We conclude that ubiquitin-dependent regulation of translation is an important feature of cell-fate determination.

Cullin-RING ligases (CRLs), the largest class of ubiquitylation enzymes, have critical roles in metazoan development. CRLs recognize their substrates through ∼300 adaptor proteins, several of which are differentially expressed during development. Although mutations in CRL adaptors have been linked to human pathology, little is known about how distinct CRLs ensure robust differentiation into specialized cell types.

To discover CRLs with crucial roles in development, we employed genome-wide transcript analysis of differentiating human embryonic stem cells (hESCs). These experiments revealed a strong reduction in the abundance of the vertebrate-specific CUL3 adaptor KBTBD8 during hESC differentiation (Extended Data Fig. 1a–c), which we confirmed for KBTBD8 messenger RNA and protein by quantitative reverse transcription PCR (qRT–PCR) and western blot analysis (Extended Data Fig. 1d–g). Consistent with evolutionary conservation, downregulation of KBTBD8 was observed in differentiating mouse embryonic stem cells (Extended Data Fig. 1h, i), as well as during Xenopus tropicalis development.

Depletion of KBTBD8 did not affect the cell cycle, survival, or pluripotency programs of hESCs (Extended Data Fig. 2a–e). Instead, gene expression profiles of hESCs subjected to embryoid body differentiation suggested that KBTBD8 was required for neural crest specification (Extended Data Fig. 2f and Supplementary Table 1). qRT–PCR experiments confirmed that loss of KBTBD8 reduced expression of neural crest markers, including FOXD3 and SOX10, which was accompanied by an increase in transcripts associated with

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Figure 1 | CUL3KBTBD8 drives neural crest specification. a, hESCs stably depleted of KBTBD8 were subjected to neural conversion and analysed by qRT–PCR (mean of 3 technical replicates, ±s.e.m.). b, Depletion of KBTBD8 results in loss of neural crest cells, as determined by western blot analysis (full scans available in Supplementary Fig. 1). NC 9d, neural conversion, day 9; molecular weight is given in kDa. c, KBTBD8-depleted hESCs were subjected to neural conversion and analysed by immuno-fluorescence microscopy (mean of 3 biological replicates, ±s.e.m.; ∼1,500 cells per condition). d, Xenopus tropicalis embryos injected with translation-blocking morpholinos against KBTBD8 were analysed by in situ hybridization. e, Model of the CUL3KBTBD8-controlled developmental switch.
centrally nervous system (CNS) precursor and forebrain identity (FOXG1, SIX3; Extended Data Fig. 2g).

On the basis of these observations, we subjected hESCs to dual-SMAD inhibition (‘neural conversion’), which directs differentiation towards CNS precursors and neural crest cells.37 As seen during embryoid body differentiation, depletion of KBTBD8 caused a striking loss of neural crest cells and an increase in CNS precursors (Fig. 1a, b), which was seen for multiple short hairpin RNAs (shRNAs) and was rescued by shRNA-resistant KBTBD8 (Fig. 3b and Extended Data Fig. 3g). We corroborated these results with single-cell resolution using the neural crest marker SOX10 (Fig. 1c) or AP2, p75 and HNK1, which are co-expressed in mouse neural crest cells (Extended Data Fig. 3a). KBTBD8 was required for early neural crest specification, with CNS precursor and forebrain identity (CNS) precursors and neural crest cells.37 As seen during embryoid body differentiation, depletion of KBTBD8 caused a striking loss of neural crest cells and an increase in CNS precursors (Fig. 1a, b), which was seen for multiple short hairpin RNAs (shRNAs) and was rescued by shRNA-resistant KBTBD8 (Fig. 3b and Extended Data Fig. 3g). We corroborated these results with single-cell resolution using the neural crest marker SOX10 (Fig. 1c) or AP2, p75 and HNK1, which are co-expressed in mouse neural crest cells (Extended Data Fig. 3a). KBTBD8 was required for early neural crest specification, with CNS precursor and forebrain identity markers were first detected in control experiments (Extended Data Fig. 3a). KBTBD8 was required for early neural crest specification, with CNS precursor and forebrain identity markers being expressed in most neural crest cells (Extended Data Fig. 3a). KBTBD8 was accordingly critical for differentiation of neural crest cells into glia, mesenchymal cells, melanocytes, or chondrocytes (Extended Data Fig. 3a). KBTBD8 was required for early neural crest specification, with CNS precursor and forebrain identity markers being expressed in most neural crest cells (Extended Data Fig. 3a). KBTBD8 was accordingly critical for differentiation of neural crest cells into glia, mesenchymal cells, melanocytes, or chondrocytes (Extended Data Fig. 3a).

KBTBD8 recognition and monoubiquitylation of TCOF1 and NOLC1 revealed that KBTBD8, but neither KBTBD8(W579A) nor KBTBD8(Y74A), induced the robust monoubiquitylation of TCOF1 and NOLC1 (Fig. 2a). Using western blot analysis, we confirmed binding of TCOF1 and NOLC1 to KBTBD8 but not KBTBD8(W579A) (Fig. 2a). Western blot analysis confirmed binding of TCOF1 and NOLC1 to KBTBD8 but not KBTBD8(W579A) (Fig. 2a).

To isolate essential targets of CUL3KBTBD8, we used CompPASS mass spectrometry to capture proteins that bound wild-type KBTBD8 but not variants with a mutant substrate-binding domain (KBTBD8(W579A); Extended Data Fig. 5a–d). These interaction networks identified the paralogues NOLC1 and TCOF1 as predominant interactors of KBTBD8, which were not recognized by KBTBD8(W579A) (Fig. 2a). Using western blot analysis, we confirmed binding of TCOF1 and NOLC1 to KBTBD8 but not KBTBD8(W579A) (Fig. 2a). Western blot analysis confirmed binding of TCOF1 and NOLC1 to KBTBD8 but not KBTBD8(W579A) (Fig. 2a).

Similar to loss of KBTBD8, hESCs expressing only KBTBD8(W579A) or KBTBD8(Y74A) failed to support neural crest specification and showed increased abundance of CNS precursors.

