SUPPLEMENTARY INFORMATION

Regulation of the MDM2-P53 Pathway and Tumor Growth by PICT1/GLTSCR2 via Nucleolar RPL11

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Supplementary Figure 1. Generation of Pict1-deficient mice and ES cells.

(a) Left, top: A portion of the mouse Pict1 WT locus showing HindIII (HIII) sites. The 5.9 kb HindIII fragment diagnostic of the WT allele is indicated. Middle: Targeting vector showing neo in antisense orientation to Pict1 transcription. The neo cassette replaced exons 1 and 2 of Pict1. Bottom: Mutated Pict1 locus indicating the diagnostic 6.3 kb HindIII fragment. The 5'-flanking probe is also shown. Right: Southern blot of HindIII-digested genomic DNA from Pict1+/– and Pict1–/– ES cells. Hybridization was to the 5' flanking probe (top) or neo probe (bottom).

(b) Absolute numbers of neonates (P0) and embryos at the indicated stages of pregnancy after Pict1+/– mouse intercrossing.

(c) Pict1+/– and Pict1–/– embryos (n = 25) were explanted at E2.5 (day 0), cultured for
the indicated number of days, and viewed under an inverted microscope. Bar, 20 μm.

(d) Left, top: A portion of the mouse Pict1 WT locus showing the BamHI (BHI) sites. The 9.5 kb BamHI fragment diagnostic of the WT allele is indicated. Middle: Targeting vector with the hygromycin resistance gene (Hyg) in reverse orientation to Pict1 transcription. Exons 3–5 were flanked by two loxP sequences (arrowheads), with a third loxP sequence flanking Hyg. Bottom: Mutated Pict1 locus indicating the diagnostic 5.3 kb BamHI fragment. The 3'-flanking probe is also shown. Right: Southern blot of BamHI-digested genomic DNA from WT (+/+), and Pict1^{flox/+} (f+/+) ES cells. Hybridization was to the 3' flanking probe (top) or hyg probe (bottom).

(e) Pict1 and IRES-linked EGFP expression vectors were driven by a Tet-regulated promoter that was suppressed by the addition of doxycycline (Dox) (Tet-off system). After generating Pict1Tg^+; Pict1^{3loxP/–} ES cells, the endogenous Pict1 locus was completely deleted by adding Cre to generate Pict1tetTg^+; Pict1^{Δ/–} ES (Pict1ES) cells. Dox was added to shut off the exogenous Pict1 allele and generate Dox^+ (Pict1-deficient) cells.

(f) FACS analysis of EGFP expression in Pict1ES cells before (pink) and 24 h after (green) Dox addition.

(g) Pict1ES cells and WT ES cells were treated with or without 1,000 pg ml⁻¹ Dox for the indicated times. Proliferation was determined by MTS assay. Results are the mean cell growth (OD 490nm) of three cultures per dose per timepoint.

(h) Dox- Pict1ES and WT ES cells were (top) stained with PI for cell cycle analysis, and (bottom) stained with TUNEL to measure apoptosis. Left: Representative FACS profiles. Right: Mean % ± SEM (n = 3) of G₀-G₁ phase ES cells and mean % ± SEM (n = 3) of TUNEL⁺ ES cells. Results are representative of four trials.
Supplementary Figure 2. The cell cycle arrest and apoptosis of Pict1-deficient ES cells are p19^{Arf}-independent.

(a) Immunoblot detecting the indicated proteins in Pict1ES cells that were transfected with scramble siRNA or siRNAs targeting Trp53 (left) or Mdm2 (right). Transfected cells were left untreated (−) or treated with 5 ng ml⁻¹ Dox⁺ for 48 h prior to IB. Actin, loading control.

(b–d) Pict1ES cells were transfected with scramble siRNA or p19^{Arf} siRNA for 24 h and treated with or without 5 ng ml⁻¹ Dox for an additional two days (b, c) or four days (d). (b) p53 measured by IB. (c) Cell cycle arrest measured by PI staining. (d) Apoptosis measured by TUNEL. For (c) and (d), left panels are FACS profiles. Right panels are the mean % ± SEM (n = 5) of G0–G1 phase ES cells (c), and the mean % ± SEM (n = 5) of TUNEL⁺ ES cells (d).
(e) Top: Gross appearance of thymi from mice of the indicated genotypes ($n = 3$ per group; five weeks old). Bar, 5 mm. Bottom: Total thymocyte numbers. (N.S., not significant).

