inhibit cell proliferation by targeting common senescence pathways [17,18]. Other miRNAs also contribute to regulate cell proliferation through common signaling pathways of senescence (Fig. 1). Notably, miRNAs regulate actin cytoskeleton structure changes that contribute to the enlarged and flattened cell morphology characteristic of senescence phenotype. Our previous study demonstrates that miR-22 induces senescent-like morphology in SiHa cells, accompanied with cytoskeleton remodeling such as actin fiber formation [17]. miRNAs control key molecules in the multiple processes governing the cell cycle during senescence induction, which allows these miRNAs to affect the cell cycle regulatory process as whole rather than at just one checkpoint or stage [79]. Because the capability of cells to adhere to extracellular matrix is linked to cytoskeleton remodeling and cell shape, the changes in cell adhesion regulation may contribute to the morphological changes induced in senescence. Senescent cells are known to acquire resistance to apoptotic stimuli, whereas miRNAs may sway the cellular decision to commit to senescence instead of apoptosis. Alteration of protein metabolism and modification is responsible for the increase in cell volume and mass resulting in the enlarged and flattened morphology. So far, various studies support that miRNAs could facilitate the complex cellular changes required to establish the senescent phenotype, associated with inhibition of cell proliferation, cellular metabolic processes, negative regulation of apoptosis, stimulation of cell adhesion and cytoskeleton remodeling. Here, we summarize the effects of several miRNAs, either up or down in senescence, on biological processes changed during cellular senescence (Table. 2).

Exosomes are secreted into surrounding body fluids, targeting cells to transfer exosomal molecules including proteins, mRNAs, and miRNAs that can aid cell proliferation, immune response and other cellular process. For example, exosomes released by prematurely senescent prostate cancer cells were enriched in B7-H3 protein [19], which is a member of the B7 family of proteins that are capable of modulating CD4 T-cell responses and antitumoral immunity [81]. Secreted factors IL-6/8 from senescent fibroblasts in SASP are involved in the induction of a classic epithelial–mesenchyme transitions and invasiveness in human breast cancer cell lines [62]. Previous studies have found miRNAs circulating in the peripheral blood and have identified exosomes as mediators of cell–cell communication. It is reported that macrophage-secreted exosomes would deliver oncogenic miRNAs to breast cancer cells, contributing to the regulation of the invasiveness of breast cancer cells [82]. Therefore, exosome formation, cargo content, and delivery to surrounding cells are thought to be of immense biological interest in cellular processes. Little is known about the role of exosomes in vivo aging; however, these in vitro experimental data give the biological importance of exosomes in aging.

6. Potential of exosomes and miRNAs in aging and cancer

Aging is a well-recognized risk factor in the development of many diseases such as neurodegenerative disorders, cardiovascular disease, diabetes and cancer, which are the primary cause of death and disability in the elderly population. The process of aging impacts on a wide range of functions within the human body, displaying age-related changes occur in cellular, molecular and physiological functionality of tissues and organs. Nowadays, it is a well-known fact that some age-related molecular changes (e.g. DNA methylation, telomere shortening) can be detected in several types of colorectal cancers [83]. The understanding connection of aging process to age-related diseases is of great clinical importance regarding prevention and modern therapeutic strategies.

Exosomes are believed to play a role in various physiological and pathological conditions. Senescence-associated exosomes could transfer many regulatory factors including proteins and miRNAs, which contribute to the aging process in an autocrine, paracrine and even endocrine fashion. Although the relationship between miRNA and aging is not fully understood, the accumulating evidence suggests that exosomes act as significant communicator in human body. Exosomes have been implicated in the spread of neurodegenerative diseases including age-related macular degeneration (AMD) and Alzheimer's disease (AD). Age-related macular degeneration (AMD) is a major cause of loss of central vision in the elderly. Wang et al. demonstrated that increased autophagy and the release of intracellular proteins via exosomes by the aged retinal pigment epithelium may contribute to the formation of drusen that is a risk factor for developing AMD [84]. Alzheimer's disease (AD) is an irreversible age-related degenerative disorder, characterized by amyloid plaques, neurofibrillary tangles, inflammation, and neuronal damage in the brain. Accumulation of β-amyloid peptides (Aβ) in the senile plaques is a hallmark of the progression of disease. Interestingly, Aβ peptides formed by the β cleavage of amyloid precursor protein (APP) by two unknown proteases, β-secretase and γ-secretase, are secreted from the cells in association with exosomes [85]. Similar observation was found in prion diseases, which are fatal and infectious neurodegenerative disorders characterized by the accumulation of an abnormally folded form

Table 2
Analysis of biological processes changed during cellular senescence.

	Up-regulated miRNAs			Down-regulated miRNAs		
Biological processes	Let-7	miR-22	miR-34a	miR-17	miR-21	miR-155
Cell proliferation	Inhibited	Inhibited	Inhibited	Promoted	Promoted	Promoted
Apoptosis	Inhibited	Inhibited	Induced	Inhibited	Induced	Inhibited
Cell cycle arrest	G1/S arrest G2/M arrest	G1/S arrest	G1/S arrest G2/M arrest	Inhibitor of miR-17 induces G0/G1 arrest	Antisense targeting miR-17 induces G1 arrest	Antisense targeting miR-155 induces G0/G1 arrest
Cell differentiation	Involved	Involved	Involved	Involved	Involved	Involved
Metabolic processes	Involved	Inhibited	Inhibited	Involved	Involved	ND
Protein metabolism and modification	Involved	Involved	Involved	Involved	Involved	Involved
Cell adhesion	ND	Involved	decreased	decreased	Involved	decreased
Cytoskeleton remodeling	ND	Increased	ND	ND	Involved	ND
Cell migration and cell motility	Inhibited	Inhibited	Inhibited	decreased	Involved	Inhibited

Note: all the data are summarized from published articles. ND means not determined.

Please cite this article as: D. Xu, H. Tahara, The role of exosomes and microRNAs in senescence and aging, Adv. Drug Deliv. Rev. (2012), doi:10.1016/j.addr.2012.07.010

of glycosylphosphatidylinositol-linked prion protein (PrP), scrapie PrP [86–88]. These results suggest that exosomes might affect the microenvironment of these age-related diseases.