Figure 2 | CUL3KBTBD8 monoubiquitylates TCOF1 and NOLC1. a, High-confidence interactors of wild-type (WT) or mutant KBTBD8. Left: normalized total spectral counts (TSC) per interactor of wild-type KBTBD8 (sum of 3 biological replicates per condition). Right: heat map depicting binding relative to wild-type KBTBD8. b, Verification of KBTBD8 interactions in 293T cells by anti-Flag immunoprecipitation and western blot analysis. IP, immunoprecipitation; molecular weight is given in kDa. c, Immunoprecipitation of KBTBD8 from hESCs (full scans available in Supplementary Fig. 1). d, Ubiquitylated (Ubi) HA-tagged TCOF1 detected after denaturing Ni-NTA purification in 293T cells reconstituted with KBTBD8 variants. e, Monoubiquitylation of HA–NOLC1 by CUL3KBTBD8 in 293T cells. f, Monoubiquitylation of endogenous TCOF1 and NOLC1 in 293T cells reconstituted with KBTBD8 variants and His–ubiquitin(1–73P).

Figure 3 | CUL3KBTBD8 controls neural crest specification through TCOF1 and NOLC1. a, hESCs were reconstituted with shRNA-resistant KBTBD8 variants or depleted of KBTBD8-binding partners, subjected to neural conversion (9 d), and analysed by qRT–PCR and unsupervised clustering. b, Protein expression during neural conversion of hESCs reconstituted with shRNA-resistant KBTBD8 variants (full scans available in Supplementary Fig. 1). Molecular weight is shown in kDa. c, Protein expression in hESCs stably depleted of KBTBD8, TCOF1, or NOLC1 and subjected to neural conversion. d, hESCs were stably depleted of the indicated combinations of KBTBD8, TCOF1, or NOLC1, subjected to neural conversion (9 d), and analysed by qRT–PCR.

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(Fig. 3a, b and Extended Fig. 6a, b). The same aberrant differentiation program was observed if we depleted TCOF1 or NOLC1 (Fig. 3a, c and Extended Data Fig. 6a, d), but not other KBTBD8-binding partners (Fig. 3a and Extended Data Fig. 6e, f). Demonstrating that these proteins act in a common pathway, co-depletion of KBTBD8 and TCOF1 or NOLC1, respectively, mirrored the differentiation program of singly depleted hESCs (Fig. 3d). We therefore conclude that TCOF1 and NOLC1 are critical monoubiquitylation substrates of CUL3KBTBD8 during neural crest specification. Consistent with this notion, mutations in TCOF1 cause Treacher Collins syndrome, a craniofacial disorder characterized by loss of cranial neural crest cells.

To understand how CUL3KBTBD8 drives neural crest specification, we identified proteins that selectively recognized ubiquitylated, but not unmodified, TCOF1 using cells that were reconstituted with either wild-type KBTBD8, inactive KBTBD8(Y74A), or empty vector. Notably, NOLC1 emerged as the major effector that was recruited to either wild-type KBTBD8, inactive KBTBD8(Y74A), or empty vector.

On the basis of these results, we established a sequential affinity purification protocol to determine the composition of ubiquitylation-dependent TCOF1–NOLC1 complexes. We found that TCOF1–NOLC1 assemblies engaged RNA polymerase I; the H/ACA complex catalysing rRNA pseudouridylation; and the SSU processome controling maturation and modification of the small ribosomal subunit (Fig. 4c, d and Extended Data Fig. 7c). Accordingly, ubiquitylation by CUL3KBTBD8 brought endogenous RNA polymerase I into complexes with the SSU processome (Fig. 4e), which required TCOF1 and NOLC1 (Extended Data Fig. 7d). Similar observations were made in hESCs, where a robust interaction between RNA polymerase I and SSU processome was lost upon depletion of KBTBD8 (Fig. 4f).

Thus, CUL3KBTBD8 induces the ubiquitin-dependent formation of TCOF1–NOLC1 complexes that serve as a platform to connect RNA polymerase I with enzymes responsible for ribosomal processing and modification (Fig. 4g). This observation supports a role of ubiquitylation in neural crest specification, as mutations in RNA polymerase I also cause Treacher Collins syndrome.

Although KBTBD8 targets proteins linked to ribosome biogenesis, its depletion did not affect the abundance of rRNAs or mRNAs encoding ribosomal proteins; levels of ribosomal proteins; processing of precursor rRNAs; nucleolar integrity; export of the small ribosomal subunit; ribosome binding to mRNA judged by polysome gradient analysis; global mRNA translation detected by metabolical labelling; or cell survival (Fig. 5a, d and Extended Data Fig. 8a–h). Accordingly, a global reduction in translation caused by rapamycin did not phenocopy the loss of KBTBD8 (Extended Data Fig. 9a, b). Depletion of TCOF1 also did not affect RNA synthesis, p53 activation, or cell survival at the time of neural crest specification (Extended Data Fig. 9c–e), although consistent with previous work, it reduced rRNA levels and triggered cell death at late stages of neural conversion.

**Figure 4 | Ubiquitylation-dependent TCOF1–NOLC1 complexes couple RNA polymerase I to ribosome modification enzymes.** a, Interactors of TCOF1 in 293T cells reconstituted with KBTBD8 or KBTBD8(Y74A) (sum of 3 biological replicates per condition). b, Validation of CUL3KBTBD8-dependent formation of TCOF1–NOLC1 complexes. c, CompPASS mass spectrometry analysis of sequential immunoprecipitation of Flag–TCOF1/HA–NOLC1 complexes. d, Validation of sequential affinity purification of KBTBD8-dependent TCOF1–NOLC1 complexes (full scans available in Supplementary Fig. 1). e, Immunoprecipitation of RNA polymerase I from 293T cells reconstituted with KBTBD8 variants. f, Immunoprecipitation of RNA polymerase I from hESCs depleted of KBTBD8. g, Model of ubiquitin-dependent formation of a TCOF1–NOLC1 platform. Molecular weight is given in kDa.
We therefore considered the possibility that CUL3KBTBD8-dependent assembly of a ribosome modification platform might produce ribosomes with distinct translational output. Indeed, as seen by RNA sequencing and ribosome profiling, depletion of KBTBD8 changed the translational program of cells undergoing neural conversion, whereas it had no effect on protein synthesis in hESCs (Fig. 5b and Extended Data Fig. 10a). Similar observations were made for TCOF1, and the translation efficiency profiles of differentiating hESCs lacking KBTBD8 or TCOF1 were correlated (Extended Data Fig. 10b). Loss of KBTBD8 caused changes in translation immediately after differentiation, and thus, before specification of hESCs into neural crest or CNS precursor cells (Fig. 5c).

