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The Zinc Finger Transcription Factor PLAGL2 Enhances Stem Cell Fate and Activates Expression of *ASCL2* in Intestinal Epithelial Cells

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SUMMARY

Intestinal epithelial stem cell (IESC) fate is promoted by two major transcriptional regulators, the TCF4/β-catenin complex and ASCL2, which drive expression of IESC-specific factors, including *Lgr5*, *Ephb2*, and *Rnf43*. Canonical Wnt signaling via TCF4/β-catenin directly transactivates *Ascl2*, which in turn auto-regulates its own expression. Conversely, Let-7 microRNAs antagonize the IESC lineage by repressing specific mRNA targets. Here, we identify the zinc finger transcription factor PLAGL2 as a Let-7 target that regulates IESC fate. PLAGL2 drives an IESC expression signature, activates Wnt gene expression, and enhances a TCF/LEF reporter in intestinal organoids. In parallel, via cell-autonomous mechanisms, PLAGL2 is required for lineage clonal expansion and directly enhances expression of *ASCL2*. PLAGL2 also supports enteroid growth and survival in the context of Wnt ligand depletion. PLAGL2 expression is strongly associated with an IESC signature in colorectal cancer and may be responsible for contributing to the aberrant activation of an immature phenotype.

INTRODUCTION

Intestinal epithelial stem cells (IESCs) reside in intestinal crypts and give rise to all endodermally derived lineages of the epithelium. The IESC consists of at least two distinct populations. The first is actively dividing, expresses high levels of the GPCR class A receptor LGR5 (Barker et al., 2007), is located at the base of intestinal crypts (Barker et al., 2007), and is highly dependent on Wnt signaling (de Lau et al., 2011; Yan et al., 2017). A second quiescent IESC is located near the base of crypts (Li et al., 2014; Sangiorgi and Capecchi, 2008; Takeda et al., 2011; Tian et al., 2011; Yan et al., 2012) and has properties of a secretory progenitor (Buczacki et al., 2013). Wnt signaling is necessary for the establishment of IESCs in post-natal intestinal development in mice (Korinek et al., 1998; Pinto et al., 2003). *Ascl2* is also required for IESC specification (van der Flier et al., 2009), and maintenance of IESCs, through cooperation with TCF4 and β-catenin to transactivate genes such as *Lgr5* and *Sox9* (Schuijers et al., 2015).

Let-7 microRNAs (miRNAs) are key regulators of cellular proliferation and differentiation in a variety of contexts and organisms, from *C. elegans* (Lee and Ambros, 2001; Slack et al., 2000) to humans. Let-7 frequently acts to repress stem cell fate or proliferation, as observed in fetal hematopoietic stem cells (Copley et al., 2013; Oshima et al., 2016; Rowe et al., 2016), neural stem cells (Rybak et al., 2008; Zhao et al., 2010), primordial germ cells (Tran et al., 2016; West et al., 2009), and IECs (Madison et al., 2015). The RNA-binding proteins, LIN28A and LIN28B, directly inhibit Let-7 in stem and progenitor cells (Hagan et al., 2009; Rahkonen et al., 2016). LIN28 proteins block Let-7 miRNA function by preventing Let-7 post-transcriptional maturation (Hagan et al., 2009; Heo et al., 2008; Piskounova et al., 2008; Viswanathan et al., 2008). Depletion of Let-7 miRNAs is frequently observed in cancer, and directly contributes to epithelial transformation in colorectal cancer (CRC) (King et al., 2011), while depletion in the mouse intestine via transgenic LIN28A/B expression drives the formation of spontaneous, aggressive adenocarcinomas (Madison et al., 2013; Tu et al., 2015). LIN28 proteins are expressed in the developing mouse gut, but only LIN28B is detectable in the adult intestine, exhibiting nuclear localization in the epithelial crypt compartment (Madison et al., 2013). In mouse models, overexpression of LIN28B in the intestinal epithelium augments the expression of stem cell markers and enhances colony-forming potential of small intestinal organoids (enteroids) (Madison et al., 2013, 2015). Consistent with this, levels of Let-7a and Let-7b miRNAs are inversely proportional to mRNA levels of *LGR5* and *EPHB2* in human CRC, which represent classical IESC markers (Madison et al., 2015). Further examination of Let-7 targets that mediate these effects revealed that the canonical Let-7 target *Hmga2* is required for LIN28B-driven enhancement of colony-forming potential in mouse enteroids (Madison et al., 2015).

However, HMGA2 overexpression in mouse enteroids does not alter the abundance of any IESC marker and only drives a modest enhancement of colony-forming potential (Madison et al., 2015).

Here we identify *PLAGL2* as a Let-7 target that is strongly associated with an IESC signature. *PLAGL2* encodes a zinc finger transcription factor found within a genomic region at 20q11.21 that is frequently amplified in CRC (Carvalho et al., 2009; He et al., 2003; Hermsen et al., 2002). *PLAGL2* is expressed at high levels in various tissues of the developing fetus and placenta and plays a critical role in late intestinal epithelial differentiation (Van Dyck et al., 2007). We have reported that PLAGL2 levels are enhanced by overexpression of LIN28B in the intestinal epithelium (Madison et al., 2015), consistent with its inverse correlation with Let-7 levels in CRC (Madison et al., 2015). We find here that *PLAGL2* is a direct Let-7 target that drives stem cell fate and is required for stem cell function in organoids. One mechanism involves the direct downstream activation of the IESC lineage factor *ASCL2,* where we find that PLAGL2 binds to a conserved consensus sequence in the proximal *ASCL2* promoter.