It is found that miRNAs can be stably detected in circulating plasma and serum, since miRNAs are packaged by exosomes to be protected from RNA degradation [13]. Ago2 and HDL that are associated with miRNAs are also stably found in serum, but their biological significance is still being discussed [89–91]. While miRNA profiling between cells and exosomes seems to be different, similar miRNA profiling was observed in exosomes containing miRNAs and Ago2 binding miRNAs. Therefore, the functional regulation of exosome secretion to deliver specific miRNAs may exist during aging and in human diseases. Recent studies have indicated that circulating exosomal miRNAs can serve as surrogates for the tumor cell-associated counterparts, extending their diagnostic potential to asymptomatic individuals [92]. Considering the high relevance between exosomes and miRNAs in many aspects, we suppose that circulating miRNAs will have a potential role in aging and age-related diseases.

Loss of immune function is typical symptom in human aging. Among human body fluids, one of major body fluid is blood, and a potential major in vivo source of exosomes is the B cell. Little is known about the function and the possible targets of B cell-derived exosomes. Exosomes derived from B cells in both human and mice elicit immune response by inducing antigen-specific MHC class II-restricted T-cell responses [93]. The frequency and number of bone marrow common lymphocyte progenitors (CLPs) are considerably reduced by 7 months of age. Furthermore, while about 90% of CLPs numbers is reduced in 20 month old mice, age-dependent reduction of the frequency and number of pro-B cell is observed. Due to recombination of impaired V-DJ heavy chain gene, the transition of pro-B cell into pre-B cells also decreases with aging. It is possible that these alterations may reflect on profiling and secretion of exosomes from these cells.

Studies on tumor derived exosomes reveal that exosomes may help establish an oncogenic niche systemically via delivery of protein, mRNA, and miRNAs that can aid tumor growth and metastasis. The presence of HLA-G in exosomes secreted by melanoma cells might imply a role in creating a tumor tolerance associated response [94]. Utilizing VEGF and cytokines cargo, exosomes enhance recruitment of hematopoietic and endothelial precursor cells to enhance neo-angiogenesis in the tumor [95]. Additionally, exosomes are known to trigger apoptotic reduction in anti-tumor immune cells through FasL and TNF-pathways and influence planar cell polarity and the extracellular matrix to allow tumor cell mobilization [96].

In summary, exosomes allow for reciprocal communication between different tissues, as shown in Fig. 3. Exosome production and secretion are altered during in vivo aging and in cancer, which has biological significance in metabolism, glycolysis, renal function, immunocompetence, tumor progression and brain memory function. Secretory factors are circulating in the tissues through the blood, so they may impact on the phenotypes by coordinate alteration of miRNAs, exosome proteins, lipids and cytokines. Furthermore, it is possible that individual difference in the production of secretory factors during aging may reflect the cancer risk as well as the cure rate. To date, limited information is available for understanding the mechanism and function of age-associated exosomes. We suppose that alteration of circulating miRNA, RNA, lipids and proteins via exosomes may coordinately influence many biological processes in human aging and cancer.

There is an increasing interest in using miRNAs as biomarker in the diagnosis of diseases and also as a promising biomedicine in the therapy of human diseases. Recent study shows that the sensitivity of miRNA amplification from human biologic fluids such as serum and saliva can be improved by exosome isolation [97], indicating exosomal miRNAs might become a potential starting point for earlier

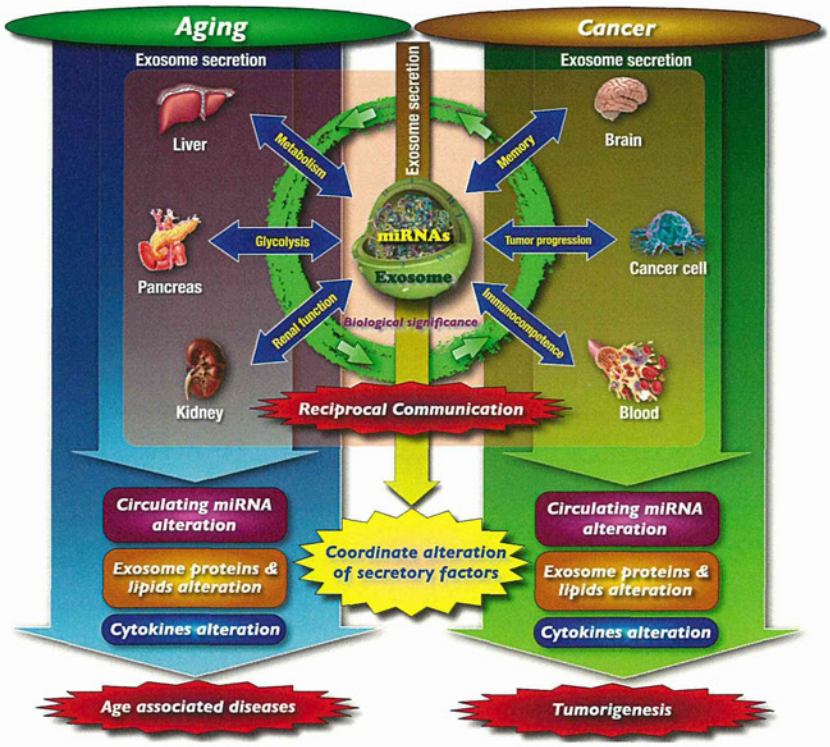

Fig. 3. Exosomes that allow for reciprocal communication between different tissues are critical in coordinating regulation of cellular function through miRNAs and proteins transfer. Epigenomic alterations during aging or in cancer may increase exosome secretion, accompanied with alteration in expression of circulating miRNAs, exosome proteins and cytokines, which are involved in cell-to-cell communication via exosomes. Therefore, exosomes play an important role in age-associated diseases and tumorigenesis.

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biomarker studies to reduce the probability of false negative results in clinic diagnosis of human diseases.

Nowadays, therapeutic potential of RNA drug to the clinic has been realized. With the development of nonimmunogenic delivery technologies, it is reported that exosomes can be used as nanocarriers to mediate siRNA delivery to the brain in mice, demonstrating efficient knockdown of BACE1, a therapeutic target in Alzheimer's disease [98]. In conclusion, exosomes provide a novel biomarker discovery and therapeutic platform for delivery of a variety of therapeutic modalities with great promise.

Acknowledgements

This work is supported by Project for Development of Innovative Research on Cancer Therapeutics and Grant-in-Aid for Scientific Research (KAKENHI), Ministry of Education, Culture, Sports, Science and Technology (H. Tarara) in Hiroshima University and "the Fundamental Research Funds for the Central Universities 2012TD027" in Dalian Maritime University.