(f) Immunoblot to detect the indicated proteins in thymocytes from mice of the indicated genotypes. Thymocytes lacking expression of p19$^{Arf}$ alone show a lower level of p53 protein than WT thymocytes. However, the increased p53 expression in Pict1-deficient thymocytes was not reduced by concomitant p19$^{Arf}$ deficiency. Results are representative of three trials.
Supplementary Figure 3. Supplementary Figures related to Figure 2.

(a) Absolute numbers of pups of the indicated genotypes obtained from the intercrossing of Pict1+/– Trp53+/– × Pict1+/– Trp53+/– mice.

(b) Immunoblot detecting Pten in Pict1ES cells that were treated with or without 5 ng ml−1 Dox for 24 h. Cycloheximide (CHX; 100 μg ml−1) was added for the indicated times after Dox treatment.

(c) Immunoblot detecting the indicated proteins in Pict1ES cells treated with or without 5 ng ml−1 Dox for the indicated times. Results are representative of three trials.
Supplementary Figure 4. Binding of endogenous p53 and Mdm2.

Left: Input. Immunoblot detecting endogenous p53 and Mdm2 proteins in Pict1ES cells that were treated with or without 5 ng ml⁻¹ Dox for 48 h. MG132 was added as indicated for the final 3 h of Dox treatment. Middle: Lysates (5 mg) were IP’d using Mdm2-specific Ab or control IgG, resolved by SDS-PAGE, and IB’d with p53-specific Ab. Right: Samples of the same lysates were IP’d using p53-specific Ab or control IgG, and IB’d with Mdm2-specific Ab.
Supplementary Figure 5. Supplementary Figures related to Figure 4.

(a) Confocal microscopy of 293T cells that were transfected with exogenous Pict1-EGFP and Npm-DsRed. The cells were counterstained with DAPI. Bar, 5 μm.

(b) Immunoblot detecting the indicated proteins in Pict1ES cells treated with or without 5 ng ml⁻¹ Dox for the indicated days.

(c) The localizations of endogenous Nsm, Npm and Nln proteins in the untreated (Day 0) and Dox-treated (Day 2) Pict1ES cells from (b) were determined by immunohistochemistry and confocal microscopy. Bar, 5 μm.

(d) LC-MS/MS identification of Pict1-binding proteins. Flag-Pict1 cDNA was transfected into 293T cells and Flag-associated protein complexes were isolated by IP. Immunoprecipitates were digested with Achromobacter protease I and peptide products were analyzed by LC-MS/MS as described¹.

(e) Top: Immobilized GST-RP fusion proteins were incubated separately with in...
vitro-translated, $^{35}$S-methionine-labeled Pict1 protein. Eluates were washed and analyzed by autoradiography. GST-PTEN, positive control. Bottom: Input as evaluated by Coomassie Brilliant Blue R-250 (CBB) staining.

(f) Immunoblot of 293T cells that were transfected with 5 µg total of the indicated combinations of plasmids expressing Flag-Pict1 [Pict1(F)], Myc-RPL5 [RPL5(M)], Myc-RPL11 [RPL11(M)], Myc-RPL23 [RPL23(M)], or Myc-RPS7 [RPS7(M)]. At 48 h post-transfection, lysates were IP’d with anti-Flag and IB’d with anti-Myc to detect RPs.

(g) Immunoblot of Pict1ES cells that were transfected with scrambled siRNA, Trp53 siRNA (positive control), or four different Rpl11 siRNAs (Rpl11 -A, -B, -C, -D). At 24 h post-transfection, cells were treated with or without 5 ng ml$^{-1}$ Dox. At 24 h post-Dox, lysates were IB’d to detect the indicated proteins. Results are representative of three trials.
**Supplementary Figure 6. Changes to RP localization following loss of Pict1.**

(a–c) Confocal microscopy of Pict1ES cells that were transfected with \textit{RPL5-DsRed} (a), \textit{RPL23-DsRed} (b), or \textit{RPS7-DsRed} (c). At 16 h post-transfection, cells were treated with or without 5 ng ml\(^{-1}\) Dox for 48 h. Endogenous Npm was detected by immunostaining. Because the expression level of RPL23-DsRed was very low, DsRed-specific Ab was used to enhance the fluorescent signal in (b). Bar, 5 μm.