Analysis of regulated mRNAs showed that KBTBD8 suppressed the production of proteins specifying CNS precursors, whereas it did not affect translation of mRNAs connected to neural crest specification (Extended Data Fig. 10c). In this manner, KBTBD8 or TCOF1 delayed the accumulation of CNS precursor proteins, including ATRX and PCM1, until neural crest specification had occurred (Fig. 5d and Extended Data Fig. 10d). Underlining the role of translational control, KBTBD8 enforced the correct timing of ATRX and PCM1 production without regulating their mRNA levels or protein stability (Fig. 5c, d and Extended Data Fig. 10e). The depletion of KBTBD8 also reduced the translation of mRNAs encoding histones or ribosomal components (Fig. 5b), yet as expected from their long half lives\textsuperscript{21,22}, the levels of the corresponding proteins were not diminished during our differentiation experiment (Fig. 5d). Thus, the CUL3\textsuperscript{KBTBD8}-dependent formation of a ribosome modification platform alters translation of specific mRNAs, which delays accumulation of CNS precursor proteins until hESCs have accomplished neural crest specification.

Our work documents an important role for ubiquitylation in remodelling translational programs during differentiation, and defines an early function for the CUL3\textsuperscript{KBTBD8} substrate and Treacher Collins syndrome-associated protein TCOF1 in neural crest specification (Fig. 5e). We hypothesize that CUL3\textsuperscript{KBTBD8} and TCOF1 may govern the production of differentially modified ribosomes, potentially including post-transcriptional changes in rRNA pseudouridylation and base methylation, or phosphorylation and ubiquitylation of ribosomal proteins or ribosome-associated factors. Such modifications may affect the interactions of ribosomes with select mRNAs, with factors that deliver specific mRNAs to the ribosome, or with proteins that control the synthesis or degradation of distinct mRNAs\textsuperscript{23–25}.

Together with studies implying specific functions for ribosomal proteins during differentiation\textsuperscript{19,20}, developmental switches controlled by ribosomal regulation might explain why mutations in ribosome biogenesis factors result in tissue-specific ribosomopathies\textsuperscript{2,19,20,28–30}. Manipulating such switches could lead to therapeutic strategies for paediatric diseases: as Treacher Collins syndrome is caused by mutation of a single TCOF1 allele, increasing the efficiency of KBTBD8-dependent ubiquitylation of the remaining wild-type TCOF1 might reconstitute ribosomal regulation and neural crest formation.

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Author Contributions A.W. designed and performed most experiments and helped to write the manuscript; S.I. performed ribosomal profiling and RNA processing experiments; C.A.M. and S.M.-R. performed Xenopus experiments; N.T. performed binding studies; J.I. wrote code for analysis of CompPASS mass spectrometry; N.T.I. supervised ribosomal profiling studies; M.R. helped design experiments and wrote the manuscript.

Author Information Raw data for microarray, RNA-seq and ribosome profiling was deposited in the NCBI Gene Expression Omnibus under accession numbers GSE62123 (microarray data) and GSE62247 (RNA seq and foot-printing data). Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.R. (mrape@berkeley.edu).
Plasmids, shRNAs, siRNAs, morpholinos. Full-length KBTBD8 was cloned into pcDNA3.1 using a C-terminal 3’ Flag tag, or a C-terminal HA tag, for expression in human cells, into pMAL with an N-terminal MBP tag for expression in *Escherichia coli* and into pFastBac with an N-terminal 6× His tag for expression in Sf9 ES insect cells using the Bac-to-Bac baculovirus expression system (Invitrogen). For expression in human embryonic stem cells, KBTBD8 was cloned into pENTR1A with a C-terminal Flag tag and recombined into pLenti-PGK-Hygro. Point mutants in KBTBD8’s BTB-BACK domain (Y74A, M78D, L73D), were constructed using site-directed mutagenesis and digestion of parent DNA with DpnI. Full-length coding sequences of TCOFI and NOLCI were cloned into pCMV-3×Flag or pCS2- HA vectors for expression in human cells and in vitro transcription/translation-IVT) reverting, respectively, as described11. His–ubiquitin was cloned into a pCS2 vector for expression in cells. A L7SP mutation was introduced in this vector by site-directed mutagenesis as described above. pLOK1-Puro Mission shRNA constructs targeting KBTBD8 (#1, TRCN000103280; #2, TRCN0001028536; NOLCI (#1, TRCN000061971; #2, TRCN0002198179), TCOFI (#1, TRCN0000008360; and BRD2 (#1, TRCN000006310; #2, TRCN0000381017). D1D5X (#1, TRCN000000026; #2, TRCN000425479), PNT1 (#1, TRCN000016252; #2, TRCN0000162998) and ANKUBI (#1, TRCN0000336664) were purchased from Sigma. siRNAs were from Dharmacon and cloned into pLKO1-hygro-shRNA plasmids (UAGCAUAGUAACAUCAGUU, AR61, #1, CGGAGAAGUUUGGAGGAAGUU; #2, UCUGAUCGUGAAUAU, AR62, #1, CAACAGAGCUAGCUGGAAG; #2, CUGAUUGACUGCAAGGAUAU, AR63, #1, CCAGAAGGGCUGCGGAAAU, #2, GAAAGGCUCGCCGAGGAUAU; #3, GGAAGACAGCACGAGCGGAUAU). Four morpholino oligonucleotides (Gene Tools, LLC) were used: kbtbd8 translation blocking, 5’-CTGAGGAGAACGTCTACTTCTC-3’; kbtbd8 splice blocking, 5’-TCTCCCAGCAGCAACACCTCA-3’; cul3 splice blocking, 5’-AACAGGTATATCTCATGCTCA-3’; and cul3 translation blocking, 5’-GCCGTCGACACATCAGCTCA-3’. Dominant-negative CUL3 (N-terminal 250 amino acids of human CUL3) was cloned into pCS2+ and mRNA was synthesized for injection using an in vitro transcription system.