RESULTS

Interrogation of TCGA CRC RNA sequencing (RNA-seq) datasets reveals that *PLAGL2* expression correlates highly with multiple lineage factors specific for—or highly enriched in—CBC IESCs (Munoz et al., 2012; Sato et al., 2011), including *ASCL2*, *EPHB2*, *NOTCH1*, *RNF43*, and *MYC* (Figure S1A). Among patient-derived CRC xenograft lines (Uronis et al., 2012), this trend is also evident, with significant correlation between *PLAGL2* and *ASCL2*, *RNF43*, and *NOTCH1* (Figure S1B). In a dataset of human colorectal adenomas (Sabates-Bellver et al., 2007), we also observe the co-expression of *PLAGL2* with CBC IESC markers, which are coordinately upregulated together in adenomas relative to normal tissue (Figure S1C).

We used human intestinal organoids to examine the relationship of LIN28B-Let-7, PLAGL2, and effects on stem cells. As expected, LIN28B overexpression in organoids enhances colony-forming potential (Figure 1A). *PLAGL2*, along with the validated Let-7 target, *HMGA2*, is upregulated in a pattern similar to *ASCL2* in these organoids (Figure 1B). *PLAGL2* upregulation in the intestinal epithelium, downstream of LIN28B, is also observed in our mouse models of LIN28B overexpression (Madison et al., 2015). Thus, *PLAGL2* activation is a downstream feature of LIN28B-mediated enhancement of stem cell activity.

To definitively classify *PLAGL2* as a Let-7 target, we pursued validation in *in vitro* models. Transfection of a Let-7b miRNA mimic into DLD1 CRC cells caused a significant depletion of *PLAGL2* mRNA (Figure 1C). We also developed a heterologous reporter system with destabilized GFP and RFP proteins for real-time evaluation of Let-7 miRNA repression (Figure 1D). In the context of a synthetic array of seven Let-7 target sites, reporter activity is repressed 60%–70% by Let-7a (Figure 1E). Likewise, assays of the *PLAGL2* 3′ UTR reveal a 40% decrease of reporter activity by Let-7a (Figure 1F) that is completely abrogated by mutations in each of two Let-7 recognition sequences in the *PLAGL2* 3′ UTR (Figure 1F). Thus, this supports our hypothesis that *PLAGL2* is directly repressed by Let-7.

To model the aberrant upregulation of *PLAGL2* that is likely within the context of Let-7 loss, *LIN28B* de-repression, or 20q11.21 amplification, we generated murine enteroids constitutively overexpressing hemagglutinin-tagged PLAGL2. This yielded modest expression of PLAGL2 protein in two separate clones (Figure 2A). PLAGL2 expression causes a marked increase in cyst-like enteroids (Figures 2B–2D). PLAGL2-expressing enteroids also exhibit a dose-dependent increase in colony-forming potential (Figures 2E and 2F). Similar effects were observed upon PLAGL2 expression in human colonic organoids, with an augmentation of colony-forming potential (Figure 2G). In human organoids, we observe enhancement of proliferation as measured by 5-ethynyl-2′-deoxyuridine incorporation (Figures 2H–2J). This effect is only evident when organoids are cultured in medium lacking Wnt3a. Inactivation of *Plagl2* in mouse enteroids with CRISPR/Cas revealed defects in colony-forming potential of mutants (Figures 2K–2M). However, no significant changes in enteroid growth were observed (Figure 2N). To gain insight into the cell-autonomous function of PLAGL2, we performed lineage-tracing experiments in enteroids stably expressing the $Cas9^{D10A}$ nuclease followed by stable transfection with a transposon constitutively expressing both GFP and gRNAs against *Plagl2*. Following transfection, the majority of surviving organoids do not contain GFP-positive transfected cells. However, a minority (~2%) show evidence of clonal expansion of GFP-positive clones, while targeted inactivation of *Plagl2* causes a reduction in these lineage-tracing events (Figure 2O), as observed over 7 days following the transfection (Figure 2P). Thus, PLAGL2 supports the stem cell lineage in a cell-autonomous manner in enteroids.

To gain insight into pathways operating downstream of PLAGL2 we performed RNA-seq in two mouse enteroid clones overexpressing PLAGL2 (Figure 3A). Because PLAGL2 appears to drive stem cell fate in enteroids, we performed gene set enrichment analysis (GSEA) (Subramanian et al., 2005) on PLAGL2-induced mRNAs in comparison with an expression dataset for GFP-sorted CBC IESCs from *Lgr5-EGFP* mice (Barker et al., 2007; Munoz et al., 2012). This reveals significant enrichment (Figure 3B), indicating that PLAGL2 drives a CBC stem cell expression

