or apoptosis. As such, small-molecule inhibitors of RIPK3 may have limited therapeutic benefit because of their potential to promote apoptotic cell death.

Nec-1 blocked necrosis induced by TNF/zVAD.fmk (Fig. S5C), was protective in an inflammatory disease model (15), and does not induce apoptotic cell death, suggesting that inhibition of RIPK1 rather than RIPK3 may have therapeutic benefit. To mimic RIPK1 inhibition in the whole animal, we generated Ripk1 knockout mice with Asp386 of the HKD motif necessary for kinase activity mutated to Asn (fig. S6, A and B). Whereas Ripk1+/− mice died soon after birth, Ripk1D138N/+ mice were viable. Consistent with a critical role for the kinase activity of RIPK1 in TNF-induced necrosis, Ripk1D138N/D138N bone marrow–derived macrophages (BMDMs) and E1A-immortalized MEFs were as resistant as Ripk1−/− cells to killing by TNF/zVAD.fmk (Fig. 4A and fig. S6C). Ripk1D138N/D138N mice expressed normal amounts of RIPK3 and MLKL (Fig. S6D). Ripk1D138N/D138N mice also resembled Ripk3−/− mice in their systemic response to TNF, exhibiting less hypothermia than did their wild-type counterparts (Fig. 4B). Unlike RIPK1 loss, RIPK1 D138N did not rescue embryonic lethality caused by RIPK3 D161N (fig. S4). This result is consistent with nec-1 not protecting MEFs expressing RIPK3 D161N (fig. S5B) and indicates that the kinase activity of RIPK1 is not required for activation of caspase-8 by RIPK3 D161N.

RIPK1 is required for TNF-induced nuclear factor κB (NF-κB) and mitogen-activated protein kinase signaling (16, 17). Wild-type RIPK1 and RIPK1 D138N restored these signals in Ripk1−/− cells (18), suggesting that RIPK1 has an essential scaffold function in this setting, whereas its kinase activity is dispensable. Indeed, Ripk1D138N/D138N and wild-type BMDMs were indistinguishable in their phosphorylation of inhibitor of NF-κB α (IκBα), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) in response to TNF (Fig. 4C). These results, together with the viability of Ripk1D138N/D138N mice, are encouraging because they suggest that inhibiting the kinase activity of RIPK1 has no deleterious effects, at least in the short term. These Ripk1D138N/D138N mice can be used to explore the contribution of RIPK1 and necrosis to various mouse models of human disease.

References and Notes

Highly Multiplexed Subcellular RNA Sequencing in Situ

Je Hyuk Lee,1,2,4† Evan R. Daugharty,1,2,44 Jonathan Scheiman,1,2 Reza Kalhor,2 Joyce L. Yang,2 Thomas C. Ferrante,4 Richard Terry,4 Sauveur S. F. Jeanty,4 Chao Li,1 Ryoji Amamoto,3 Derek T. Peters,3 Brian M. Turczyk,1 Adam H. Marblestone,1,2 Samuel A. Inverso,1 Amy Bernard,1 Prashant Mali,2 Xavier Rios,2 John Aach,2 George M. Church1,2,4†

Understanding the spatial organization of gene expression with single-nucleotide resolution requires localizing the sequences of expressed RNA transcripts within a cell in situ. Here, we describe fluorescent in situ RNA sequencing (FISSEQ), in which stably cross-linked complementary DNA (cDNA) amplicons are sequenced within a biological sample. Using 30-base reads from a simulated wound-healing assay. FISSEQ is compatible with tissue sections and whole-mount embryos and reduces the limitations of optical resolution and noisy signals on single-molecule detection. Our platform enables massively parallel detection of genetic elements, including gene transcripts and molecular barcodes, and can be used to investigate cellular phenotype, gene regulation, and environment in situ.