**Proteins.** KBTBD8, KBTBD8(Y74A) and KBTBD8(W579A) were purified from hESCs by nickel affinity chromatography and were analyzed for purity by western blotting using anti-HA (clone C29F4; Cell Signaling, 1:2,000 in IB), anti-HA (clone 12C10; Cell Signaling, 1:100 in IB), anti-rRNA 5.8S (ab534174, clone Y10bc, Abcam, 1:1000 in IF), anti-TCOFI (11003-1-AP, Proteintech, 1:250 in IF), anti-TCOFI (sc-49529, Santa Cruz, 1:1000 in IF), anti-FBL (ab8521, Abcam, 1:1000 in IF), anti-NOLCI (11815-1-P, Proteintech, 1:1000 in IF), anti-ARRB1/2 (#4674, clone D2H9, Cell Signaling, 1:1000 in IF), anti-PKIN (610687, clone 49/PK1, BD Biosciences, 1:1000 in IB), anti-CUL3 (Bethyl, 1:1000 in IF), anti-NANOG (#3580, Cell Signaling, 1:1000 in IF), anti-OCT4 (sc-8628, Santa Cruz, 1:100 in IF), anti-SNAI2 (#9585, clone C919G7, Cell Signaling, 1:1000 in IF), anti-GAPDH (#2118, clone 14G10, Cell Signaling, 1:1000 in IF), anti-BRD4 (#5848, clone D889B, Cell Signaling, 1:500 in IF), anti-PCNA (Santa Cruz, 1:5000 in IF), anti-PAI1 (Santa Cruz, 1:100 in IB, 1:5000 in IF), anti-RAB2 (sc-17913, Santa Cruz, 1:100 in IF), anti-RP1 (sc-5943, Santa Cruz, 1:100 in IF), anti-DKCI (sc-48794, Santa Cruz, 1:1000 in IF), anti-NF-L (sc-6057, Santa Cruz, 1:1000 in IF), anti-HA (clone C29F4; Cell Signaling, 1:5000 in IB), anti-HA (clone C29F4; Cell Signaling, 1:1000 in IB, anti-Flag (F1804, clone M2, Sigma, 1:2000 in IB), and anti-Flag (F7425, Sigma, 1:2000 in IB) antibodies were commercially purchased.

Mammalian cell culture and transfections. Human embryonic kidney (HEK) 293T cells were maintained in DMEM with 10% fetal bovine serum. Plasmid transfections of HEK 293T cells were with calcium phosphate and siRNA transfections were with Lipofectamine RNAIMAX (Invitrogen) according to the manufacturer’s instructions using 10 nM for each siRNA.

HEs cell culture, lentiviral infections and HEs differentiations. Human embryonic stem (hES) H1 cells were from the Wisconsin stem cell bank, routinely characterized for mycoplasma contamination, and maintained under feeder-free conditions on Matrigel-coated plates (#354277; BD Biosciences) in mTeSR1 (#05871-05852, StemCell Technologies Inc.) and were routinely passaged with collagenase (#07909, StemCell Technologies Inc.) and ReLeR (#05872, StemCell Technologies Inc.).

Lentiviruses were produced in 293T cells by co-transfection of lentiviral constructs with packaging plasmids (Addgene) for 48–72 h. Transduction was carried out by infecting 30% confluent HEs cells with lentivirus in the presence of 6 mg ml⁻¹ Polybrene (Sigma). After 7 days of selection with appropriate antibiotic (0.5 μg ml⁻¹ puromycin for pLOK1-puro-shRNA constructs, 500 μg ml⁻¹ hygromycin for pLOK1-hygro-shRNA or pLenti-Hygro constructs), HEs H1 cells were analyzed, and used in differentiation experiments.

Embryoid body formation from hES H1 cells and HEs H1 cells expressing control shRNA or shRNAs targeting KBTBD8 was performed using AggreWell 800 plates (#27865, StemCell Technologies Inc.) and APEL medium (#05210, StemCell Technologies Inc.) for differentiation. HEs cells at the early outgrowth stage were subjected to the neural induction medium supplemented with 10 μM Y-27632 ROCK inhibitor (Calbiochem). 24 h after seeding, EBs were harvested and transferred into ultra-low adherence culture dishes (Corning) and cultured free well per well of a 6-well plate and differentiated in APEL medium for 3 and 6 days. Medium was replaced every other day.

Induction of HEs H1 cells expressing different shRNA constructs was performed using STEMdiff Neural Induction Medium (#05831, StemCell Technologies Inc.) in combination with a monolayer culture method according to the manufacturer’s technical bulletin (#28044) and as previously described19. In brief, single-cell suspensions were prepared by treatment of HEs cells with accutase (#07920, StemCell Technologies Inc.) and 1 × 10⁶ cells were seeded per well of an AggreWell 800 plate in APEL medium supplemented with 10μM Y-27632 ROCK inhibitor (Calbiochem). 24h after seeding, EBs were harvested and transferred into ultra-low adherence culture dishes (Corning) and cultivated free well per well of a 6-well plate and differentiated in APEL medium for 3 and 6 days. Medium was replaced every other day.

Neural induction of HEs H1 cells expressing different shRNA constructs was performed using STEMdiff Neural Induction Medium as described above for 43 days. Medium was changed daily until 18 days, then every other day.

Long-term neural conversion experiments to assess spontaneous differentiation of neural crest cells into derivatives, HEsCs were subjected to the neural conversion protocol in the presence of 50 or 100 nM rapamycin.

For long-term neural conversion experiments to assess spontaneous differentiation of neural crest cells into derivatives, HEsCs were subjected to neural conversion using STEMdiff Neural Induction Medium as described above for 43 days. Medium was changed daily until 18 days, then every other day.

Microinjections and in situ hybridizations. Morpholinos (20 ng) and mRNA (100 pg) with 2 ng of tracer morpholino were injected into the animal cap in 1 blastomere of 2-cell stage *Xenopus tropicalis* embryos. At stage 10–14, embryos were sorted by left or right injection side via tracer morpholino fluorescence. Embryos were developed to stage 16–18 and fixed for 4–6 h in MEMFA at room temperature. In *in situ* hybridization of *X. tropicalis* embryos with digoxigenin-labelled RNA probes was performed using a multi-basket method as described previously15. Sorting and imaging were performed on a Zeiss SteREO Lumar.V12 microscope.
Cycloheximide (CHX) chase assays. For cycloheximide chase assays, control or KBTBD8-depleted hES H1 cells and cells that had undergone neural induction for 3 days were treated with 40 μg ml⁻¹ CHX for 2, 4 and 6 h. Cells were lysed in 8 M urea, 50 mM sodium phosphate, pH 8.0. Lysates were diluted in SDS loading buffer, sonicated, and were analysed by immunoblotting. For quantification, immunoblot signals for respective proteins were quantified using ImageJ (NIH, http://rsbweb.nih.gov/ij/) and normalized to GAPDH or β-actin.