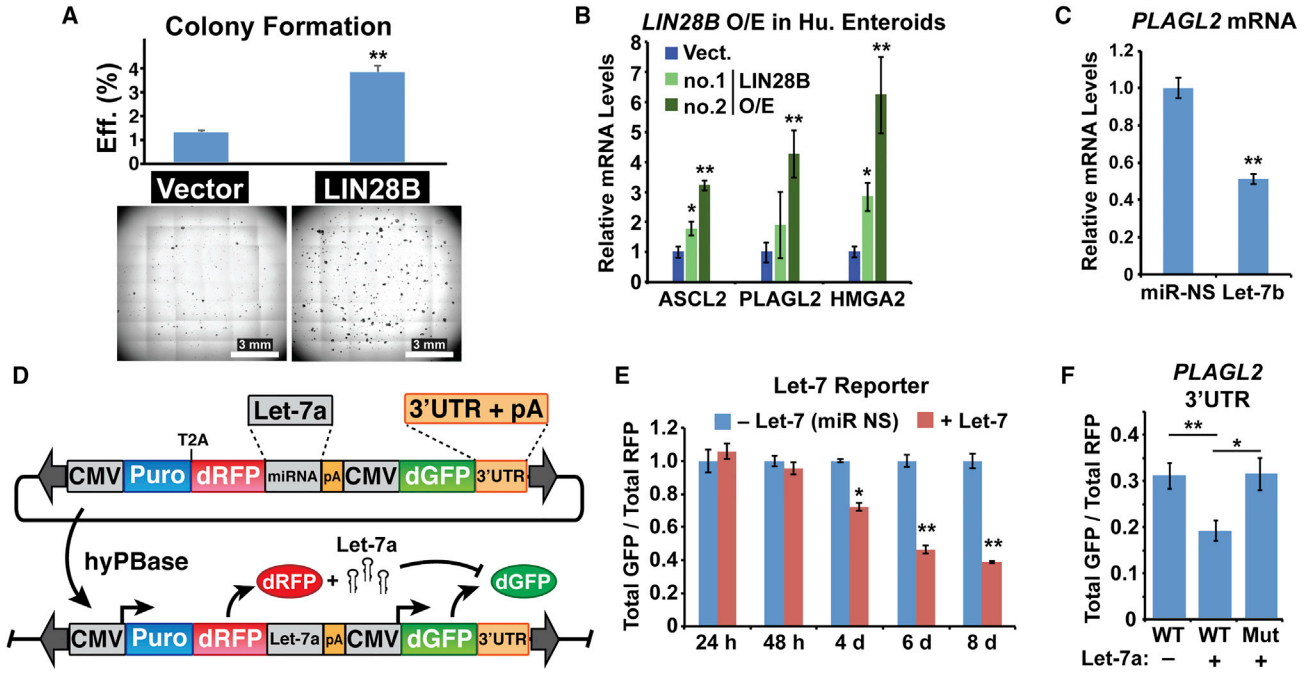

Figure 1. PLAGL2 Is Directly Repressed by Let-7 miRNAs

(A) Human organoids were plated as single cells in Matrigel for a colony-forming assay, in quadruplicate. Colonies were counted after 7 days in culture.

(B) Expression levels of *PLAGL2*, *HMGA2*, and *ASCL2* were assayed in two human organoid clones constitutively expressing LIN28B (LIN28B O/E).

(C) Transient transfection of DLD1 cells with a Let-7b miRNA mimic causes the depletion of endogenous *PLAGL2* mRNA, as assayed by RT-PCR 72 hr after transfection.

(D) Schematic of a transposon miRNA reporter vector for assaying effects of Let-7a on the *PLAGL2* 3′ UTR.

(E) Validation of the miRNA reporter vector containing a synthetic Let-7 target with seven repeats of the Let-7 target seed sequence.

(F) The miRNA reporter vector containing the *PLAGL2* 3′ UTR and a non-specific miRNA or Let-7a. Mutation (Mut) of both Let-7 target seed sequences in the *PLAGL2* 3′ UTR renders the reporter resistant to Let-7.

Student's one-tailed t test was performed to evaluate significance between means of replicates, where *$p < 0.05$ and **$p < 0.01$.

signature. This was true for both PLAGL2 clone no. 1 (false discovery rate [FDR] $q < 0.001$) and no. 2 (FDR $q < 0.02$). An unbiased GSEA query of all molecular function gene ontology gene sets reveals that PLAGL2-upregulated transcripts are enriched for genes associated with Frizzled binding (Figure 3C), both for PLAGL2 clone no. 1 (FDR $q = 0.164$) and no. 2 (FDR $q = 0.317$). Further examination reveals several Wnt ligands (*Wnt9b*, *Wnt4*, *Wnt10a*, and *Wnt5a*) are upregulated by PLAGL2 (Figure 3D), which was validated by RT-PCR (Figure 3E). Decreased expression of Wnt target genes, *Cd44* and *Axin2*, in *Plagl2* null enteroids also supported a positive role for PLAGL2 in driving canonical Wnt signaling (Figure 3F).

To gauge effects on Wnt we generated enteroids with a stable TCF/LEF reporter transgene driving expression of nuclear localized tdTomato (TOP-tdT, Figure 3G), which exhibited the expected sensitivity to manipulation of Wnt signaling with GSK3β and Porcupine inhibitors (Figures 3H and 3I). Transgenic co-expression of PLAGL2 in enteroids caused a marked increase in the number of TOP-tdT-positive cells (Figures 3J–3O), although upregulation of TOP-tdT reporter activity within each individual cell appeared variable (Figure 3P) and did not correlate with levels of overexpressed *PLAGL2* mRNA (Figure 3Q). To better measure possible effects of secreted signals on TOP-tdT reporter activity, GFP-labeled wild-type (WT) TOP-tdT enteroids were co-cultured with PLAGL2-expressing enteroids (Figure 3R). Reporter activity in GFP-labeled co-cultured enteroids was enhanced by these PLAGL2 enteroids (Figure 3S). Consistent with an enhancement of Wnt signaling, colony-forming potential was augmented in WT *ROSA26*^{mTmG} enteroids co-cultured with PLAGL2 enteroids (Figure 3T). Thus, PLAGL2 drives modest enhancement of canonical Wnt signaling, non-cell-autonomously.

To determine if Wnt signaling is the primary underlying driver of the PLAGL2 phenotype in enteroids, we treated