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Please cite this article as: D. Xu, H. Tahara, The role of exosomes and microRNAs in senescence and aging, Adv. Drug Deliv. Rev. (2012), doi:10.1016/j.addr.2012.07.010

nature genetics

Mutations in *UVSSA* cause UV-sensitive syndrome and destabilize ERCC6 in transcription-coupled DNA repair

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UV-sensitive syndrome (UV^{S}S) is an autosomal recessive disorder characterized by photosensitivity and deficiency in transcription-coupled repair (TCR), a subpathway of nucleotide-excision repair that rapidly removes transcription-blocking DNA damage[1]. Cockayne syndrome is a related disorder with defective TCR and consists of two complementation groups, Cockayne syndrome (CS)-A and CS-B, which are caused by mutations in *ERCC8* (*CSA*) and *ERCC6* (*CSB*), respectively[2]. UV^{S}S comprises three groups, UV^{S}S/CS-A, UV^{S}S/CS-B and UV^{S}S-A, caused by mutations in *ERCC8*, *ERCC6* and an unidentified gene, respectively[3–6]. Here, we report the cloning of the gene mutated in UV^{S}S-A by microcell-mediated chromosome transfer. The predicted human gene *UVSSA* (formerly known as *KIAA1530*)[7] corrects defective TCR in UV^{S}S-A cells. We identify three nonsense and frameshift *UVSSA* mutations in individuals with UV^{S}S-A, indicating that *UVSSA* is the causative gene for this syndrome. The UVSSA protein forms a complex with USP7 (ref. 8), stabilizes ERCC6 and restores the hypophosphorylated form of RNA polymerase II after UV irradiation.

To clone the gene responsible for UV^{S}S-A, mouse chromosomes were randomly transferred to Kps3 cells (UV^{S}S-A) by microcell-mediated chromosome transfers (MMCTs)[9] using mouse A9 cells as the donor. Kps3 cells fused with A9 microcells were UV irradiated periodically over 6 weeks. We obtained four independent UV-resistant cellular clones (15A-7, KAB1-14, KAGA2-6 and KAGB2-4) that exhibited normal levels of UV sensitivity and recovery of RNA synthesis after UV irradiation (UV-RRS) (**Supplementary Fig. 1**). To identify the mouse genomic DNA that was integrated in each clone, a comparative genomic hybridization (CGH) array analysis was performed. An intact mouse chromosome 5 was found in KAB1-14, whereas chromosome 5 with segments of chromosomes 12 and 17 was found in KAGA2-6. Only a few fragments of chromosome 5 were found in the 15A-7 and KAGB2-4 clones (**Fig. 1a**). These results indicated that the causative gene was located within the common 600-kb region of chromosome 5 that was integrated in the 15A-7 and KAGB2-4 clones (**Fig. 1b**). There are 11 known genes in that region (**Fig. 1c**). Six BAC clones[10] encompassing the 600-kb region were introduced into Kps3 cells (**Fig. 1c**). Kps3 clones transfected with BAC 476L03 acquired normal levels of UV resistance, whereas transfection with BAC 154C21 failed to complement UV-sensitive cells (**Fig. 1d**). These results indicate that *4933407H18Rik* is a complementing gene for UV^{S}S-A. The *4933407H18Rik* gene is the mouse homolog of a predicted human gene, *KIAA1530*, which has subsequently been renamed *UVSSA* (encoding UV-stimulated scaffold protein A) in light of this finding, with support from the Human Gene Nomenclature Committee (HGNC).

Sequencing of *UVSSA* cDNA in UV^{S}S-A cells revealed that Kps3 (Japanese)[4] and XP24KO (Japanese)[11] cells had a homozygous c.367A>T transversion, resulting in premature termination at amino acid 123 (p.Lys123*). TA-24 (Iranian)[3] cells had a homozygous deletion, c.87delG, that caused a frameshift at position 29, leading to premature termination at amino acid 39 (p.Ile31Phefs*9) (**Fig. 2a,b**). In addition, the expression of cDNA encoding Flag-HA-UVSSA successfully conferred normal levels of UV-RRS and UV resistance to Kps3 cells (**Fig. 2c,d**). Taken together, these results indicate that *UVSSA* is the causative gene for UV^{S}S-A.

The *UVSSA* gene is located on human chromosome 4 at p16.3 and is 40.7 kb long. The transcript of the gene is 4,336 nt long and is composed of 14 exons, encoding a protein that consists of 709 amino acids with a deduced molecular weight of 81 kDa[7,12]. Analysis of the amino-acid sequence of UVSSA revealed that there is a Vps27, Hrs and STAM (VHS) domain and a structurally similar epsin NH_2-terminal homology (ENTH) domain in the N-terminal region and a well-conserved domain of unknown function (DUF) 2043 domain in the C-terminal region (**Fig. 2a**). It was reported that the VHS/ENTH domains of SCAF8 and NRD1 interact with the C-terminal domain of RNA polymerase II (RNA Pol II)[13,14], suggesting that UVSSA also interacts with RNA Pol II. Homologs of UVSSA have been identified in *Arabidopsis thaliana*, rice and *Caenorhabditis elegans*, but there are no reports of their function.

To elucidate the function of UVSSA in TCR, we first examined its interaction with other TCR factors (**Fig. 3**). The UVSSA protein complex was affinity purified from the soluble or chromatin

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Received 26 September 2011; accepted 29 February 2012; published online 1 April 2012; doi:10.1038/ng.2228