Gene expression analysis by microarray. To compare gene expression profiles of hES-H1 cells (control and Flag-HA-KBTBD8) versus KBTBD8-depleted hES H1 cells, and gene expression profiles of control versus KBTBD8-depleted embryoid bodies (6 days), we isolated and purified total RNA from respective samples using the RNeasy Mini Kit (Qiagen, catalogue no. 74104). Microarray analysis was performed in biological triplicates by the Functional Genomics Laboratory (UC Berkeley) using the Affymetrix HUMAN GENE 1.0 ST ARRAY.

Quantitative real-time PCR (qRT–PCR) analysis. For qRT–PCR analysis, total RNA was extracted and purified from cells using the RNeasy Mini Kit (Qiagen, catalogue no. 74104) and transcribed into cDNA using the RevertAid first strand cDNA synthesis kit (#K1621, Thermo Scientific). Gene expression was quantified by Maxima SYBR Green/Rox qPCR (#K0221, Thermo scientific) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Nonspecific signals caused by primer dimers were excluded by dissociation curve analysis and use of non-template controls. To normalize for loaded cDNA, β-actin or RPS6 was used as endogenous control. Gene-specific primers for qRT–PCR were designed by using NCBI Primer-Blast or ordered pre-designed from Integrated DNA Technologies. Primer sequences can be found in Supplementary Table 2.

Cluster analysis. To determine shRNA treatments that caused similar effects on nuclear conversion of hESCs, we performed cluster analysis of mRNA expression profiles. We stably transduced H1 hESCs with lentiviruses expressing various shRNAs and/or shRNA-resistant cDNAs and subjected these cells to neural conversion by dual SMAD inhibition for 9 days. We then measured the mRNA abundance of neural progenitor and neural crest markers by RT-qPCR. Data sets were clustered using the heatmap.2 function of the gplots package on R.

In vivo ubiquitylation assays. To detect ubiquitylation of ectopic TCOF1 or NOLC1, HEK 293T cells were transiently transfected with 6× His-tagged ubiquitin and HA-tagged TCOF1 or NOLC1. For detection of ubiquitylation of endogenous TCOF1 or NOLC1, HEK 293T cells were transiently transfected with 6× His-tagged ubiquitin-173P. Cells were harvested, washed with PBS, lysed in 8 M urea, 50 mM sodium phosphate, pH 8.0 and sonicated. His-ubiquitin conjugates were purified using Ni-NTA agarose (Qiagen) and ubiquitylated NOLC1 or TCOF1 was detected by immunoblotting using anti-HA, anti-TCOF1, or anti-NOLC1 antibodies. For analysis of the influence of β-arrestin proteins on KBTBD8-mediated ubiquitylation, HEK 293T cells were transfected with control shRNAs or a pool of shRNAs targeting ARRB1 (#1 and #2) and ARRB2 (#1 and #2) 24 h before plasmid transfection.

Immunoprecipitations for mass spectrometry. Anti-Flag immunoprecipitations (IPs) for mass spectrometry analysis were performed from extracts of HEK 293T cells transiently expressing KBTBD8–3Flag or versions or 3Flag–TCOF1 in the presence and absence of KBTBD8 versions (20–15 × 1 cm dishes per condition). Lysis was in two pellet volumes of 20 mM HEPES pH 7.3, 150 mM NaCl, 110 mM KOAc, 2 mM Mg(OAc)₂, 5 mM EDTA, 5 mM EGTA, condition). Lysis was in two pellet volumes of 20 mM HEPES pH 7.3, 150 mM NaCl, 110 mM KOAc, 2 mM Mg(OAc)₂, 5 mM EDTA, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 100 mM Tris (pH 8.5), followed by reduction with 5 mM TCEP (Sigma), alkylation with 10 mM iodoacetamide (Sigma), and overnight digestion with trypsin (0.5 mg ml⁻¹, Fisher). Samples were analysed by the Vincent J Coates Proteomics/Mass Spectrometry Laboratory at UC Berkeley and compared with ~100 reference immunoprecipitations against different Flag-tagged bait proteins using a Java script programmed according to the CompPASS software suite. For determination of the KBTBD8 interaction network, three independent KBTBD8–Flag IPs were compared as replicates against the reference IPs. Thresholds for high confidence interaction partners (HCIPs) were top 5% of interactors with highest Z-score and highest WD score. To narrow down putative substrates of KBTBD8 in the interaction map, we compared relative total spectral counts for each HCIP found in wild-type KBTBD8 immunoprecipitates to the ones found in KBTBD8(Y74A), KBTBD8(F550A) and KBTBD8(W579A) immunoprecipitates. For identification of effector proteins recruited to TCOF1 upon ubiquitylation, we determined the TCOF1 interaction network as described above for KBTBD8 and compared relative total spectral counts for each TCOF1 HCIP to those found upon co-expression of KBTBD8 or KBTBD8(Y74A). We then plotted relative TSC changes upon KBTBD8 expression against the difference of relative TSC changes upon KBTBD8 and KBTBD8(Y74A) expression.

Immunoﬂuorescence microscopy. For immunoﬂuorescence analysis, hES H1 cells or hES H1 cells expressing different shRNA were seeded on Matrigel-coated coverglasses using accutase, ﬁxed with 3.7% formaldehyde for 10 min, permeabilized with 0.1% Triton for 20 min, and stained with indicated antibodies or Hoechst. Images were taken using Zeiss LSM 710 confocal microscope or Olympus IX81 microscope, deconvolved using Metamorph, and processed using ImageJ.

Determination of average nucleolar size. To analyse nucleolar integrity, we performed indirect immunoﬂuorescence microscopy using antibodies against ﬁbrillarin, an established nucleolar marker. We stained nucleoli of control and KBTBD8-depleted hESCs or of hESCs that were subjected to neural conversion for 3 days. Images were taken for each condition using a Zeiss LSM 710 confocal microscope with a 20× objective followed by quantification of average nucleolar and nuclear size using ImageJ. Average nucleolar size was expressed relative to average nucleolar area. Error bars represent standard deviation of three different images (~100 cells per image).