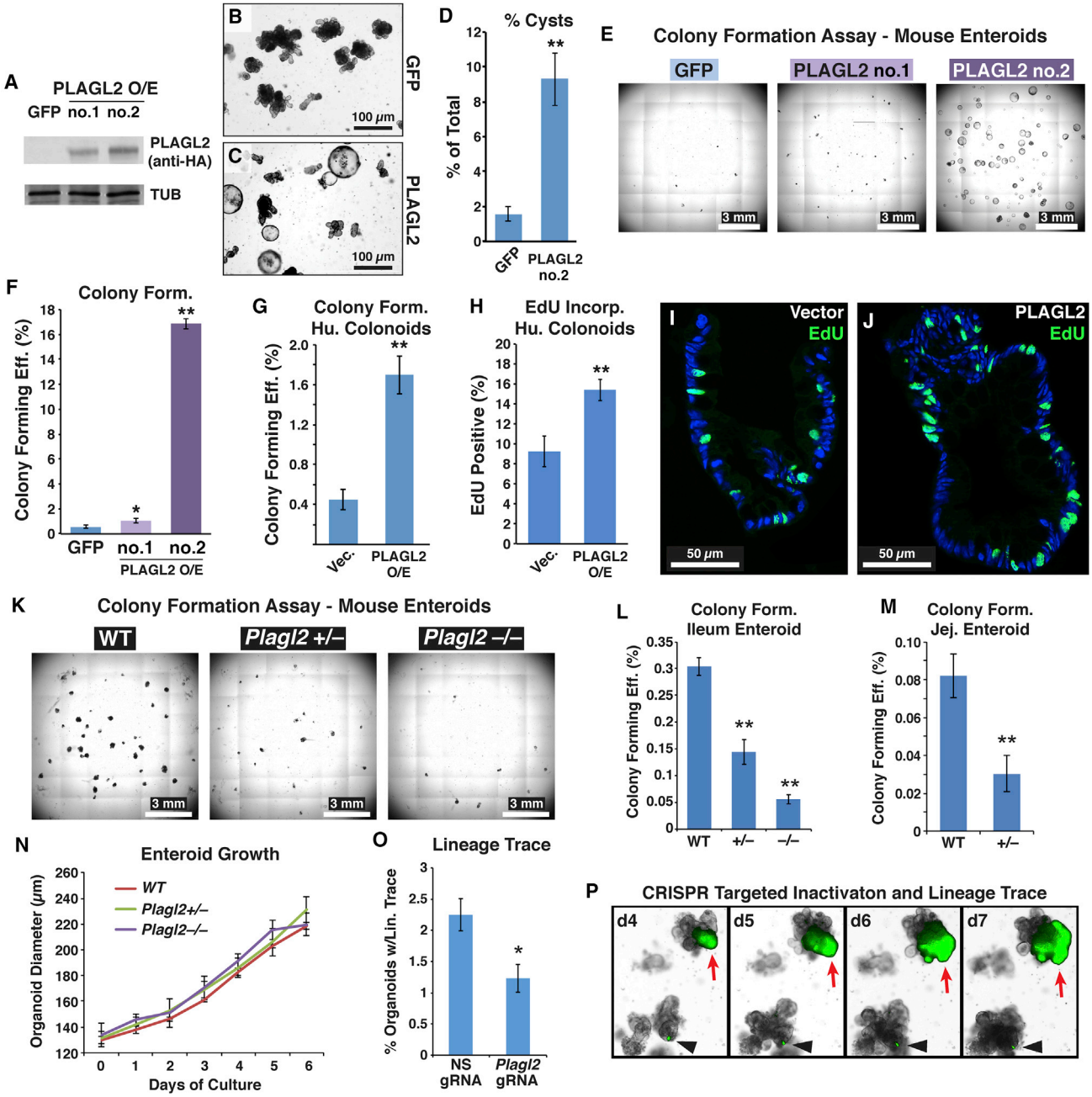

Figure 2. PLAGL2 Drives Stem Cell Potential in Enteroids

(A) Mouse enteroids stably overexpressing (O/E) hemagglutinin-tagged *PLAGL2* or GFP were evaluated by immunoblot.

(B–D) GFP-expressing enteroids appeared morphologically similar to un-transfected parental enteroids (not shown) (B), whereas *PLAGL2* O/E enteroids (clone no. 2) frequently formed large cysts (C), quantified in (D).

(E and F) Stitched representative microscopic images of colony-forming assay (CFA) of *PLAGL2* O/E mouse enteroids, performed in quadruplicate (E), which are quantified in (F).

(G) Quantification of colony-forming assay of *PLAGL2*-expressing human colonoids.

(H–J) 5-Ethynyl-2′-deoxyuridine (EdU) incorporation in human colonoids as quantified in sections (H), with representative images for empty vector (I) and *PLAGL2*-expressing human colonoids (J).

(K–M) Representative images of CFA of *Plagl2* mutant mouse ileum enteroids, performed in quadruplicate (K), which are quantified in (L), while *Plagl2* mutant mouse jejunum enteroids are quantified in (M).

(N) *Plagl2* mutant mouse enteroid size over 6 days of culture.

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cultures with IWP-2, a small molecule that blocks Wnt palmitoylation and secretion through inhibition of Porcupine (Chen et al., 2009). After 5–7 days of IWP-2 treatment the vast majority of control (GFP-expressing) enteroids die, while surviving enteroids eventually regress (Figure 4A). However, many PLAGL2-expressing enteroids survive IWP-2 treatment, either as cysts or budding structures (Figure 4B), which is never observed in controls (Figure 4C). To further parse Wnt dependency, PLAGL2 enteroids with the TOP-tdT reporter were treated with IWP-2 and closely monitored for reporter activity (Figures 4D–4F). Despite Wnt inhibition, PLAGL2 enteroids maintain higher expression levels of the IESC lineage markers *Ascl2* and *Lgr5* (Figure 4G), but do not drive higher expression of other Wnt target genes (Figure 4H). To see if PLAGL2 maintains stem cell potential in the face of Wnt inhibition, we continued IWP-2 treatment of enteroids (from Figures 4E and 4F) for 2 additional days, confirmed complete loss of the TOP-tdT reporter in each enteroid, and then re-plated remaining enteroids in fresh medium lacking IWP-2. PLAGL2-expressing enteroids yielded significantly more numerous new colonies, relative to the initial number of enteroids (Figures 4I–4K). Thus, PLAGL2 can drive intestinal epithelial growth and support stem cell potential despite severe Wnt depletion.