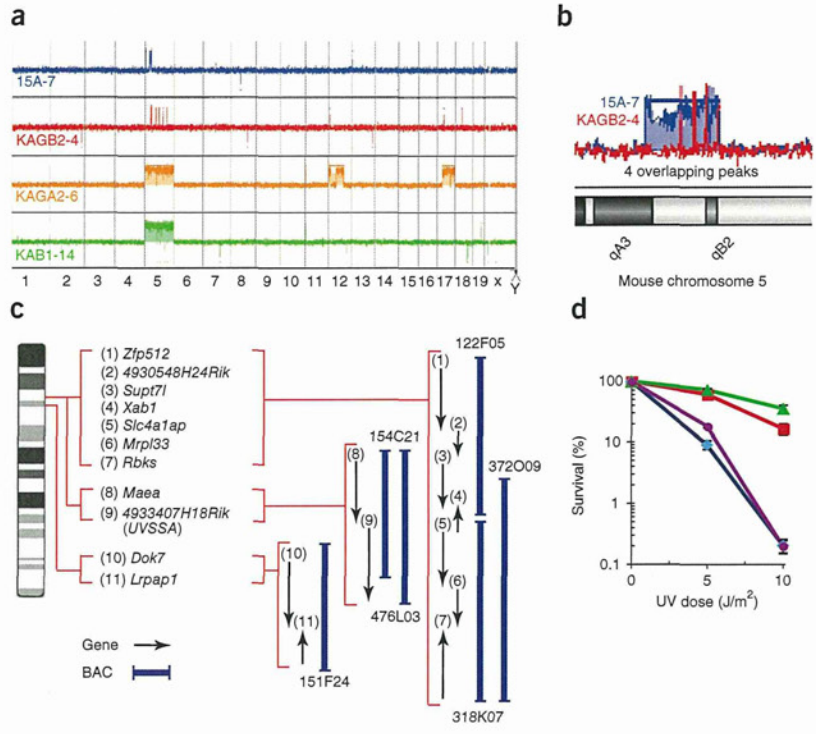

Figure 1 CGH array analysis and identification of BAC clones encompassing mouse genomic DNA integrated in the 15A-7 and KAGB2-4 clones. (**a**) CGH array analysis for identification of mouse genomic DNA integrated into the Kps3 clones containing mouse A9 chromosome segments. Kps3 cells were used as a control. (**b**) Enlarged view of the mouse chromosome 5 region integrated into the 15A-7 and KAGB2-4 clones. (**c**) Schematic of BAC clones encompassing the common 600-kb region of mouse genomic DNA integrated in clones 15A-7 and KAGB2-4. Genes included in the region are listed in positional order with a corresponding number. Chromosomal regions in red brackets correspond to peaks 2 to 4, from the left side, in **b**; there are no genes in peak 1. Arrows indicate the direction of the genes. (**d**) UV survival of Kps3 cells transfected with BAC 476L03 (green triangles) or BAC 154C21 (purple circles), compared to normal human WI38VA13 cells (red squares) and parental Kps3 cells (blue rhombuses). Error bars, s.d. calculated from three independent experiments.

fraction of extracts derived from Kps3 cells expressing Flag- and HA-tagged wild-type UVSSA. In the soluble fraction, ERCC8 was coimmunoprecipitated with UVSSA, irrespective of UV irradiation, but not with RNA Pol II or ERCC6 (**Fig. 3a**). In the chromatin fraction, ERCC6, ERCC8 and RNA Pol II were coimmunoprecipitated with UVSSA after UV irradiation (**Fig. 3b**).

Next, we searched for new TCR proteins that interact with UVSSA. The wild-type UVSSA protein fused with Flag and HA tags was stably expressed in HEK293 cells. Whole-cell extract was used for affinity purification of the UVSSA complex with antibodies to Flag and HA. Mass spectrometric analysis of the UVSSA complex identified USP7 as an interacting protein (**Supplementary Fig. 2**). USP7 is a ubiquitin-specific protease that recognizes and removes ubiquitin from proteins. Numerous proteins have been identified as potential substrates of USP7, including Mdm2, p53, claspin, Chfr and histone H2B[8,15]. To determine whether USP7 is involved in TCR, wild-type cells were treated with *USP7* small interfering RNA (siRNA) (**Fig. 3c**). *USP7* siRNA–treated cells showed decreased UV-RRS and UV survival, implicating USP7 in TCR (**Fig. 3d,e**). In the *USP7* siRNA–treated cells, not only USP7 protein levels, but also those of UVSSA, were decreased (**Figs. 3c** and **4a**). The amount of *UVSSA* mRNA was not affected in *USP7* siRNA–treated cells (**Supplementary Fig. 3**). Conversely, knockdown of *UVSSA* did not result in decreased USP7 protein levels (**Fig. 4a**), probably because of a much greater quantity of USP7 relative to UVSSA in the cells. The amount of ERCC8 protein was not affected in *USP7* siRNA–treated cells (**Fig. 4b**). These results indicate that UVSSA forms a stable complex with USP7 and transiently binds ERCC8. Also, the UVSSA-USP7 complex was coimmunoprecipitated with ERCC8, irrespective of UV irradiation in the soluble fraction, and it was coimmunoprecipitated with ERCC6, ERCC8 and RNA Pol II in the UV-damaged chromatin fraction (**Fig. 3a,b**). Coimmnunoprecipitation was also detected in the cells expressing a normal level of *UVSSA* (**Supplementary Fig. 4**). These results indicate that the UVSSA-USP7 complex and ERCC8 are recruited to the sites of RNA Pol II and ERCC6 in the UV-damaged chromatin fraction. In the accompanying paper by Schwertman *et al.*, it was shown that both ERCC6 and UVSSA were recruited to the spot locally irradiated with 254-nm UV-C light, although the recruitment of ERCC6 and UVSSA was much weaker than that of the global genome-repair protein XPC[16]. These results suggest that only a small portion of cellular UVSSA is involved in TCR.

It is known that ERCC6 has an essential role in recruiting TCR factors to the stalled RNA Pol II complex[17,18]. To examine whether the binding of UVSSA, USP7 and ERCC8 to RNA Pol II is dependent on ERCC6, the Flag- and HA-tagged UVSSA protein was expressed in CS1AN (CS-B) cells, and immunoprecipitation of the cell extracts was performed using antibodies to Flag and HA. USP7 and ERCC8 were coimmunoprecipitated with UVSSA in the chromatin fraction, irrespective of UV irradiation, but RNA Pol II was not, even after UV irradiation (**Fig. 3f**). These results suggest that UVSSA, USP7 and ERCC8 are loaded onto RNA Pol II in an ERCC6-dependent manner.

Next, to examine whether the binding of UVSSA and USP7 to ERCC6 and RNA Pol II is dependent on ERCC8, the Flag- and HA-tagged UVSSA protein was expressed in CS3BE (CS-A) cells, and immunoprecipitation of the cell extracts was performed using antibodies to Flag and HA. USP7 was coimmunoprecipitated with UVSSA in the chromatin fraction, irrespective of UV irradiation, but RNA Pol II and ERCC6 were not coimmunoprecipitated, even after UV irradiation (**Fig. 3g**). These results suggest that ERCC8 is required for recruitment of the UVSSA-USP7 complex to ERCC6 and RNA Pol II in the UV-irradiated chromatin fraction. In the accompanying paper by Schwertman *et al.*, it was shown that GFP-UVSSA accumulated at sites of UV damage caused by a 266-nm UV-C laser in CS-A and CS-B cells, and a UV-independent interaction between hyperphosphorylated RNA Pol II (RNA Pol IIo) and UVSSA was detected in CS-B cells by chromatin immunoprecipitation (ChIP) using cross-linking reagent[16]. We assume that ERCC6 is required for stable integration of UVSSA, USP7, ERCC8 and RNA Pol II into a functional TCR complex.

