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Loss of CSL Unlocks a Hypoxic Response and Enhanced Tumor Growth Potential in Breast Cancer Cells

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SUMMARY

Notch signaling is an important regulator of stem cell differentiation. All canonical Notch signaling is transmitted through the DNA-binding protein CSL, and hyperactivated Notch signaling is associated with tumor development; thus it may be anticipated that CSL deficiency should reduce tumor growth. In contrast, we report that genetic removal of CSL in breast tumor cells caused accelerated growth of xenografted tumors. Loss of CSL unleashed a hypoxic response during normoxic conditions, manifested by stabilization of the HIF1α protein and acquisition of a polyploid giant-cell, cancer stem cell-like, phenotype. At the transcriptome level, loss of CSL upregulated more than 1,750 genes and less than 3% of those genes were part of the Notch transcriptional signature. Collectively, this suggests that CSL exerts functions beyond serving as the central node in the Notch signaling cascade and reveals a role for CSL in tumorigenesis and regulation of the cellular hypoxic response.

INTRODUCTION

In most cellular contexts Notch signaling acts as a gatekeeper to differentiation, promoting maintenance of stem or progenitor cell fates (Andersson et al., 2011; Guruharsha et al., 2012). Modulation of Notch signaling is used to control stem or progenitor cell differentiation in vitro, for example toward neural, intestinal, or hematopoietic lineages (Lowell et al., 2006; Schmitt et al., 2004; Yin et al., 2014). Deregulated Notch signaling is increasingly linked to cancer, and Notch receptor mutations are found in, for example, T cell leukemia, non-small cell lung cancer, and breast cancer as well as in several types of tumor cell lines (Mutvei et al., 2015; Robinson et al., 2011; Weng et al., 2004; Westhoff et al., 2009). Notch signaling is also frequently hyperactivated in a range of tumors, including breast cancer (for review see Andersson and Lendahl, 2014).

Notch signaling ensues when transmembrane Notch ligands of the Jagged or Delta-like type interact with Notch receptors on a juxtaposed cell. This results in proteolytic cleavage and liberation of the intracellular domain of the Notch receptor (Notch ICD), which relocates to the cell nucleus and interacts with the DNA-binding protein CSL (also known as RBP-Jk or CBF1), thus making CSL the central node in the signaling cascade for all four Notch receptors (Notch 1–4) (Andersson et al., 2011). In the "Notch off" state, CSL acts as a repressor and binds a number of transcriptional co-repressors, such as SHARP/MINT, KDM5A, and KyoT2 (for review see Borggrefe and Oswald, 2014). In the "Notch on" state, i.e., upon binding to Notch ICD, CSL sheds the co-repressors and instead recruits co-activators, such as p300 and PCAF, converting it to an activator. The interaction between Notch ICD and CSL is stabilized by the MAML protein, and the ternary Notch ICD/MAML/CSL complex induces expression of Notch downstream genes (Nam et al., 2006; Wilson and Kovall, 2006). It has traditionally been assumed that CSL serves as a DNA-bound repressor in the absence of Notch, and in line with this, CSL can bind to DNA in the absence of Notch and remains bound to DNA even during mitosis (Lake et al., 2014). Recent studies, however, provide support for a more dynamic view whereby CSL is recruited to the DNA by Notch ICD (Castel et al., 2013; Krejcí and Bray, 2007).

It is an open question whether CSL only transmits the signal from the Notch receptors or also plays a role in other, non-Notch-related signaling transductions. Gene-targeting experiments show that phenotypes resulting from targeting of Notch ligands or receptors in some situations are phenocopied by targeting of CSL, for example during somitogenesis (Conlon et al., 1995; Oka et al., 1995) or in memory T cells (Maekawa et al., 2015), which is in line with CSL functioning exclusively as the central hub in the Notch signaling cascade (Guruharsha et al., 2012). On the other hand, there are also an increasing number of proteins, such as CTCF, EBNA3c, interferon regulatory factor 4, and RITA (see Collins et al., 2014 and references therein), which are not part of the Notch signaling mechanism but interact