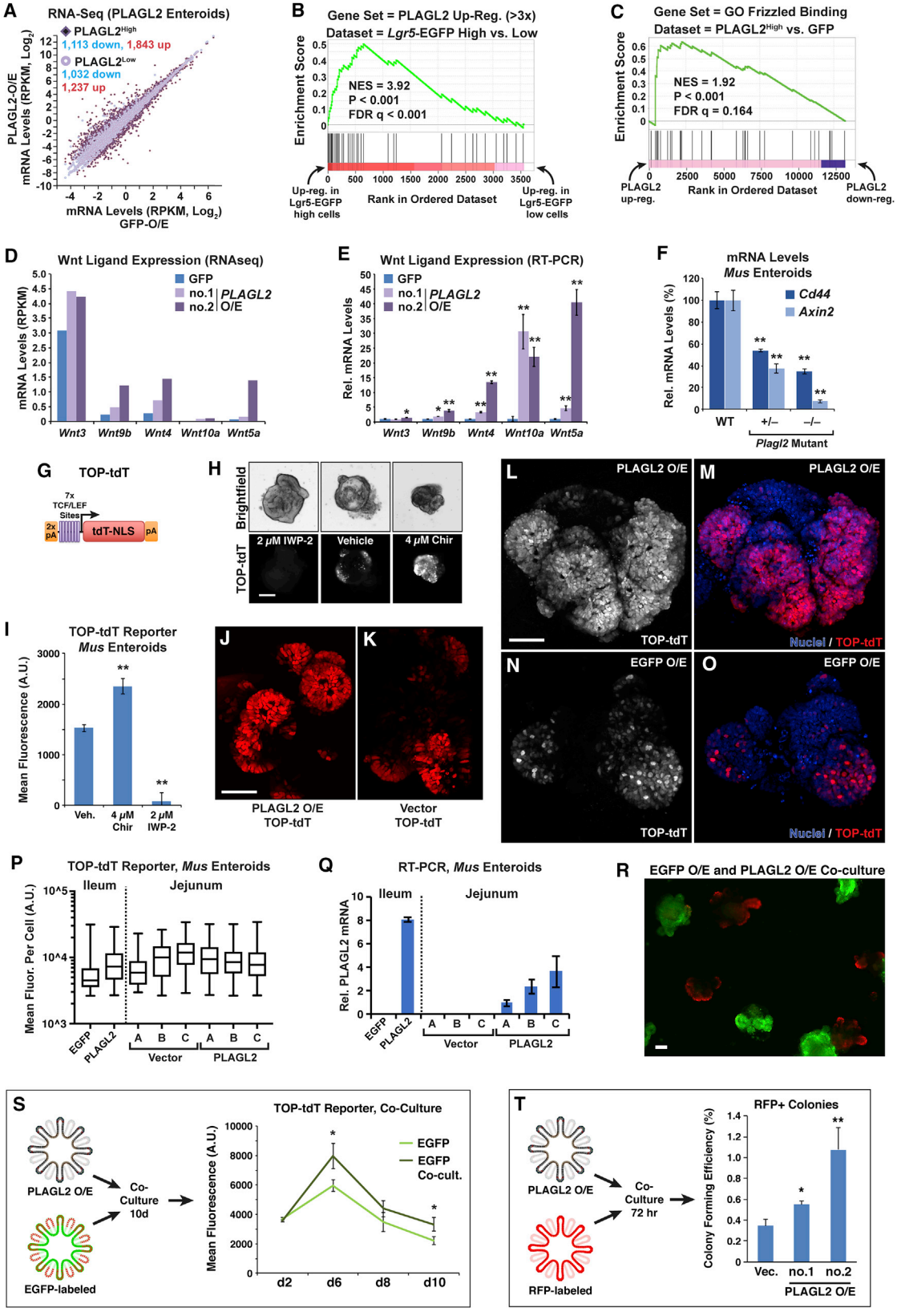
Oddly, despite the apparent augmentation of Wnt signaling by PLAGL2, TCF4/β-catenin target genes are not induced by PLAGL2 overexpression (Figure 5A). However, RNA-seq did reveal that ASCL2 target genes (as previously described by Schuijers et al., 2015) were modestly induced, in a dose-dependent manner, in PLAGL2-expressing enteroids (Figure 5A). RT-PCR for canonical Wnt target genes *Axin2* and *Cd44* confirmed no increase of these transcripts (Figure 5B). Expression analysis in both mouse (Figure 5C) and human (Figure 5D) organoids confirmed that PLAGL2 augments expression of *ASCL2*, in a dose-dependent manner. Using a gene set of ASCL2-induced transcripts (Schuijers et al., 2015) we compared PLAGL2-modulated transcripts using GSEA, which reveals enrichment of an ASCL2 signature in PLAGL2-upregulated genes (Figure 5E). Decreased expression of *Ascl2* and the ASCL2 target, *Lgr5*, in *Plagl2* null enteroids also suggests a positive role for PLAGL2 in supporting ASCL2 expression (Figure 5F).

Although *ASCL2* is a TCF4/β-catenin target gene (Giakountis et al., 2016; Schuijers et al., 2015) that could be activated by increased levels of Wnt ligands, we investigated a possible Wnt-independent relationship between *PLAGL2* and *ASCL2*. We looked first at available RNA-seq data for CRC tumors, which frequently possess Wnt-activating mutations. RNA-seq data from CRC tumors were parsed according to common Wnt-activating mutations (truncating mutations in *APC* or *AXIN2*, or missense mutations in *CTNNB1*). This revealed a strong relationship between *PLAGL2* and *ASCL2* expression, regardless of these hallmarks of aberrant canonical Wnt pathway activation (Figure 5G). We then examined this relationship in a CRC tumor cell line, DLD1, which has inactivating mutations in *APC*. Inactivation of the endogenous *PLAGL2* gene in DLD1 CRC cells (Figure S2) causes a stepwise loss of *ASCL2* mRNA (Figure 5H), suggesting dependency on PLAGL2, despite constitutive Wnt pathway activation in these cells. Thus, PLAGL2 is a necessary component of the regulatory mechanisms operating upstream of ASCL2.