Analysis of cell cycle progression. For DNA content analysis, control or KBTBD8-depleted hES H1 cells or control or KBTBD8-depleted cells that had undergone neural induction for 3 or 6 days were ﬁxed in 70% ethanol in PBS overnight. Cells were pelleted and resuspended in PBS containing 1 mg ml⁻¹ RNase (Sigma) and 10 μg ml⁻¹ propidium iodide (PI), incubated at room temperature for 30 min, then analysed using a Beckman-Coulter EPICS XL Flow Cytometer (575 nm band pass ﬁlter).

Determination of the division rate of control and KBTBD8-depleted hES cells, we seeded 3×10⁶ cells per well of a 6-well plate using accutase. Cells were accutased at 2, 3 and 4 days post seeding and counted using a haemacytometer.

RNA sequencing and ribosome proﬁling. RNA-seq libraries were prepared with Tru-seq Ribo-zero gold kit (Illumina). The preparation of ribosome proﬁling library and the data analysis were performed according to the method previously described. The libraries were sequenced on a HiSeq 2000 Illumina. The reads were aligned to the hg19 human genome reference and the resulting aligned reads were mapped to UCSC known reference genes. Based on length of each footprint, we obtained each read coverages for 14 for 26–28 nucleotides and 15 for 29–31 nucleotides. For mRNA fragments, we used offset 14. For measuring footprint density and mRNA fragments between samples, we restricted our analysis to genes that have at least 128 summed counts in each sample, only including the genomic positions 15 codons following the start codon and the position 5 codons preceding the stop codon. DESeq was used to calculate fold change enrichment of genes by KBTBD8 or TCOF1 knockdown at each time point after neural induction.

Polysome proﬁling. Cells lysate was prepared as described. Lysate containing 3 μg total RNA was loaded on to 10–50% linear sucrose gradients containing 20 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTT, 100 μg ml⁻¹ cycloheximide, and 2 μM 1× SUPERase in RNase inhibitor and centrifuged at 56,000 rpm for 2.5 h at 4 °C with SW41 rotor (Beckman Coulter). UV absorbance of fractionated gradient with Gradient station (Bio-comp) was detected by ECONO UV monitor (Biorad). Monosome/Polyosome ratio was determined by integration of the area under the respective peaks using Igor Pro software (WaveMetrics).
Metabolic labelling. To determine global mRNA translation in control or KBTBD8-depleted hESCs, hESCs subjected to embryoid body formation for 3 days, or hESCs subjected to neural conversion for 3 days, we employed metabolic labelling. Cells were pre-equilibrated in labelling medium (RPMI without methionine containing 10% dialysed FBS) for 15 min, followed by preparation of single-cell suspensions by accutase treatment. 0.6 x 10^6 cells for hESCs and hESCs that were subjected to neural conversion for three days, and ~300 EBs (initial seeding cell number: 3,000 cells per EB) were pulsed with 100 μl labelling medium containing 0.1 mCi ml^-1 [35S]-methionine at 37 °C for 20 min. After washing with PBS, cells were lysed in 8 M urea, 100 mM Tris pH 8.0, diluted in SDS loading buffer, sonicated, and lysates were analysed by SDS–PAGE and autoradiography. [35S]-methionine incorporation was quantified using Image J software, normalized to total protein amount, and expressed relative to the control hESC sample (set to 1). Error bars denote standard deviation of three biological replicates.
Extended Data Figure 1 | KBTBD8 is a developmentally regulated CUL3 adaptor. a, Gene expression analysis by microarray of hESCs differentiated into embryoid bodies (EB) for 6 days (n > 30,000 transcripts, mean of 3 biological replicates, analysis of variance (ANOVA) P value < 0.05; blue, downregulated genes; red, upregulated genes). b, Expression analysis of all CRL substrate adaptors, including KBTBD8, with data derived from the experiment described above. c, Expression analysis of CUL3 adaptors during hESC differentiation into hEBs (blue, downregulation; yellow, upregulation). d, mRNA levels of pluripotency markers and KBTBD8 during hESC differentiation into EBs, as determined by qRT–PCR (mean of 3 technical replicates ± s.e.m.). e, Protein levels of KBTBD8 during hESC differentiation into EBs, as seen by western blot (OCT4, NANOG: pluripotency; PAX6: CNS precursors; TFAP2: neural crest marker). f, KBTBD8 is expressed in hESCs, but not in somatic cell lines, as determined by qRT–PCR (mean of 3 technical replicates ± s.e.m.). g, Abundance of KBTBD8 in H9 hESCs, D3 mESCs, or somatic cell lines was determined by western blot analysis. h, KBTBD8 expression is downregulated during mouse embryonic stem cell (mESC) differentiation into mouse embryoid bodies, as determined by qRT–PCR (mean of 3 technical replicates ± s.e.m.). i, KBTBD8 protein levels are reduced during mESC differentiation, as shown by western blot analysis.
Extended Data Figure 2 | KBTBD8 controls neural crest formation.

a, Stable depletion of KBTBD8 from H1 hESCs, as determined by western blot analysis. KBTBD8 depletion does not significantly change the cell cycle profile of hESCs, as determined by propidium iodide staining and FACS. 

b, Control or KBTBD8-depleted hESCs were counted at indicated times after seeding (mean of 3 biological replicates, ± s.d.). KBTBD8 depletion does not induce apoptosis in hESCs, as shown by immunostaining against cleaved caspase 3 (red) or DNA (blue) (200 cells per condition; scale bar, 10 μm).

c, KBTBD8 depletion does not affect the gene expression profile of hESCs, as determined by microarray analysis (genes > 2.5-fold change, n > 30,000; mean of 3 biological replicates, ANOVA P-value < 0.05).

d, Loss of KBTBD8 causes a decrease in the expression of neural crest cell markers during EB formation, as shown by comparative microarray analysis (genes > 2.5-fold change, n > 30,000; mean of 3 biological replicates, ANOVA P-value < 0.05). mRNA levels of pluripotency and differentiation markers in EBs stably expressing control or KBTBD8 shRNAs were measured by qRT–PCR (3 technical replicates ± s.e.m.).
Extended Data Figure 3 | KBTBD8 controls neural crest specification.