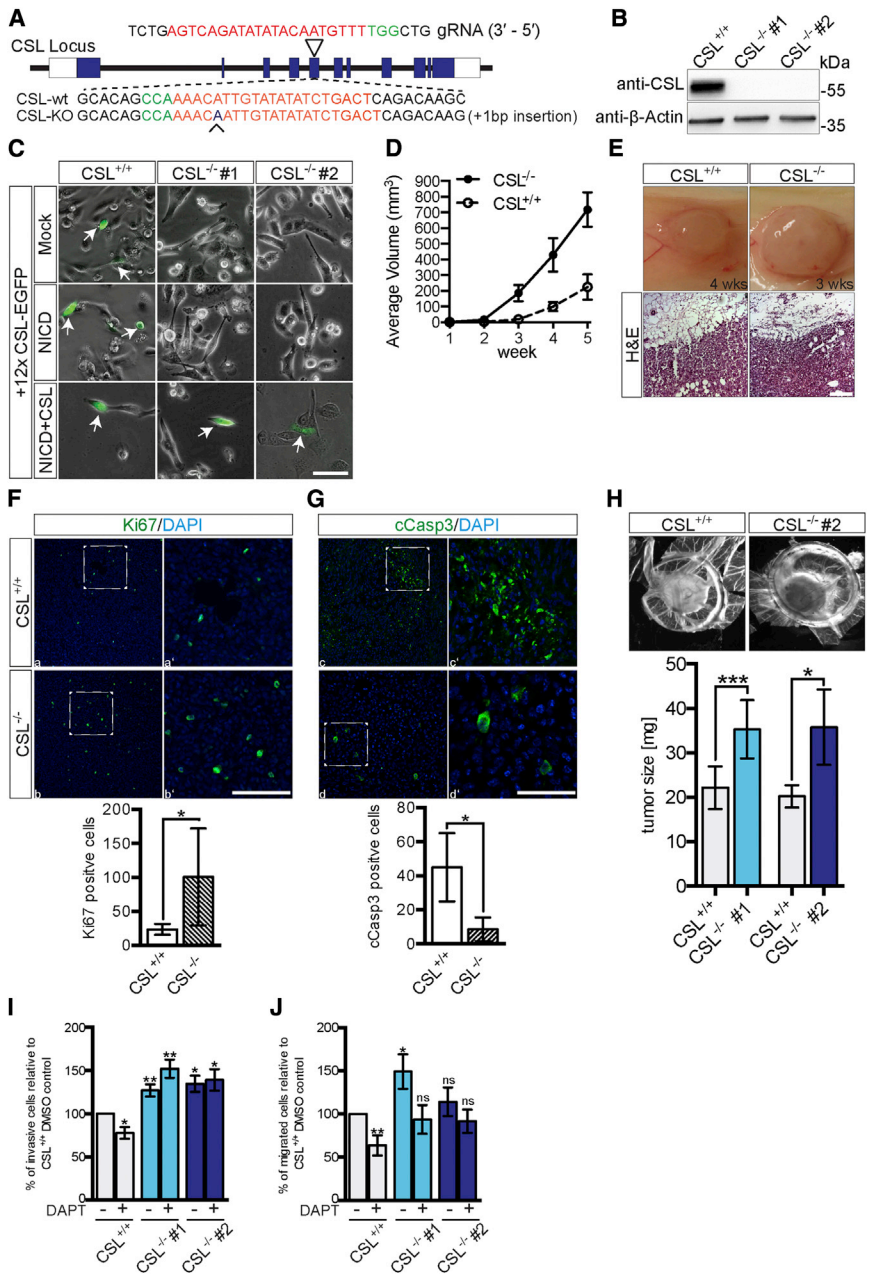

Figure 1. CSL-Deficient Cells Accelerate Tumor Growth In Vivo

(A) Schematic representation of CRISPR/Cas9 targeting of the CSL locus. The triangle points to the targeted exon. Red letters represent the guide RNA sequence and green letters the PAM sequence.

(B) Western blot of CSL and β-actin (loading control) in control ($CSL^{+/+}$) and two clones of CSL-deficient MDA-MB-231 cells ($CSL^{-/-}$).

(C) Notch reporter (12x CSL-EGFP) activity in control and CSL-deficient cells after transfection of 12xCSL-EGFP, Notch1-ICD (NICD), and CSL, as indicated. White arrows indicate cells expressing EGFP.

(D) Average tumor volume at different time points after xenografting $CSL^{+/+}$ or $CSL^{-/-}$ cells. Eight tumors of four mice per group were analyzed.

(E) Representative images and H&E stainings of control and CSL-deficient tumors.

(F and G) Analysis of Ki67 (F) and cleaved Caspase-3 (cCasp3) (G) expression in MDA-MB-$231^{CSL+/+}$ and CSL-deficient tumor sections (enlarged images to the right). At the bottom of each figure, the number of positive cells is quantified. Signals of at least four randomly chosen images from one tumor sample of each kind were counted.

(H) Analysis and quantification of tumor growth in the chick chorioallantoic membrane (CAM) assay for $CSL^{+/+}$ and $CSL^{-/-}$ cells. At least five different tumors of each kind were measured.

(I and J) Invasion and migration assays for $CSL^{+/+}$ and $CSL^{-/-}$ cells. This analysis is based on at least three independent experiments. Data are shown as percent of wild-type MDA-MB-231 DMSO control cells (set to 100%).

Data are presented as mean ± SEM. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. ns, not significant. Scale bars: 100 μm (C), 200 μm (E lower), 100 μm (F and G), and 75 μm (F and G inset).

with CSL, suggesting that CSL has a broader range of actions extending beyond only transmitting Notch signaling.

In this study, we address the question of possible additional roles for CSL and report the unexpected discovery that transplanted breast tumor cells in which CSL was genetically ablated caused rapid tumor growth, a phenotype opposite to blocking Notch function at the receptor level. The phenotype was accompanied by acquisition of a hypoxic response during normoxia and a polyploid giant-cell, cancer stem cell-like, morphology.

RESULTS

Loss of CSL Promotes Tumor Growth In Vivo

To explore the role of CSL in a breast tumor context, we targeted both CSL alleles by CRISPR/Cas9 genome editing in MDA-MB-231 cells (Figure 1A), a breast tumor cell line with active Notch signaling and which promotes tumor growth when transplanted in vivo (Holliday and Speirs, 2011; Jin et al., 2013). In the two independent MDA-MB-$231^{CSL-/-}$ clones selected for further analysis, there was as expected no detectable CSL protein (Figure 1B), and