(d) Confocal microscopy of Pict1ES cells that were treated with or without 5 ng ml\(^{-1}\) Dox for 24 h and double-immunostained using Mdm2-specific Ab and Nln-specific Ab. Bar, 5 μm.

(e) Immunoblot detecting the indicated proteins in Pict1ES cells that were treated with or without 5 ng ml\(^{-1}\) Dox for the indicated times and IP’d with control IgG or anti-Mdm2 Ab. Results are representative of three trials.
Supplementary Figure 7. Prognostic significance of PICT1 mRNA expression in human cancer samples.

(a) The human glioma cell lines U251 and SF295, colorectal cancer cell lines DLD1 and HCT15, the ovarian cancer cell line TYK-nu, and the uterine cancer cell line HeLa were transfected with scramble shRNA or the indicated PICT1 shRNAs. Top: MTS cell growth assay. Bottom: Immunoblot detecting the indicated proteins. TP53 is mutated in U251, SF295, DLD1, HCT15, and TYK-nu cells. In HeLa cells, P53 is inactivated via the E6-E6AP complex rather than inhibited by MDM2². Results are representative of three trials.

(b) Plot of the ratio of PICT1 mRNA level to GAPDH mRNA level in tumors from 181 individuals with colorectal cancer (left) and in 81 individuals with esophageal cancer (right) as determined using real-time RT-PCR. Horizontal line, arbitrary cut-off value
distinguishing high from low $PICT1$ expression.

(e) Univariate and multivariate analyses were performed for the prognoses of 181 individuals with colorectal cancer and 81 individuals with esophageal cancer. Risk ratio (RR) and the 95% confidence interval (95% CI) were assessed using the Cox proportional hazards model.
Supplementary Figure 8. Nucleolar stress stimuli inhibit Pict1 expression and increase p53 protein.

Immunoblot detecting the indicated proteins in WT ES cells treated with 5 nM actinomycin D (ActD) or 10 μM mycophenolic acid (MPA) for the indicated times. Tubulin, loading control. Results are representative of three trials.
SUPPLEMENTARY METHODS

Generation of Pict1 null mutant (Pict1Δexon1-2/Δexon1-2) mice

The targeting vector was designed to replace a 1.3 kb genomic fragment containing part of murine Pict1 exons 1 and 2 with the PGK-neo resistance expression cassette in antisense orientation. The resulting targeting vector was electroporated into E14K ES cells and homologous recombinants were confirmed by Southern blotting using a 5’-flanking probe and a neo-specific probe. Pict1+/− mice were generated using standard procedures and backcrossed to C57BL/6 mice five times before intercrossing to generate Pict1−− progeny. The primer set used to detect the wild type (WT) Pict1 allele was: 5’-AGCCTACTCCCTACCAACCCGA-3’ and 5’-CGCTTCTCACCGTCCCTGTTAC-3’ (497 bp product); and to detect the neo-containing Pict1 allele was: 5’-AGCCTACTCCCTACCAACCCGA-3’ and 5’-CCAGCTCATTCCTCCCACTCA TGATC-3’ (437 bp product).

Targeting of the second Pict1 allele (Pict1flox)

A conditional targeting vector based on the Cre-loxP system was constructed to delete a genomic fragment containing exons 3–5 of the murine Pict1 gene. Pict1 exons 3–5 encode amino acids that bind to PTEN. We introduced two loxP sites into Pict1 intron 2, and one loxP site into intron 5, such that Pict1 exons 3–5 were flanked by the intron 2 and intron 5 loxP sites. The PGK-Hyg resistance expression cassette was inserted in antisense orientation between the two loxP sites in intron 2.