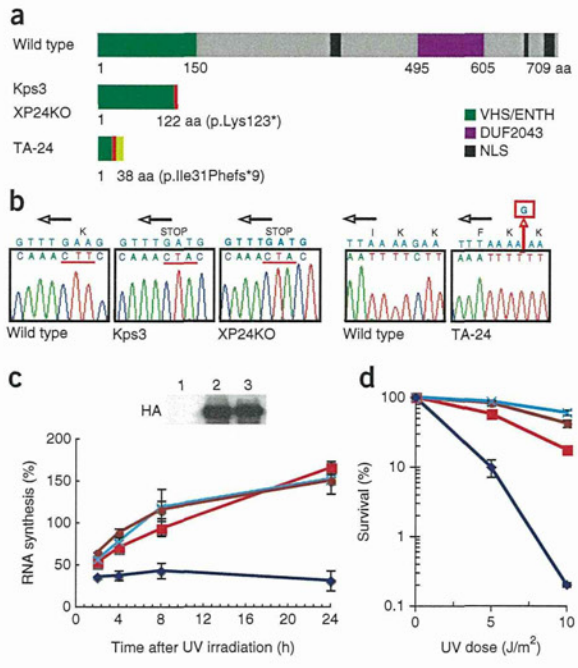

Figure 2 Mutations within the *UVSSA* gene in three subjects with UV^{S}S-A. (**a**) Schematic of the UVSSA protein and of amino-acid changes in Kps3, XP24KO and TA-24 cells. Amino-acid changes are shown in red. (**b**) Sequence chromatograms showing mutations in Kps3, XP24KO and TA-24 cells. The homozygous c.367A>T transversion is shown in Kps3 and XP24KO cells, and the homozygous c.87delG mutation is indicated in TA-24 cells. Translated amino acids are labeled above the cDNA sequence. Arrows represent the direction in which the sequence is read. (**c**) UV-RRS of Kps3 clones stably expressing *UVSSA* cDNA. Upper, protein blot of the Kps3 clones stably expressing Flag- and HA-tagged UVSSA using antibody to HA: lane 1, parental Kps3; lane 2, UVSSA-corrected Kps3 clone 1; lane 3, UVSSA-corrected Kps3 clone 2. Lower, UV-RRS of clones 1 (brown circles) and 2 (aqua crosses) and of WI38VA13 (red squares) and parental Kps3 cells (blue rhombuses). Error bars, s.d. calculated from three independent experiments. (**d**) UV survival of the Kps3 clones stably expressing Flag- and HA-tagged UVSSA. Symbols for each cell line are the same as in **c**. Error bars, s.d. calculated from three independent experiments.

We have found that the ERCC8 protein is translocated to the nuclear matrix after UV irradiation in an ERCC6- and transcription factor IIH (TFIIH)-dependent manner and that it colocalizes with Pol IIo[17,19]. Using a cell-free system[19], we found that UVSSA enhances UV-induced translocation of ERCC8 to the nuclear matrix (**Supplementary Fig. 5**).

It is known that inhibition of transcription after UV irradiation not only results from a blockage of transcription elongation by DNA damage on the transcribed strand but also can be caused by a reduction in the levels of the hypophosphorylated form of RNA polymerase II (RNA Pol IIa), which is required for the initiation of transcription[20]. The restoration of RNA Pol IIa levels and transcription after UV irradiation are deficient in CS-A and CS-B cells[20]. Consistent with this, we found that RNA Pol IIa disappeared after UV irradiation (10 J/m^2) in both the parental CS3BE (CS-A) cells and in ERCC8-corrected CS3BE cells. Approximately 16 h after UV irradiation, RNA Pol IIa reappeared in the ERCC8-corrected CS3BE cells but not in the parental CS3BE cells (**Supplementary Fig. 6**). We then examined the phosphorylation status of RNA Pol II in whole-cell lysates from Kps3 cells and UVSSA-corrected Kps3 cells after 10 J/m^2 of UV irradiation (**Fig. 4c**). RNA Pol IIa disappeared in both the parental and UVSSA-corrected Kps3 cells. However, it reappeared 8 h after UV irradiation in the UVSSA-corrected Kps3 cells but not in the parental Kps3 cells, indicating that UVSSA is required, in addition to ERCC6 and ERCC8, for the reappearance of RNA Pol IIa after UV irradiation.

Moreover, the amount of ERCC6 protein decreased in the non-irradiated parental Kps3 cells compared to the amount in the UVSSA-corrected Kps3 cells, and a greater decrease was detected after UV irradiation in the parental Kps3 cells, whereas there was little decrease in the UVSSA-corrected Kps3 cells (**Fig. 4c**). These results indicate