The spatial organization of gene expression can be observed within a single cell, tissue, and organism, but the existing RNA localization methods are limited to a handful of genes per specimen, making it costly and laborious to localize RNA transcriptome-wide (1–3). We originally proposed fluorescent in situ sequencing (FISSEQ) in 2003 and subsequently developed methods to sequence DNA amplicons on a solid substrate for genome and transcriptome sequencing (4–7); however, sequencing the cellular RNA in situ for gene expression profiling requires a spatially structured sequencing library and an imaging method capable of resolving the amplicons.

We report here the next generation of FISSEQ. To generate cDNA amplicons within the cell (fig. S1), RNA was reverse-transcribed in fixed cells with tagged random hexamers (fig. S2A). We incorporated aminooxycarbonyl 5′-triphosphate (dUTPP) during reverse transcription (RT) (fig. S2B) and refixed the cells using BS(PEG)9, an amine-reactive linker with a 4-nm spacer. The cDNA fragments were then circularized before rolling circle amplification (RCA) (fig. S2C), and BS(PEG)9 was used to cross-link the RCA amplicons containing aminooxycarbonyl dUTPP (fig. S2, D and E). We found that random hexamer-primed RT was inefficient (fig. S3A), but cDNA circularization was complete within hours (fig. S3, B to D). The result was single-stranded DNA nanoballs 200 to 400 nm in diameter (fig. S4A), consisting of numerous tandem repeats of the cDNA sequence. BS(PEG)9 reduced nonspecific probe binding (fig. S4B), and amplicons were highly fluorescent after probe hybridization (fig. S4C). As a result, the amplicons could be rehybridized many times, with minimal changes in their signal-to-noise ratio or position (fig. S4, D and E). Using SOLID sequencing by ligation (fig. S5), the signal overlap over 27 consecutive sequencing reactions was ~600 nm in diameter (fig. S4F). In induced pluripotent stem (iPS) cells, the amplicons counterstained subcellular structures, such as the plasma membrane, the nuclear membrane, the nucleolus, and the chromatin (fig. 1A, fig. S6, and movies S1 to S3). We were able to generate RNA sequencing libraries in different cell types, tissue...
sections, and whole-mount embryos for three-dimensional (3D) visualization that spanned multiple resolution scales (Fig. 1, B and C).

High numerical aperture and magnification are essential for imaging RNA molecules in single cells (8–10), but many gene expression patterns are most efficiently detected in a low-magnification and wide-field mode, where it typically becomes difficult to distinguish single molecules because of the optical diffraction limit and low sensitivity (11). To obtain a spot density that is high enough to yield statistically significant RNA localization, and yet sufficiently low for discerning individual molecules, we developed partition sequencing, in which preselected sequencing primers are used to reduce the number of molecular sequencing reactions through random mismatches at the ligation site (Fig. 2A). Progressively longer sequencing primers result in exponential reduction of the observed density, and the sequencing primer can be changed during imaging to detect amplion pools of different density.

Fluorescence microscopy can be accompanied by tissue-specific artifacts and autofluorescence, which impede accurate identification of objects. If objects are nucleic acids, however, discrete sequences, rather than the analog signal intensity, can be used to analyze the image. For FISSEQ, putative nucleic acid sequences are determined for all pixels. The sequencing reads are then compared with reference sequences, and a null value is assigned to unaligned pixels. With a suitably long read length (L), a large number of unique sequences (n) can be used to identify transcripts or any other objects with a false-positive rate of approximately \( n^{4/2} \) per pixel. Because the intensity threshold is not used, even faint objects are registered on the basis of their sequence, whereas background noise, autofluorescence, and debris are eliminated (Fig. 2B).

We applied these concepts to sequence the transcription start site of inducible mCherry mRNA in situ, analogous to S′ rapid amplification of cDNA ends–polymerase chain reaction (RACE-PCR) (12). After RT and molecular amplification of the S′ end followed by fluorescent probe hybridization (Fig. 2A), we quantified the concentration- and time-dependent mCherry gene expression in situ (Fig. 2B). Using sequencing-by-ligation, we then determined the identity of 15 contiguous bases from each amplicon in situ, corresponding to the transcription start site (Fig. 2C). When the sequencing reads were mapped to the vector sequence, 7472 (98.7%) amplicons aligned to the positive strand of mCherry, and 3967 (52.4%) amplicons mapped within two bases of the predicted transcription start site (Fig. 2D).