Generation of T cell-specific Pict1-deficient mice
The linearized Pict1<sup>fl</sup> targeting vector described above was electroporated into E14K ES cells. Correctly-targeted clones were transiently transfected with pMCI-Cre to delete the loxP-flanked PGK-Hyg gene. Progeny clones that became sensitive to hygromycin were subjected to Southern blot analysis to detect those retaining exons 3–5 flanked by two loxP sites (Pict1<sup>fl</sup> allele) and those lacking exons 3–5 (Pict1<sup>△</sup> allele). Pict1<sup>fl/+</sup> ES clones were microinjected into C57BL/6 blastocysts to generate Pict1<sup>fl/+</sup> mice. To generate T cell-specific Pict1-deficient mice, Pict1<sup>fl/+</sup> mice were mated to LckCre Tg mice (C57BL6/J background)<sup>3</sup>. Offspring carrying LckCre plus two floxed Pict1 mutations (LckCrePict1<sup>fl/ff</sup>), LckCre plus Pict1<sup>fl/+</sup> (LckCrePict1<sup>fl/+</sup>), and LckCre plus the WT Pict1 allele (LckCrePict1<sup>+/+</sup>) were used in the analysis as homozygous mutant, heterozygous mutant and WT mice, respectively. Neonates were genotyped by Southern blotting.

To generate LckCrePict1<sup>+</sup>/Trp53<sup>−/−</sup> and LckCrePict1<sup>+</sup>/p19<sup>Arf</sup> double knockout (DKO) mice, LckCrePict1<sup>fl/ff</sup> mice were mated to Trp53<sup>−/−</sup> mice<sup>4</sup> or p19<sup>Arf</sup><sup>−/−</sup> mice<sup>5</sup>, respectively.

**In vitro culture of preimplantation embryos**

Pict1<sup>−/−</sup> males and females were intercrossed, and embryonic day 2.5 (E2.5) embryos were collected by flushing oviducts of plugged females. Eight-cell stage embryos were individually cultured in micro-drops in modified Whitten's medium under mineral oil in 5% CO<sub>2</sub> at 37°C. Cultured embryos were photographed daily. After three days in culture, the morphology of the embryos was noted and genotypes were determined by PCR amplification using DNA extracted from whole embryos (Supplementary Fig. 1c).
**Proliferation, cell cycle, and TUNEL assays**

ES cells were seeded on 96-well plates at a density of 5×10^2 cells per well. After 24 h, cells were treated with 0–1,000 pg ml\(^{-1}\) Dox (Sigma) (Fig. 1b). Cell proliferation was evaluated by MTS assay using the CellTiter 96 AQ\(_{ueous}\) One Solution Cell Proliferation Assay Kit (Promega) according to the manufacturer’s instructions. For cell cycle analysis, ES cells were fixed in 70% ethanol at –20˚C, resuspended in phosphate-citrate buffer [192 mM Na\(_2\)HPO\(_4\), 4 mM citric acid], and incubated for 20 min in PBS containing 10 µg ml\(^{-1}\) propidium iodide (PI) and 10 µg ml\(^{-1}\) RNase A. Data were collected on a FACSCalibur flow cytometer (Becton Dickinson), and the sub-G\(_1\) (apoptotic) population was gated out of the analysis so as to compare the cell cycle status of each population more easily. TUNEL assays of ES cells and E3.5 embryos were performed using the *In situ* Cell Death Detection kit (Roche) according to the manufacturer’s instructions. Embryonic tissues were fixed with 2% PFA, stained with TUNEL, and counterstained with DAPI. For a positive control, tissues from WT embryos were fixed, permeabilized, and treated with DNase (3 U ml\(^{-1}\)) for 10 min.

**Southern and Northern blot analyses**

Genomic Southern blots were performed as described\(^6\). For Northern blots, total RNA was isolated from ES cells using Trizol reagent (Invitrogen), and 20 µg was loaded onto a 1% formaldehyde agarose gel containing 2.2 mM formaldehyde, 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA. Formaldehyde agarose gels were transferred to nylon membranes (GE Healthcare) and hybridized to radio-labeled *Trp53*, *Pict1*, *Mdm2* or *Gapdh* cDNA probes (Fig. 3a).
**Sequences of siRNA and shRNA duplexes**

The siRNA duplexes used in this study were based on the coding region of the gene of interest, designed to contain dTdT overhangs, and were obtained from Hokkaido System Science. Sequences were:

- **Trp53-A**, 5'-GCCAAGUCGUUAUGUGCAG UACU-3’;
- **Trp53-B**, 5’-CCAUCACUACAAAGUACGUUA-3’;
- **p19**, 5’-CAC CGGAAUCCUGACCAG-3’;
- **Rfwd2**, 5’-AACUGAUCAAGAUAACCUUGA-3’;
- **Rchy1**, 5’-AUUUAGCCUAACCACGAA-3’;
- **Huwe1**, 5’-UGCUAUGUGUCUGG GACA-3’;
- **Mdm2**, 5’-GCACCTCACAGATCCACGC-3’;
- **Rpl5**, 5’-GUCAGAAUG UGGCAGACUA-3’;
- **Rpl11-A**, 5’-AAGGUGCGGGAGUAUGUGUUG-3’;
- **Rpl11-B**, 5’-AAGCAUUGGGAUCUACGCGCCU-3’;
- **Rpl11-C**, 5’-GCCAACACAGAAUC AGCA-3’;
- **Rpl11-D**, 5’-GGCAUAAAAUACGACCACCA-3’;
- **Rpl23**, 5’-GCAAC CAGAACUAAGGAAU-3’;
- **Rps7**, 5’-AGGCAAGGAGGUUGUUUUU-3’.

Scrambled negative control, 5’-UUCUCCGAACGUGUGACGUTT-3’.

For shRNA: Scrambled, 5’-TACGACCTGAACGCTTTAGGA-3’;
- **hPICT1 shRNA1**, 5’-GCTGACAAAGAAGAACAAAGAAA-3’;
- **hPICT1 shRNA2**, 5’-AAGTCCAGAAGA GTCACTGC-3’.

**Ubiquitin ligase activity assay**

H1299 cells in a 9-cm diameter dish were infected for three days with lenti-shRNA as described. Infected cells were co-transfected with **pCAG-HA-Ubiquitin (Ub)** (3.5 μg), **pcDNA3.1-Myc-P53** (3.5 μg), and/or **pCMV-T7-MDM2** (3.5 μg) using Fugene HD (Roche). The total amount of plasmid DNA in each transfection was adjusted to 10.5 μg using empty **pcDNA3.1** vector as needed. At 2 days post-transfection, cells were treated
with 20 μM proteasome inhibitor MG132 (Sigma) for 6 h. Cells were boiled for 10 min in 100 μl SDS lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1% SDS and 1 mM dithiothreitol), and lysates were diluted in 1 ml 0.5% Nonidet P40 buffer. Ubiquitinated protein was immunoprecipitated (IP) from cell lysates using 2 μg HA-specific Ab (Roche), resolved by SDS-PAGE, and immunoblotted (IB) with Myc-specific Ab (Santa Cruz).

To examine the ubiquitination of endogenous p53 protein, Pict1ES cells were transfected with siRNA against Mdm2 and cultured for 16 h. Transfected cells were left untreated or treated with 5 ng ml⁻¹ Dox for 48 h. MG132 (20 μM) was added to the samples indicated in Fig. 3e for the final 3 h of Dox treatment. Cells were lysed by sonication in RIPA buffer (1 % Nonidet P40, 0.1% SDS, Tris-HCl pH 7.8, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, and proteinase inhibitors). p53 protein levels in each sample were quantified by IB, adjusted to equality, and subjected to IP using 2 μg p53-specific Ab (Santa Cruz). IPs were resolved by SDS-PAGE and IB’d with Ubiquitin-specific Ab (Santa Cruz) and p53-specific Ab.

**Protein identification by LC-MS/MS analysis**

Pict1-associated proteins in 293T cells were digested with *Achromobacter* protease I. The resulting peptides were analyzed with a nanoscale liquid chromatography-tandem mass spectrometry (LC-MS/MS) system as described previously¹.