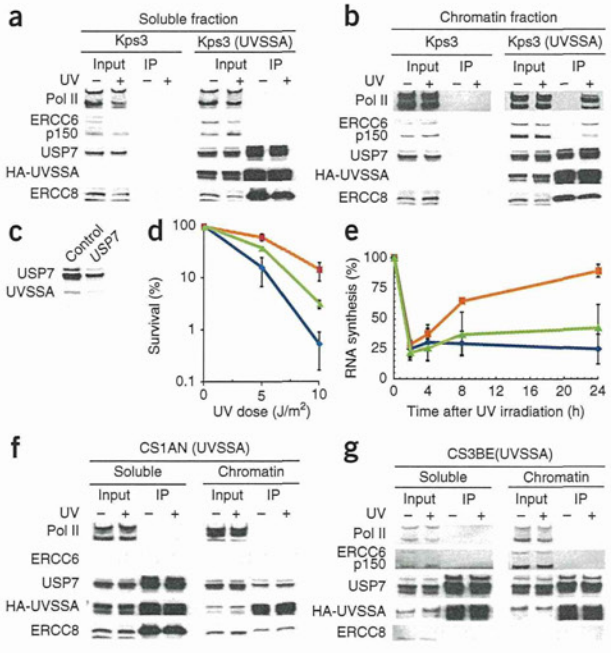

Figure 3 Interaction of UVSSA with RNA Pol II, ERCC6, ERCC8 and USP7. (**a**,**b**) Parental Kps3 cells and Flag- and HA-tagged wild-type UVSSA-corrected Kps3 cells were either irradiated with 20 J/m^2 of UV or were not irradiated and were incubated for 30 min. The UVSSA protein complex (IP) was then affinity purified from the soluble (**a**) or chromatin (**b**) fraction of each cell extract using anti-FLAG M2 agarose and anti-HA agarose and was subjected to protein blotting with antibodies to HA (UVSSA), RNA Pol II, ERCC6, ERCC8 and USP7. p150 corresponds to the CPFP protein (fusion protein consisting of N-terminal ERCC6$^{1-465}$ and the piggyback transposon)[32]. (**c**) Protein blot analysis of USP7 and UVSSA in UVSSA-corrected Kps3 cells and in isogenic cells transfected with *USP7* siRNA. The upper band of USP7 may be a modified form. (**d**) UV survival of parental Kps3 cells (blue rhombuses), UVSSA-corrected Kps3 cells (orange squares) and UVSSA-corrected Kps3 cells transfected with *USP7* siRNA (green triangles). Error bars, s.d. calculated from three independent experiments. (**e**) RNA synthesis after 10 J/m^2 of UV irradiation in the three cell lines in **d**. The symbols for each cell line are the same as in **d**. (**f**) Interaction of UVSSA with RNA Pol II, ERCC8 and USP7 in CS-B cells. Flag- and HA-tagged UVSSA protein was expressed in CS1AN (CS-B) cells, and immunoprecipitation of the cell extracts was performed using antibodies to Flag and HA. (**g**) Interaction of UVSSA with RNA Pol II, ERCC6 and USP7 in CS-A cells. Flag- and HA-tagged UVSSA protein was expressed in CS3BE (CS-A) cells, and immunoprecipitation of the cell extracts was performed using antibodies to Flag and HA. Error bars, s.d. calculated from three independent experiments.

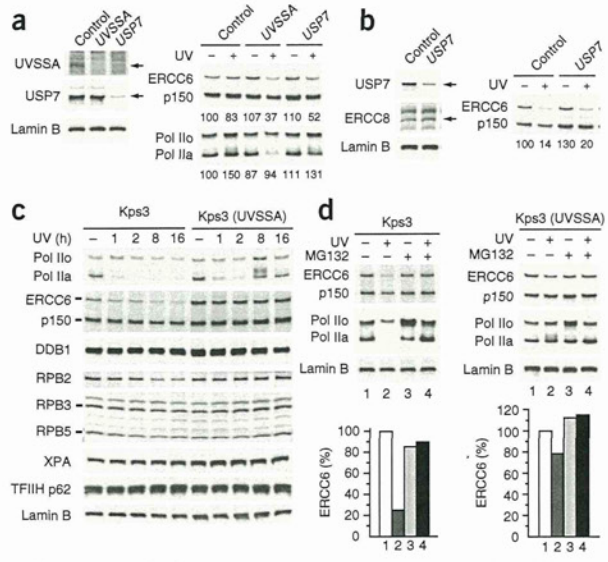

Figure 4 Degradation of ERCC6 in parental Kps3 cells, UVSSA-corrected Kps3 cells and normal cells transfected with *USP7* siRNA after UV irradiation. (**a**) UV-induced degradation of ERCC6 and RNA Pol IIo in normal cells transfected with control siRNA or siRNA targeting *UVSSA* or *USP7*. Knockdown of *UVSSA* and *USP7* was confirmed by protein blotting of cell lysates using the indicated antibodies. Normal cells transfected with each siRNA were irradiated with 10 J/m^2 of UV or not irradiated and were incubated for 16 h. Samples lysed with SDS gel-loading buffer were then analyzed by protein blotting with antibody to ERCC6. Numbers below the blots indicate the amounts of ERCC6 (upper) and RNA Pol IIo (lower) relative to their amounts in non-irradiated normal cells transfected with control siRNA. (**b**) UV-induced degradation of ERCC6 in Kps3 cells transfected with control siRNA or with siRNA targeting *UVSSA* or *USP7*. Knockdown by siRNA and protein blotting were performed as in **c**, except for an 8-h incubation after UV irradiation. Numbers below the blot indicate the amount of ERCC6 relative to the amount in non-irradiated Kps3 cells transfected with control siRNA. (**c**) Parental Kps3 cells and UVSSA-corrected Kps3 cells were irradiated with 10 J/m^2 of UV and incubated for the indicated times. Samples lysed with SDS gel-loading buffer were analyzed by protein blotting using the indicated antibodies. DDB1, UV-damaged DNA binding protein 1; RPB2, second largest subunit of RNA Pol II; RPB3, third largest subunit of RNA Pol II; RPB5, fifth largest subunit of RNA Pol II; XPA, xeroderma pigmentosum group A protein. Only RPB2 was decreased to some extent in UV-irradiated Kps3 cells. (**d**) Inhibition of the degradation of ERCC6 and the reappearance of RNA Pol IIa after UV irradiation in Kps3 cells in the presence of MG132. Parental Kps3 cells and UVSSA-corrected Kps3 cells were either irradiated with 10 J/m^2 of UV or not irradiated and were incubated for 8 h in the presence (10 μM) or absence of the proteasome inhibitor MG132. Samples lysed with SDS gel-loading buffer were analyzed by protein blotting using the indicated antibodies. Histograms indicate the amount of ERCC6 relative to the amount in non-irradiated cells in the absence of MG132.

that UVSSA has an important role in stabilizing ERCC6 in TCR. In addition, ERCC6 was degraded in the *USP7* siRNA–treated cells, as in the *UVSSA* siRNA–treated cells, but not in control siRNA–treated cells (**Fig. 4a**). Kps3 cells that lack *UVSSA* were then treated with *USP7* siRNA. No significant difference in UV-induced degradation of ERCC6 was detected between control siRNA– and *USP7* siRNA–treated Kps3 cells (**Fig. 4b**). These results indicate that UVSSA and USP7 cooperate to protect ERCC6 from UV-induced degradation in TCR.

When the Kps3 cells were UV irradiated in the presence of the proteasome inhibitor MG132, the reduction in ERCC6 levels after UV irradiation was suppressed, and RNA Pol IIa reappeared 16 h after UV irradiation (**Fig. 4d**). These results suggest that, in UV-irradiated Kps3 cells, ERCC6 is ubiquitinated and degraded by the ubiquitin-proteasome pathway, and the recovery of RNA Pol IIa is blocked, leading to the inhibition of UV-RRS.

The amount of RNA Pol IIo decreased in parental Kps3 cells (**Fig. 4c**) and in *UVSSA* siRNA–treated normal cells (**Fig. 4a**), although the decrease in RNA Pol IIo was not significant compared with that of ERCC6. RNA Pol IIo levels did not decrease in the UVSSA-corrected Kps3 cells and in control siRNA–treated cells (**Fig. 4a,c**). In the *USP7* siRNA–treated cells, the amount of RNA Pol IIo slightly decreased after UV irradiation when compared to the amount in control siRNA–treated cells (**Fig. 4a**). Taken together, these results suggest that UVSSA and USP7 are involved in the stabilization of RNA Pol IIo, but the effect might be indirect.

