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SUPPLEMENTAL MATERIALS & METHODS

Inducible RNAi and cDNA expression

Lentiviral supernatants were generated by transient transfection of 293T cells using TransIT-293 transfection reagent (Mirus) according to the manufacturer's protocol and were harvested 48 h after transfection. Stable cell lines expressing IPTG-inducible shRNAs or doxycycline-inducible CDK4 cDNA were generated by lentiviral transduction in the presence of 8 μg/mL polybrene. Cells were selected using puromycin.

Western blot analyses

Cells were treated for the duration of time indicated, washed with ice cold PBS, and lysed with RIPA buffer containing Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Lysates were clarified by centrifugation at 15,000 x g for 10 min at 4 °C. Total protein concentration was quantified by BCA protein assay (Thermo Scientific). Proteins were resolved on 10% Tris-HCl pre-cast gels using the Criterion system (Bio-Rad) or on 10% Bis-Tris pre-cast gels using the Bolt Mini Gel system (Life Technologies). The following antibodies were used at the dilutions indicated: CDK4 (Abcam, ab7955; 1:2000), CDK6 (Cell Signaling Technology, 3136; 1:2000), cyclin D1 (Cell Signaling Technology, 2926; 1:1000), FOXM1 (Santa Cruz Biotechnology, sc-500; 1:500), GAPDH (Santa Cruz Biotechnology, sc-25778; 1:1000), MYH (Santa Cruz Biotechnology, sc-20641; 1:300), myogenin (Santa Cruz Biotechnology, sc-576; 1:500), p16 (BD Pharmingen, 51-1325GR; 1:500), RB (Cell Signaling Technology, 9309;

1:1000), phospho-RB Ser795 (Cell Signaling Technology, 9301; 1:500), and phospho-RB Ser780 (Cell Signaling Technology, 9307; 1:500).

FISH

Sections (5 mm) of formalin-fixed paraffin-embedded tissue blocks were deparaffinized and rehydrated. Antigen retrieval was performed with IHC-Tek Epitope Retrieval Solution (IHC World) with steaming for 25 min. After cooling, slides were subjected to 50 mg/ml pepsin treatment at 37 ^{o}C, rinsed in PBS solution followed by dehydration in an ethanol series. Co-denaturation of the probe and target DNA at 73 ^{o}C in HYBrite (Abbott Molecular) for 5 min was followed by overnight hybridization at 37 ^{o}C. The next day, slides were washed at 72 ^{o}C in 0.4X SSC/0.3% Tween-20 for 2 min and then cooled in 2X SSC/0.1% Tween-20 to room temperature. The slides were counterstained and mounted with DAPI/Antifade (Vector Laboratories).

Expression microarray analysis of CDK4 knockdown

Raw gene expression data were normalized with RMA method using the Bioconductor oligo package. Hierarchical cluster analysis was performed with R statistical software using agglomerative clustering with Euclidean distance and complete linkage. To identify genes that were downregulated in response to CDK4 knockdown, we used a two-step selection procedure: 1) for each individual gene, we calculated the ratio of gene expression in Rh30 cells expressing NT control shRNA treated with IPTG versus vehicle ($G_{Control} = G_{shControl + IPTG} / G_{shControl + Vehicle}$). Likewise, the ratio of gene expression in shCDK4-expressing cells treated with IPTG versus vehicle was calculated ($G_{shCDK4} = G_{shCDK4 + IPTG} / G_{shCDK4 + Vehicle}$). 2) We defined gene expression change upon CDK4 knockdown as the relative ratio of $G_{shCDK4} / G_{Control}$.

Microarray analysis of tissues with and without 12q13-q14 amplification

Affymetrix HuGene 1.0 GeneChip gene expression data were normalized with RMA with the Bioconductor oligo package. Heat maps of differential gene expression of E2F target genes were created using the R-package gplots. The visualized data comprised gene by sample expression levels, and expression was normalized to Z-score on a gene-by-gene basis to convert all genes to the same scale.