We next examined whether the cell-autonomous defect in lineage tracing following mutagenesis of *Plagl2* (Figure 2O) could be rescued by ASCL2. In enteroids transfected with the stable gRNA transposon, we co-transfected an ASCL2-expressing transposon. ASCL2 rescued the lineage-tracing defect in *Plagl2*-mutated enteroids (Figure 5I). Both T7E1 assays (Figure 5J) and Illumina-based genotyping for the targeted region of *Plagl2* (Figure 5K) revealed similar levels of mutagenesis in both populations, but not enteroids targeted with a non-specific gRNA.

To investigate the importance of PLAGL2 in the transcriptional activation of *ASCL2*, we constructed a fluorescent reporter vector with the mouse *Ascl2* proximal promoter (Figure 6A). Assays in DLD1 cells revealed that constitutive expression of PLAGL2 augmented reporter activity (Figures 6B and 6C), while siRNA knockdown of endogenous PLAGL2 resulted in decreased activity (Figure 6D). To examine direct interaction, we employed chromatin immunoprecipitation (ChIP) to determine if PLAGL2 binds conserved PLAG consensus sites ($GRGGCN_{6\text{-}8}RGGK$, as previously defined by Hensen et al., 2002; Voz et al., 2000) located in the proximal mouse *Ascl2* promoter and near a 3′ lncRNA (*Wintrlinc1*) (Figure 6E). ChIP and qPCR revealed clear interaction of PLAGL2 with the *Ascl2* promoter in both PLAGL2-O/E enteroid clones (Figure 6F). ChIP also indicated interaction with the mouse *Wintrlinc1* promoter, although only in enteroids expressing higher levels of PLAGL2 (Figure 6F). Thus, PLAGL2 interacts with *cis* regulatory sequences near *ASCL2* and can drive transcription of the *ASCL2* promoter.

(O) Quantification of lineage-tracing events in stable $Cas9^{D10A}$-expressing enteroids 7 days after transfection with a transposon constitutively expressing gRNAs targeting *Plagl2* or *LacZ* (NS gRNA).
(P) Exemplary images of a lineage-tracing event from days 4 to 7 post-transfection (red arrow) described above, as observed via constitutive GFP co-expression from the gRNA-expressing transposon. Single isolated transfected cells (black arrowhead) that do not expand are not quantified as lineage-tracing events.
Student's one-tailed t test was performed to evaluate significance between means of replicates, where *$p < 0.05$ and **$p < 0.01$.



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DISCUSSION

Here, we have reported the role for the transcription factor PLAGL2 in promoting stem cell identity, in part through direct transcriptional activation of *ASCL2*, which may be responsible for Wnt-independent growth driven by PLAGL2. Previously, known regulators of *ASCL2* in the intestine were limited to the Wnt pathway transcriptional activators TCF4/β-catenin (Schuijers et al., 2015) and *WiNTRLINC1*, a long non-coding RNA (lncRNA) located 3′ of *ASCL2* that is also needed for human *ASCL2* expression (Giakountis et al., 2016). The transcriptional activation of *WiNTRLINC1* by TCF4/β-catenin is coupled with the transcriptional activation of *ASCL2,* with the lncRNA itself aiding in chromatin looping between TCF4/β-catenin-bound *WiNTRLINC1* and the *ASCL2* promoter (Giakountis et al., 2016). While we do not observe any change of the mouse *Wintrlinc1* RNA in PLAGL2-expressing mouse enteroids (data not shown), the *Wintrlinc1* promoter itself may act in a facultative fashion as an enhancer for *ASCL2,* independent of the *Wintrlinc1* RNA. lncRNA promoters have been documented to execute this type of *cis* regulation of neighboring genes (Engreitz et al., 2016).

In vivo, a role for *PLAGL2* in stem cell development and/or homeostasis remains to be determined. Early in murine post-natal intestinal development (post-natal day 3, or p3) PLAGL2 protein appears to be distributed throughout epithelial cells along the crypt-villus axis, with mRNA levels dropping gradually after p14 (Van Dyck et al., 2007). While mice homozygous for a germline null mutation in *Plagl2* die shortly after birth, the reason for this mortality is unclear, and may be due to stem cell failure, or due to epithelial lipid malabsorption, as proposed previously (Van Dyck et al., 2007). *In vivo, Plagl2*-null phenotypes caused by depletion of Wnt ligand expression may be masked by the unperturbed expression of stromal-derived Wnts. A conditional (e.g., floxed) allele is needed to determine the role of PLAGL2 in the adult mouse intestinal epithelium through conditional inactivation.

In addition to direct regulation of ASCL2, PLAGL2 also appears to drive Wnt signaling, although this effect appears minimal in our overexpression model; i.e., Wnt target genes are not globally increased, and TOP-tdT reporter activity is only modestly increased. PLAGL2 O/E appears to augment the abundance of Wnt-high cells, as gauged from our confocal microscopy, which may reflect a compartmentalized effect of PLAGL2, perhaps only within the IESC lineage. The Wnt targets *Cd44* and *Axin2*, which are not restricted to the IESC lineage (Li et al., 2016; Zeilstra et al., 2014), may only be increased in IESCs, which could be obfuscated by expression analysis of total RNA from whole enteroids. Alternatively, in the context of overexpression, PLAGL2-mediated effects on canonical Wnt signaling may depend on limiting co-factors. This could account for depletion of Wnt targets in *Plagl2* knockouts, but not increased abundance of the same targets following PLAGL2 O/E. Previous

Figure 3. PLAGL2 Drives an Lgr5^{High} Intestinal Stem Cell Signature and Wnt Activation

(A) Scatterplot of reads per kilobase of transcript per million mapped reads (RPKM) values from RNA-seq of mouse enteroids, clone no. 1 (PLAGL2-Low O/E) and clone no. 2 (PLAGL2-High O/E), compared with GFP-expressing (GFP-TG) enteroids. The number of genes down- or upregulated >2-fold for each clone is indicated.