We then sequenced the transcriptome in human primary fibroblasts in situ (Fig. 3A) and generated sequencing reads of 27 bases with a median per-base error rate of 0.64% (fig. S8). Using an automated analysis pipeline (fig. S9), we identified 14,960 amplicons with size >5 pixels, representing 4171 genes, of which 13,558 (90.6%) amplicons mapped to the correct annotated strand (Fig. 3B, fig. S10, and table S1). We found that mRNA (43.6%) was relatively abundant even though random hexamers were used for RT (Fig. 3C). Ninety genes with the highest expression counts included fibroblast markers (13), such as fibronectin (FN1); collagens (COL1A1, COL1A2, COL3A1); matrix metalloproteinases and inhibitors (MMP14, MMP2, TIMP1); osteonectin (SPARC); stanniocalcin (STC1); and the bone morphogenesis–associated transforming growth factor (TGF)–induced protein (TGFBI), representing extracellular matrix, bone development, and skin development [Benjamini-Hochberg false discovery rate (FDR) <10^{-16}, 10^{-3}, and 10^{-9}, respectively] (Fig. 3D) (14). We made Illumina sequencing libraries to compare FISSEQ to RNA-seq. Pearson’s \( r \) correlation coefficient between RNA-seq and FISSEQ ranged from 0.52 to 0.69 (\( P < 10^{-16} \)), excluding one outlier (FN1). For 854 genes with more than one observation, Pearson’s \( r \) was 0.57 (\( P < 10^{-16} \)), 0.47 (\( P < 10^{-16} \)), and 0.23 (\( P < 10^{-13} \)) between FISSEQ and RNA-seq from fibroblasts, lymphocytes, and iPSC cells, respectively (Fig. 3E). When FISSEQ was compared with gene expression arrays, Pearson’s \( r \) was as high as 0.73 (\( P < 10^{-14} \)) among moderately expressed genes, whereas genes with low or high expression levels correlated poorly (\( r < 0.4 \)) (fig. S11).
Highly abundant genes in RNA-seq and gene expression arrays were involved in translation and splicing (figs. S11 and S12), whereas such genes were underrepresented in FISSEQ. We examined 12,427 (83.1%) and 2533 (16.9%) amplicons in the cytoplasm and nuclei, respectively, and found that nuclear RNA was 2.1 [95% confidence interval (CI) 1.9 to 2.3] times more likely to be non-coding ($P < 10^{-15}$), and antisense mRNA was 1.8 [95% CI 1.7 to 2.0] times more likely to be nuclear ($P < 10^{-16}$). We confirmed nuclear enrichment of MALAT1 and NEAT1 by comparing their relative distribution against all RNAs (Fig. 3F) or mitochondrial 16S ribosomal RNA (tRNA) (table S2), whereas mRNA, such as COL1A1, COL1A2, and THBS1, localized to the cytoplasm (table S3). We also examined splicing junctions of FN1, given its high read coverage (481 reads over 8.9 kilobases).
Structure of the Mitochondrial Translocator Protein in Complex with a Diagnostic Ligand

Łukasz Jaremko,1,2* Mariusz Jaremko,1 Karin Giller,1 Stefan Becker,1† Markus Zweckstetter1,2,3†

The 18-kilodalton translocator protein TSPO is found in mitochondrial membranes and mediates the import of cholesterol and porphyrins into mitochondria. In line with the role of TSPO in mitochondrial function, TSPO ligands are used for a variety of diagnostic and therapeutic applications in animals and humans. We present the three-dimensional high-resolution structure of mammalian TSPO reconstituted in detergent micelles in complex with its high-affinity ligand PK11195. The TSPO-PK11195 structure is described by a tight bundle of five transmembrane α helices that form a hydrophobic pocket accepting PK11195. Ligand-induced stabilization of the structure of TSPO suggests a molecular mechanism for the stimulation of cholesterol transport into mitochondria.