a, Depletion of KBTBD8 from hESCs subjected to neural conversion results in loss of neural crest cells, as determined by immunofluorescence against HNK1, TFAP2 and p75 (n > 200 cells, mean of 3 biological replicates ± s.d.). b, H1 hESCs transduced with control (green) or KBTBD8 shRNAs (red) were subjected to neural conversion, and expression of neural crest markers SOX10 (circles) and SNAIL2 (boxes) was monitored by qRT–PCR (mean of 3 technical replicates ± s.e.m.). c, H1 hESCs described above were subjected to neural conversion, and abundance of CNS precursor markers SOX2 (circles) and PAX6 (boxes) was measured by qRT–PCR. d, H1 hESCs described above were subjected to neural conversion, and abundance of telencephalon markers SIX3 (circles) and FOXG1 (boxes) was measured by qRT–PCR. e, Expression of OCT4 was monitored by qRT–PCR during neural conversion in the presence or absence of KBTBD8. f, hESCs stably expressing control or KBTBD8 shRNAs were subjected to neural conversion and analysed for expression of pluripotency (OCT4, CDH1), neural crest (SOX10, SNAIL2, AP2), or CNS precursor markers (PAX6) by western blotting. To provide consistency, samples were taken from the same experiment as shown in Fig. 5d (asterisks mark blots that are also shown in Fig. 5d). g, Loss of neural crest occurs in response to KBTBD8 depletion by two independent shRNAs, as shown by western blot analysis. h, HESCs were subjected to neural conversion and analysed by immunofluorescence microscopy against SOX10 (neural crest), PAX6 (CNS precursor), and OCT4 (pluripotency) (confocal, original magnification 20×).
Extended Data Figure 4 | KBTBD8 is required for differentiation into functional neural crest cells. a, H1 hESCs stably expressing control or KBTBD8 shRNAs were subjected to neural conversion for 43 days and analysed by immunofluorescence microscopy against GFAP (glia), smooth muscle actin (SMA; mesenchymal cells), and neurofilament L (neurons). b, Control H1 hESCs or hESCs depleted of KBTBD8 were subjected to neural conversion for 43 days and expression of markers for glia (GFAP), mesenchyme (smooth muscle actin, SMA), melanocytes (TYRP1, DCT), chondrocytes (COL2A1), or CNS derivatives (PAX6, NESTIN, neurofilament L) was analysed by qRT–PCR (mean of 3 technical replicates ± s.e.m.). c, Xenopus tropicalis embryos were injected at the two-cell stage with splice-blocking morpholinos (sMO) against CUL3 or KBTBD8, or with a dominant-negative construct of CUL3 that allows KBTBD8 to bind, but not ubiquitylate, substrates. Neural crest formation was monitored by SOX10 in situ hybridization. Quantification included experiment shown in Fig. 1d (mean of 3 biological replicates ± s.d.; ~20 embryos per condition and replicate).
Extended Data Figure 5 | Biochemical characterization of the substrate adaptor role of KBTBD8. a, Domain structure of KBTBD8, including the residues mutated to generate ubiquitylation-(Y74A) and substrate-binding-deficient KBTBD8 (F550A, W579A). b, Effects of point mutations in predicted KELCH domain loops on binding of KBTBD8 to candidate substrates were determined by affinity purification and western blot analysis. c, Effects of point mutations in BTB domain on binding of KBTBD8 to CUL3 were determined by affinity purification and western blotting. Dimerization of Flag–KBTBD8 with KBTBD8–HA was analysed in the same experiment to provide a folding control. d, Binding of recombinant CUL3 to immobilized recombinant MBP–KBTBD8 variants was analysed by Coomassie. e, Binding of in vitro-transcribed/translated 35S-NOLC1 to immobilized recombinant KBTBD8 variants was analysed by autoradiography. f, Binding of in vitro-transcribed/translated 35S-TCOF1 to immobilized recombinant KBTBD8 variants was analysed by autoradiography. g, Endogenous β-arrestin proteins in reticulocyte lysates binds immobilized, recombinant KBTBD8, as detected by western blot analysis. h, 293T cells were transfected with control- or β-arrestin 1/2-siRNAs and reconstituted with Flag–KBTBD8. Binding of KBTBD8 to endogenous TCOF1 and NOLC1 was analysed by anti-Flag affinity purification and western blot analysis. i, Ubiquitylation of HA–TCOF1 in 293T cells depleted of β-arrestin 1/2 and reconstituted with KBTBD8 was determined after denaturing Ni-NTA purification by western blotting as described above. j, Ubiquitylation of HA–NOLC1 was detected in 293T cells depleted of β-arrestins and reconstituted with KBTBD8, as described above.
Extended Data Figure 6 | KBTBD8 specifies neural crest fate through TCOF1 and NOLC1.  