**In vitro studies of Pict1-RPL11 interaction**

GST alone, or full-length GST-RPL5, -RPL11, -RPL23, -RPS7, or GST-PTEN fusion proteins, were purified from lysates of *E. coli* strain BL21 bacteria expressing the *pGEX*
vector (GE Healthcare) that had been cultured for 16 h at 18°C in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Lysate proteins were isolated by binding to glutathione-Sepharose 4B beads (GE Healthcare) according to the manufacturer’s protocol. GST-RPs were incubated separately with in vitro-translated 35S-methionine-labeled Pict1 for 2 h at 4°C. After washing, bound proteins were eluted with 2×SDS sample buffer and detected by SDS-PAGE and autoradiography (Supplementary Fig. 5e).

**Cell fractionation**

Cytoplasmic, nuclear, and nucleolar fractionations were performed as previously described8 with minor modifications. Briefly, 1×10⁸ Pict1ES cells were resuspended in 5 ml hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT), and homogenized with a Dounce homogenizer (Sansyo, Tokyo). After centrifugation (218×g for 5 min), the supernatant was recovered to serve as the cytoplasmic fraction. The pellet was resuspended in 3 ml S1 buffer (0.25 M sucrose, 10 mM MgCl₂), overlaid onto 3 ml S2 buffer (0.35 M sucrose, 0.5 mM MgCl₂), and centrifuged (1430×g for 5 min). The pellet was resuspended in 3 ml S2 buffer to serve as the nuclear fraction. The nuclear fraction was sonicated (10-sec intervals for a total of 120 sec), overlaid onto 3 ml S3 Buffer (0.88 M sucrose, 0.05 mM MgCl₂), and centrifuged (3000×g for 10 min). The pellet was resuspended in RIPA buffer to serve as the nucleolar fraction. For protein detection, each cell fraction (30 µg) was IB’d with the appropriate Abs as shown in Fig. 4a and 4g. Protease inhibitor cocktail was added to all fraction samples.
Confocal microscopy

For Supplementary Fig. 5a, Pict1-EGFP and Nucleophosmin (Npm)-DsRed expression plasmids were transfected into 293T cells using Lipofectamine 2000 (Invitrogen). At two days post-transfection, cells were examined by confocal microscopy (Carl Zeiss LSM5). For Fig. 4d–f and Supplementary Fig. 6a–c, either RRL11-DsRed, RPL5-DsRed, RPL23-DsRed, or RPS7-DsRed expression plasmid was transfected into Pict1ES cells using Lipofectamine 2000. Transfected cells were grown on cover slips for one day before Dox treatment (5 ng ml⁻¹). One or two days after Dox treatment, cells were fixed in 4% formaldehyde and dehydrated with cold methanol-acetone (1:1). To detect endogenous Npm, fixed cells were immunostained with Npm-specific Ab. Because expression levels of RPL23-DsRed were very low, DsRed-specific Ab was used to enhance the fluorescent signal in Supplementary Fig. 6b. To detect endogenous Mdm2 (Supplementary Fig. 6d), Nucleolin (Nln) (Supplementary Fig. 5c and 6d), Npm, or Nucleostemin (Nsm) (Supplementary Fig. 5c), cells were incubated with primary Abs against Mdm2 (Calbiochem), Nln (Sigma), Npm (Sigma), or Nsm (Millipore), followed by incubation with Alexa568- or Alexa488-labeled secondary Abs (Molecular Probes). Nuclei were counterstained with DAPI (Sigma–Aldrich) and examined by confocal microscopy.

Real-time quantitative RT-PCR

Primers for RT-PCR were as follows. PICT1: sense primer, 5'-ACCAGTTCTGGAGACGTG-3'; antisense primer, 5'-GCAGGGACTTTGGATGTGT-3' (208 bp). GAPDH: sense primer, 5'-GTCAACGGATTTGGTCTATT-3'; antisense primer, 5'-AGTCTTCTGGGTGGATGTTATT-3' (270 bp). PCR reactions were performed in a LightCycler
480 (Roche Applied Science) using the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics). The amount of PICT1 mRNA in a sample was divided by the amount of endogenous GAPDH mRNA present in the same sample to obtain the normalized PICT1 mRNA expression level (Supplementary Fig. 7b).

**Nucleolar stress**

For Supplementary Fig. 8, WT ES cells were treated for 0, 3, 6 or 12 h with 5 nM actinomycin D (ActD; Sigma) or 10 μM mycophenolic acid (MPA; Sigma) to exert nucleolar stress.
SUPPLEMENTAL REFERENCES