The affected individuals with the UV^{S}S, UV^{S}1KO and UV^{S}1VI, had homozygous mutations in the *ERCC6* and *ERCC8* genes, respectively[5,6]. This prompted us to examine whether some individuals with Cockayne syndrome features and TCR deficiency have mutations in the *UVSSA* gene. We sequenced the ORF of *UVSSA* in three TCR-deficient Cockayne syndrome cell lines (CS7099, CS6864 and CS2760) that have no mutations in the *ERCC6* and *ERCC8* genes. We found only SNPs in *UVSSA* in these cells (**Supplementary Fig. 7**), suggesting that there are some other gene(s) involved in the Cockayne syndrome phenotype beyond *ERCC6* and *ERCC8*. To exclude the possibility that some individuals with Cockayne syndrome have mutations in *UVSSA*, it is necessary to sequence the *UVSSA* ORF in many other TCR-deficient subjects with Cockayne syndrome.

It has been reported that CS-A and CS-B cells are hypersensitive to treatment with hydrogen peroxide and potassium bromate, specific inducers of oxidative DNA damage, and are deficient in the repair of oxidative DNA damage, whereas Kps3 (UV^{S}SA) and UV^{S}S1VI (UV^{S}S/CS-A) cells are not hypersensitive to oxidative DNA damage[6,21,22]. It has also been reported that transcription by RNA Pol II was reduced in the extracts of CS-A and CS-B cells compared to transcription levels in normal cells[23,24]. Nuclear extracts of CS-B cells failed to transcribe human rDNA, whereas those of CS-B cells expressing wild-type ERCC6 showed high transcriptional activity of RNA Pol I (ref. 25). Of note, RNA Pol I transcription was proficient in UV^{S}1KO (UV^{S}S/CS-B) cells[26]. Taken together, these results suggest that marked differences in the pathological phenotypes between Cockayne syndrome and UV^{S}S are caused by differences in transcription and/or in the repair of oxidative DNA damage in affected individuals.

We also determined that ERCC6 was ubiquitinated and degraded by the ubiquitin-proteasome pathway after UV irradiation in Kps3 cells (**Fig. 4c,d**). It has been reported that ERCC6 is required for the resumption of transcription after UV irradiation and for the recruitment of RNA Pol II and other transcription factors at the promoter in UV-irradiated cells[27]. It is therefore suggested that the ubiquitination and degradation of ERCC6 in the absence of UVSSA prevents recovery of RNA Pol IIa after UV irradiation and, consequently, blocks UV-RRS (**Supplementary Fig. 8**). Kps3 cells are deficient in the removal of UV damage on transcribed DNA strands[28], indicating that UVSSA is also required for excision of DNA damage in TCR (**Supplementary Fig. 8**).

It is not yet clear which E3 ligase is involved in the ubiquitination of ERCC6 in TCR. It would be interesting to examine whether ERCC6 is ubiquitinated by ERCC8 (ref. 29), BRCA1-BARD1 (ref. 30) or the p44 subunit of TFIIH[31]. UVSSA may negatively regulate E3 ligase activities. Alternatively or simultaneously, it may positively regulate

the deubiquitinase activity of USP7 to stabilize ERCC6 in TCR. We conclude that the UVSSA-USP7 complex has an important role in TCR, whereby it controls the steady-state levels of ERCC6.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank Y. Iwamoto and I. Kuraoka for their help in DNA sequencing and microcell-mediated chromosome transfer and M. Hoshi for her help in establishing Flp-In cells and performing immunoprecipitation. We also thank M. Yamaizumi (Kumamoto University Medical School) and N.G. Jaspers (Erasmus Medical Centre) for providing Kps3 cells and TA-24 cells, respectively. We thank G. Spivak for critical reading of the manuscript. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and by Health and Labor Sciences Research Grants for Research on Intractable Diseases (to K.T.). Part of this work was carried out under the Cooperative Research Project Program of the Institute of Development, Aging and Cancer (IDAC) at Tohoku University.

AUTHOR CONTRIBUTIONS

X.Z., K.H., M.S. and K.T. conceived the experiments. K.T. and H.T. established the cell lines. X.Z., K.H. and C.I. performed microcell-mediated chromosome transfer. A.U., K.H. and M.H. performed CGH array analysis. X.Z., M.S. and S.K. performed biochemical analysis. E.G.N. diagnosed Cockayne syndrome patients. K.T., X.Z., K.H., M.S., M.H., T.N. and A.Y. analyzed the data. K.T., X.Z., M.S. and K.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cell lines. Kps3, XP24KO and TA-24 cells belong to UV^{s}S-A and were immortalized by simian virus 40 large T antigen and hTERT. FS3 and WI38VA13 are normal human cells. Mouse A9 cells were used as donors in microcell-mediated chromosome transfer. All cell lines used were cultured in DMEM containing 10% FCS, penicillin and streptomycin at 37 °C under 5% CO_2.

UV survival. Cells were inoculated in 10-cm dishes at a density of 1,000–2,000 cells per dish. After 6 h, cells were washed with PBS and irradiated with UV at 0, 5 and 10 J/m^2. Cells were then incubated for 1–2 weeks. Resulting colonies were fixed with 3.7% formaldehyde and stained with 0.1% crystal violet and were counted using a binocular microscope.

Microcell-mediated chromosome transfer. Donor A9 cells were plated onto 25-cm^2 flasks in DMEM supplemented with 10% FCS. After 1–3 d (when cells were 80% confluent), the culture medium was changed to DMEM supplemented with 20% FCS and 50 ng/ml colcemid (Sigma). After 48 h of incubation, flasks were centrifuged at 12,000*g* for 1 h in the presence of 10 μg/ml cytochalasin B (Sigma) for enucleation. The microcell pellets were resuspended in serum-free DMEM and sequentially filtered through polycarbonate membranes with pores of 8, 5 and 3 μm in diameter. Purified microcells, which were irradiated with 10 Gy of γ-irradiation in some experiments, were plated onto a monolayer of recipient Kps3 cells in a 6-cm dish with serum-free DMEM containing 50 μg/ml phytohemagglutinin P (Sigma). After 30 min of incubation, the microcells were fused with recipient cells by treating the cells with 50% polyethylene glycol 1000 (Nakarai) for 1 min. After fusion, cells were grown for 24 h in DMEM supplemented with10% FCS. Then, cells were replated onto six 10-cm dishes and incubated for 24 h. Cells were irradiated with 10 J/m^2 of UV light. Surviving cells were collected and replated onto six 10-cm dishes and allowed to grow for 7 d. Cells were then irradiated with 10 J/m^2 of UV light. In total, cells were irradiated six times at 7-d intervals. As a negative control, Kps3 cells fused in the absence of microcells were UV irradiated in the same manner.