a, mRNA levels of KBTBD8, NOLC1 and TCOF1 were determined in hESCs or differentiating cells transduced with lentiviruses expressing the indicated shRNAs by qRT–PCR (mean of 3 technical replicates ± s.e.m.).  
b, hESCs stably depleted of KBTBD8 and reconstituted with either wild-type KBTBD8, KBTBD8(W579A), or KBTBD8(Y74A) were subjected to neural conversion (9 days) and analysed for the expression of marker proteins by qRT–PCR (mean of 3 technical replicates ± s.e.m.).  
c, hESCs stably depleted of KBTBD8, TCOF1, or NOLC1 were subjected to neural conversion (9 days) and analysed for marker expression by qRT–PCR (mean of 3 technical replicates ± s.e.m.).  
d, Depletion of TCOF1 or NOCL1 from hESCs results in loss of neural crest cells, as determined by triple staining immunofluorescence against the neural crest markers HNK1, TFAP2 and p75 (n > 200 cells, mean of 3 biological replicates ± s.d.). Scale bar, 10 μm.  
e, hESCs were transduced with lentiviruses expressing control or BRD2 shRNAs, subjected to puromycin selection for 7 days, and analysed by western blot analysis.  
f, Depletion efficiency for shRNAs against various KBTBD8 binding partners, as determined by qRT–PCR (mean of 3 technical replicates ± s.e.m.).
Extended Data Figure 7 | Characterization of TCOF1 regulation by ubiquitylation. a, hESCs depleted of either KBTBD8 or TCOF1 were subjected to neural conversion and analysed for expression of indicated proteins by western blot. b, Control or KBTBD8-depleted hESCs were fixed and subjected to indirect immunofluorescence analysis against endogenous TCOF1 or NOLC1. Scale bar, 10 μm. c, Total spectral counts of proteins associated with TCOF1–NOLC1 complexes purified by sequential immunoprecipitation in the presence of KBTBD8 compared to single TCOF1 affinity purification in the absence of KBTBD8, as determined by mass spectrometry (sum of 3 biological replicates). d, 293T cells were reconstituted with CUL3KBTBD8 and depleted of TCOF1 and NOLC1 by siRNAs. Endogenous RNA polymerase I was immunoprecipitated and binding to the SSU processome (NOP58, CSK2A) was analysed by western blot.
Extended Data Figure 8 | KBTBD8 is not required for general ribosome biogenesis. a, hESCs stably depleted of KBTBD8 were subjected to neural conversion and levels of 5S rRNA, 18S rRNA and mRNAs encoding RPS6, RPS28, RPL10A and RPL28 were measured by qRT–PCR (mean of 3 technical replicates ± s.e.m.). b, hESCs stably depleted of KBTBD8 were subjected to neural conversion, and total RNA was subjected to a bioanalyzer assay to monitor processing of ribosomal RNAs. c, hESCs stably depleted of KBTBD8 were subjected to neural conversion (3 days), and nucleoli were analysed by anti-fibrillarin (original magnification: 60×, confocal) immunofluorescence microscopy. d, Quantification of nucleolar analysis described above (mean of 3 technical replicates ± s.e.m.). e, hESCs stably depleted of KBTBD8 were analysed for localization of 5.8S rRNA by anti-5.8S rRNA immunofluorescence microscopy (original magnification: 60×, confocal). f, hESCs depleted of KBTBD8 were subjected to neural conversion and analysed by anti-5.8S rRNA immunofluorescence microscopy (original magnification: 60×, confocal). g, Polysomes were purified from control or KBTBD8-depleted hESCs and differentiated counterparts subjected to neural conversion via sucrose gradient centrifugation followed by fractionation and UV detection. h, KBTBD8-depleted hESCs were subjected to neural conversion for 9 days and analysed for apoptosis by immunofluorescence analysis against cleaved caspase 3 (red) and DNA (Hoechst, blue). Cells with active caspase 3 staining were quantified (~200 cells per condition; scale bar, 10μm) (original magnification: 40×, confocal).
Figure a shows a heatmap of gene expression with fold regulation (log2) values ranging from -8.4 to 6.8.

Figure b illustrates the neural conversion of hESC to neural crest cells with different concentrations of rapamycin.

Figure c displays a bar chart showing the fold change expression of genes relative to control cells.

Figure d presents a graph comparing the neural conversion, d3, of CTRL vs shKBTBD8 and CTRL vs shTCOF1.

Figure e shows immunofluorescence images of neural conversion with cleaved caspase 3.

Figure f displays a bar chart showing the fold change expression of genes in hESC and NC9.

Figure g illustrates a comparison of neural conversion and cleaved caspase 3 in hESC with shNOLC1 and shTCOF1.
Extended Data Figure 9 | Characterization of KBTBD8- and TCOF1-depleted hESCs during neural conversion.  

**a**, hESCs were treated with increasing concentrations of rapamycin, subjected to neural conversion for 9 days, and analysed for expression of neural crest or CNS precursor markers by qRT–PCR. For comparison, effects of KBTBD8, TCOF1, or NOLC1 depletion (extracted from Fig. 3a) are shown.  

**b**, hESCs were treated with rapamycin, subjected to neural conversion, and analysed for marker expression by western blotting.  

**c**, hESCs were depleted of KBTBD8 or TCOF1, subjected to neural conversion for 3 days, and analysed for expression of 5S and 18S rRNA by qRT–PCR (mean of 3 technical replicates ± s.e.m.).  

**d**, hESCs depleted of KBTBD8 or TCOF1 were subjected to neural conversion for 3 days and analysed for p53 activation by RNA-seq against p53 targets.  

**e**, hESCs were depleted of KBTBD8 or TCOF1, subjected to neural conversion for 3 days, and analysed for apoptosis by immunofluorescence microscopy against cleaved caspase 3. Quantification shown below (~200 cells per condition).  

**f**, hESCs depleted of KBTBD8 were subjected to neural conversion for 9 days and analysed for expression levels of 5S and 18S rRNA by qRT–PCR (mean of 3 technical replicates ± s.e.m.).  

**g**, hESCs stably depleted of NOLC1 or TCOF1 were subjected to neural conversion for 9 days and analysed by immunofluorescence microscopy against cleaved caspase 3 (red) or DNA (Hoechst, blue). Quantification is shown below (~200 cells per condition; scale bar, 10 μm).
Extended Data Figure 10 | KBTBD8 controls translation. a, hESCs stably depleted of KBTBD8 were subjected to neural conversion for 3 days, and hESCs and differentiating cells were analysed by RNA deep sequencing and ribosomal profiling to determine translation efficiency. Distribution of translation efficiency changes for 7,725 mRNAs brought about by KBTBD8 depletion is shown. b, hESCs stably depleted of either TCOF1 or KBTBD8 were subjected to neural conversion for 3 days, and translation efficiency was determined by RNA-seq and ribosome profiling. c, Translation efficiency blot of differentiating hESCs transduced with control or KBTBD8 shRNAs was labelled for significantly affected transcripts in general (blue), with links to CNS precursor formation (gold), or with links to neural crest formation (green). d, hESCs stably depleted of KBTBD8 or TCOF1 were subjected to neural conversion for 3 days, and expression levels of indicated proteins were analysed by western blotting. e, hESCs stably depleted of KBTBD8 were subjected to neural conversion for 3 days, and levels of ATRX1 and PCM1 mRNA were determined by qRT–PCR (mean of 3 technical replicates ± s.e.m.). f, hESCs stably depleted of KBTBD8 were subjected to neural conversion for 3 days, and protein stability of ATRX1 and PCM1 was determined by cycloheximide chase and western blotting (mean of 3 biological replicates ± s.d., ATRX1 and PCM1 levels were normalized relative to actin levels and 0 h time point set to 100%).