(B) GSEA using a gene set consisting of 600 transcripts upregulated >3-fold in PLAGL2 no. 2 enteroids, and upregulated >1.05-fold in PLAGL2 no. 1 enteroids, relative to GFP-TG enteroids. Dataset is a ranked list of 3,566 transcripts from Lgr5-EGFP-sorted IESCs selected for up- or downregulation, relative to Lgr5-EGFP^{LOW} cells ($p < 0.05$) from a published dataset (Munoz et al., 2012).

(C) GSEA against all molecular function gene ontology (GO) terms revealed a strong "Frizzled Binding" enrichment in the RNA-seq dataset.

(D) Expression levels of Wnt ligand genes altered by PLAGL2 O/E, represented as RPKM values from RNA-seq data.

(E) RT-PCR validation of Wnt mRNA levels, expressed relative to GFP controls.

(F) RT-PCR for the Wnt target genes *Cd44* and *Axin2* in WT and *Plagl2* mutant ileum enteroids.

(G) Schematic map of Tcf/Lef reporter (TOP-tdT) transposon that expresses tdTomato with a nuclear-localization signal (tdT-NLS).

(H and I) Representative images of stable lines of WT mouse enteroids with the TOP-tdT reporter treated for 48 hr with vehicle (0.1% DMSO), 4 μM Chir99021, or 2 μM IWP-2 (H), with fluorescence quantified in (I).

(J–O) Confocal image of TOP-tdT fluorescence in jejunum enteroid clone A stably overexpressing PLAGL2 (J), with empty vector control A depicted in (K). Confocal image of TOP-tdT fluorescence in ileum PLAGL2 O/E enteroid (L) (white), and overlaid in red with a nuclear stain (M). Control enteroids (GFP O/E) are shown in (N) and (O).

(P) Enteroids from eight independent *Piggybac* transgenic lines were dissociated for quantifying fluorescence in each cell.

(Q) RT-PCR for the human PLAGL2 cDNA expressed in mouse ileum and jejunum enteroid lines, also transgenic for TOP-tdT.

(R) Images of GFP-labeled and PLAGL2 O/E ileum enteroids co-cultured together for 10 days.

(S) Enteroids overexpressing PLAGL2 or GFP were imaged for TOP-tdT reporter activity following 10 days of culture, with increased reporter activity evident in GFP-expressing enteroids co-cultured with PLAGL2-expressing enteroids.

(T) PLAGL2 O/E enhances colony-forming potential of RFP-expressing (*ROSA26^{mTmG}*) WT mouse enteroids following 72 hr of co-culture. Scale bars, 50 μm. Student's one-tailed t test was performed to evaluate significance between means of replicates, where $*p < 0.05$ and $**p < 0.01$.