Comparative genomic hybridization array analysis. The regions of segmented mouse chromosomes transferred to Kps3 cells were analyzed using a Mouse Genome CGH 244A Oligo Microarray Kit with SurePrint Technology (Agilent Technologies), according to the manufacturer's instructions with some modifications. In brief, genomic DNA derived from the 15A-7, KAGB2-4, KAGA2-6 and KAB1-14 clones was compared with genomic DNA from parental Kps3 cells on the arrays. Genomic DNA was extracted by Qiagen Gentra Puregene core Kit A. After digestion with AluI and RsaI, the genomic DNA derived from Kps3 cells was labeled with Cy3, and the genomic DNA from the 15A-7, KAGB2-4, KAGA2-6 and KAB1-14 clones was labeled with Cy5, using a Genomic DNA Enzymatic Labeling Kit (Agilent Technologies). To prevent nonspecific hybridization of human genomic DNA on the array, 50 μg of human Cot-1 DNA (Invitrogen) and 5 μg of mouse Cot-1 DNA (Invitrogen) were mixed and subjected to prehybridization. After hybridization with the labeled genomic DNA, arrays were washed and scanned with GenePix4000B. Scanned data were analyzed by Feature Extraction Software version 9.5 and DNA Analytics version 4.0 (both from Agilent Technologies).

Transfection of mouse BACs. Mouse BAC clones were amplified in *Escherichia coli* cultured in LB medium containing chloramphenicol (25 μg/ml). BAC DNA was prepared with a Midiprep Kit (Qiagen), following the manufacturer's instructions. BAC DNA (20 μg) was cotransfected with 0.6 μg of pSV2neo into recipient Kps3 cells grown on a 10-cm tissue culture dish, and cells were selected with medium containing G418 (400 μg/ml) and irradiated with 10 J/m^2 of UV twice at 4–d intervals to examine whether the BAC-transfected Kps3 clones acquired a normal level of UV resistance.

Recovery of RNA synthesis after UV irradiation. To measure RNA synthesis after UV irradiation, two sets of cells were seeded into 6-well culture plates (1×10^6 cells/well). One set was used for counting cells and the other for measuring RNA synthesis. After 6 h of incubation, cells were washed with PBS and treated with UV at 10 J/m^2. After 2, 4, 8 and 24 h of incubation, the number of cells in one set was counted. The other set of cells was washed with PBS and incubated in DMEM containing 370 kBq/ml of [^{3}H]-uridine for 30 min to quantify RNA synthesis. Labeling was terminated by the addition of sodium azide to a final concentration of 200 μg/ml. Cells were washed twice with PBS containing 200 μg/ml sodium azide and lysed in 0.8% SDS for 30 min at room temperature. An equal volume of 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate was then added to the lysates, and these were incubated on ice for 1 h. Acid-insoluble materials were collected on GF-C glass microfiber filters (Whatman), and radioactivity was measured with an LS 6500 liquid scintillation counter (Beckman Coulter). Total radioactivity was divided by the number of cells to obtain single-cell radioactivity. The ratio (as a percentage) of the radioactivity of individual UV-irradiated cells to that of non-irradiated cells was considered as a measure of the recovery of RNA synthesis after UV irradiation (UV-RRS).

Immunoprecipitation. Cells stably expressing a Flag- and HA-tagged protein were lysed with MNase buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 300 mM sucrose, 2 mM $MgCl_2$, 0.1% Triton X-100, 1 mM $CaCl_2$, 1 mM DTT and complete protease inhibitor cocktail (Roche)) at 4 °C for 10 min. Lysates were centrifuged at 3,800*g* for 5 min. Supernatant was used as the soluble fraction. The pellet was washed once with MNase buffer and incubated with 30 U/ml of micrococcal nuclease (Takara) in MNase buffer at 25 °C for 30 min. The reaction was terminated by adding EDTA to a 5 mM final concentration and centrifuged at 3,800*g* for 5 min at 4 °C. The pellet was washed with MNase buffer. Supernatants were combined and used as the solubilized chromatin fraction. Tagged protein was affinity purified from the soluble and solubilized chromatin fractions with anti-FLAG M2 antibody-conjugated agarose (Sigma) followed by anti-HA agarose (Sigma).

Knockdown experiments. siRNA (Thermo Scientific) was transfected into target cells with RNAiMAX (Invitrogen), according to the manufacturer's instructions. At 24 h after the first transfection, a second transfection was performed. Cells were allowed to grow for another 36 h before experiments were carried out.

Quantitative RT-PCR. cDNA was synthesized from fresh total RNA using a Quantitative Reverse Transcription kit (Qiagen), following the manufacturer's instructions. RT-PCR samples were prepared with TaqMan gene expression master mix (Applied Biosystems), according to the manufacturer's instructions. RT-PCR was carried out using the 7300 Real-Time PCR system (Applied Biosystems), under the following conditions: 10 s at 95 °C, 10 s at 60 °C and 20 s at 72 °C for 25 cycles. Probe sets were ordered from Applied Biosystems.

UV-induced translocation of ERCC8 to the nuclear matrix using a cell-free system. UV-induced translocation of ERCC8 in the cell-free system was examined as described previously[19]. Parental Kps3 cells and UVSSA-corrected Kps3 cells were irradiated with 20 J/m^2 of UV and incubated for 1 h and then treated with CSK-Triton buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100, 1 mM DTT, 1 mM EGTA and Complete protease inhibitor cocktail (Roche)) to prepare the insoluble (CSK-ppt) fractions. The soluble fractions (CSK-sup) were prepared from CS3BE (CS-A) cells stably expressing Flag- and HA-tagged ERCC8 by treatment with CSK-Triton buffer. The CSK-sup fraction containing HA-tagged ERCC8 was incubated with the CSK-ppt fraction and then treated with DNase I. The ERCC8 retained in the DNase I–insoluble fractions was detected by immunoblotting with antibody to HA.

Antibodies. The antibodies employed were to ERCC8 (W-16, Santa Cruz Biotechnology), ERCC6 (E-18, Santa Cruz Biotechnology), RNA Pol II (N-20 and A-10, Santa Cruz Biotechnology), KIAA1530 (106751, GeneTex) and HA (3F10, Roche).