The 18-kDa translocator protein TSPO is preferentially expressed in mitochondrial membranes of steroidogenic tissues, and its gene family is present in almost all organisms (1–3). TSPO was first described as a peripheral benzodiazepine receptor, a secondary receptor for diazepam (1, 4). TSPO was subsequently found to be responsible for the transport of cholesterol into mitochondria, thereby influencing the synthesis of neurosteroids (1, 5). TSPO also plays an important role in apoptosis (6) and stress adaptation (7). Expression of TSPO is strongly up-regulated in areas of brain injury and in neuroinflammatory conditions including Alzheimer’s and Parkinson’s diseases (2). TSPO is located at contact sites between the outer and inner mitochondrial membrane, and was suggested to be part of (together with cyclophilin D, the voltage-dependent anion channel, and the adenosine nucleotide translocator) the mitochondrial permeability transition pore (8).

TSPO ligands have potential diagnostic and therapeutic applications such as attenuation of cancer cell proliferation (6) and neuroprotective effects (2). TSPO ligands such as XBD-173 might also be useful in treating anxiety with reduced side effects relative to traditional benzodiazepine-related drugs (9). The best-characterized ligand of TSPO is 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isooquinolinecarboxamide (PK11195), which binds to TSPO with nanomolar affinity (10). The TSPO-PK11195 structure is described by a tight bundle of five transmembrane α helices that form a hydrophobic pocket accepting PK11195. Ligand-induced stabilization of the structure of TSPO suggests a molecular mechanism for the stimulation of cholesterol transport into mitochondria.

References and Notes

Structure of the Mitochondrial Translocator Protein in Complex with a Diagnostic Ligand

Łukasz Jaremko,1,2* Mariusz Jaremko,1 Karin Giller,1 Stefan Becker,1† Markus Zweckstetter1,2,3†

The 18-kilodalton translocator protein TSPO is found in mitochondrial membranes and mediates the import of cholesterol and porphyrins into mitochondria. In line with the role of TSPO in mitochondrial function, TSPO ligands are used for a variety of diagnostic and therapeutic applications in animals and humans. We present the three-dimensional high-resolution structure of mammalian TSPO reconstituted in detergent micelles in complex with its high-affinity ligand PK11195. The TSPO-PK11195 structure is described by a tight bundle of five transmembrane α helices that form a hydrophobic pocket accepting PK11195. Ligand-induced stabilization of the structure of TSPO suggests a molecular mechanism for the stimulation of cholesterol transport into mitochondria.

The 18-kDa translocator protein TSPO is preferentially expressed in mitochondrial membranes of steroidogenic tissues, and its gene family is present in almost all organisms (1–3). TSPO was first described as a peripheral benzodiazepine receptor, a secondary receptor for diazepam (1, 4). TSPO was subsequently found to be responsible for the transport of cholesterol into mitochondria, thereby influencing the synthesis of neurosteroids (1, 5). TSPO also plays an important role in apoptosis (6) and stress adaptation (7). Expression of TSPO is strongly up-regulated in areas of brain injury and in neuroinflammatory conditions including Alzheimer’s and Parkinson’s diseases (2). TSPO is located at contact sites between the outer and inner mitochondrial membrane, and was suggested to be part of (together with cyclophilin D, the voltage-dependent anion channel, and the adenosine nucleotide translocator) the mitochondrial permeability transition pore (8).

TSPO ligands have potential diagnostic and therapeutic applications such as attenuation of cancer cell proliferation (6) and neuroprotective effects (2). TSPO ligands such as XBD-173 might also be useful in treating anxiety with reduced side effects relative to traditional benzodiazepine-related drugs (9). The best-characterized ligand of TSPO is 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isooquinolinecarboxamide (PK11195), which binds to TSPO with nanomolar affinity in many species (10–13). PK11195 is used as a biomarker in positron emission tomography to visualize brain inflammation in patients with neuronal damage (2, 10). Moreover,