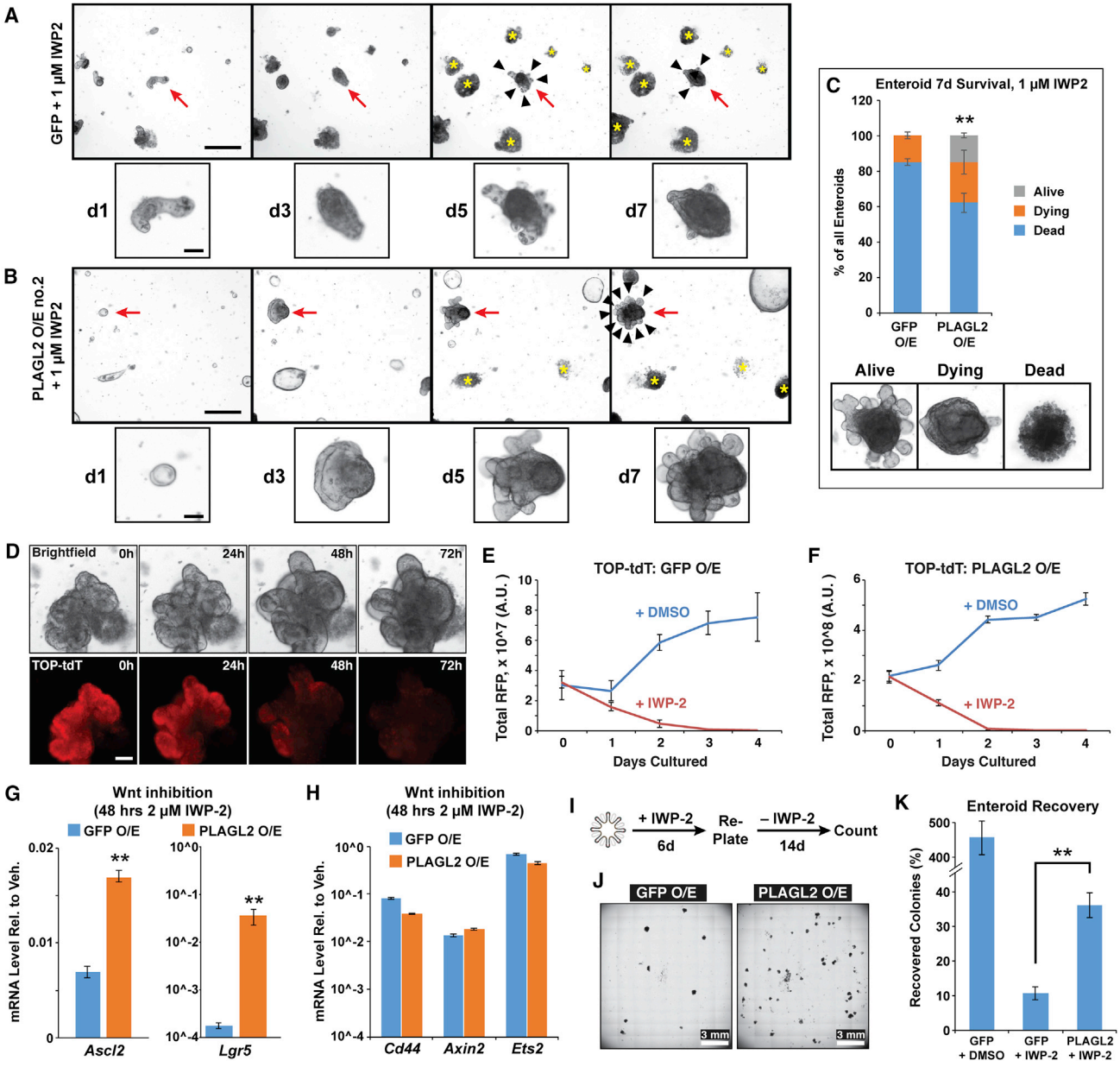

Figure 4. PLAGL2 Drives Enteroid Growth and Survival in the Absence of Wnt

(A and B) GFP-expressing (A) or PLAGL2-O/E (B) enteroids were plated in ENR medium containing DMSO or 1 μM IWP2 and monitored over 7 days for viability. The vast majority of GFP-expressing enteroids are dead by 5 days with 1 μM IWP2 treatment (A) (yellow asterisks) while rare surviving enteroids begin to regress by day 7 (A) (red arrow, and inset). Many PLAGL2-O/E enteroids survive 1 μM IWP2, mostly as cysts, with some persisting as budding enteroids (B) (red arrow, and inset). Enteroid buds are indicated with arrowheads, and yellow asterisks indicate dead enteroids.

(C) Quantification of surviving enteroids following 7 days of IWP-2 treatment, with morphological qualification of status (see Supplemental Experimental Procedures).

(D–F) Representative images of a TOP-tdT enteroid, overexpressing PLAGL2, imaged over 72 hr of treatment with 2 μM IWP-2 (D). Levels of tdT were quantified in enteroids expressing either GFP (E) or PLAGL2 (F) over 4 days of treatment, after which tdT fluorescence was not visible in any organoid treated with IWP-2.

(G) RT-PCR for stem cell markers *Ascl2* and *Lgr5* in TOP-tdT enteroids treated with 2 μM IWP-2 for 48 hr, relative to levels in enteroids treated with 0.1% DMSO (Veh.).

(H) RT-PCR for Wnt target genes, as in (G).

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effects of PLAGL2 on Wnt ligand expression have also been documented in glioma cells, although PLAGL2 drives expression of Wnt6 in this model (Zheng et al., 2010). The involvement of other PLAGL2 targets also cannot currently be excluded as possible modulators of Wnt signaling.

It is rather enigmatic that PLAGL2 robustly induces the expression of Wnt genes (*Wnt9b, Wnt4, Wnt10a,* and *Wnt5a*) when overexpressed in enteroids, yet modestly augments canonical Wnt signaling. These Wnts may be minor components compared with the larger pool of Wnt3 (or Wnt2b, *in vivo*). In addition, robust *Wnt4* and *Wnt5a* induction by PLAGL2 may trigger the activation of non-canonical Wnt signaling. These Wnts can activate non-canonical Wnt signaling pathways (Heinonen et al., 2011; Tanigawa et al., 2011; Wallingford et al., 2001; Yamanaka et al., 2002) and antagonize canonical signaling, which is observed for both Wnt5a (Bernard et al., 2008; Topol et al., 2003; Yuzugullu et al., 2009) and Wnt4 (Bernard et al., 2008; Tanaka et al., 2011). In CRC, *WNT5A* is frequently downregulated, antagonizes canonical Wnt signaling, represses epithelial-to-mesenchymal transition, and slows CRC cell line proliferation (Cheng et al., 2014). Consistent with this, Wnt5a is a stromal signal that antagonizes epithelial proliferation of wounded mucosa in the mouse colon but is necessary for regeneration (Miyoshi et al., 2012). More studies of the roles of each Wnt ligand are needed for the colon, which appears more resistant to loss of all Wnt ligands, as observed following global inactivation of Wntless (*Wls*) in the mouse (Farin et al., 2012). PLAGL2 may have differing effects in these compartments (colon versus small intestine), *in vivo*, perhaps due to unique contexts of specific Wnt ligands.

In future efforts to antagonize canonical Wnt signaling in CRC tumors for therapeutic purposes, as underway in mouse models (Cha and Choi, 2016; Fang et al., 2016; Masuda et al., 2016; Qu et al., 2016; Yamada and Masuda, 2017), it may be necessary to consider pathways that drive Wnt-independent maintenance of proliferation or a stem cell-like state. PLAGL2 appears to drive such a pathway and may also be a relevant target for therapeutic inhibition.

EXPERIMENTAL PROCEDURES

Vector Construction

BII-ChPt and BII-ChBt vectors for Piggybac-mediated transgenesis were constructed using standard molecular cloning techniques. PLAGL2 was PCR amplified and cloned, in frame, between each BsmBI restriction site (see Supplemental Information). LIN28B was cloned into the PB533A-2 vector (System Biosciences) between the XbaI and SwaI restriction sites after PCR amplification from the MSCV-PIG-LIN28B vector (King et al., 2011). The pCMV-hyPBase plasmid (Yusa et al., 2011) was a gift from Allan Bradley (Wellcome Trust Sanger Institute).

Transgenic Organoid Production

Human organoids were established from biopsies obtained from healthy patients undergoing routine colonoscopy, cultured as described previously (Matano et al., 2015), and transfected with the PB533A-LIN28B (transfected at passage 8) or BII-ChBt-PLAGL2 (transfected at passage 10) using previously described protocols (Fujii et al., 2015).

Mouse jejunum enteroids established from 6- to 8-week-old C57BL/6 mice were transfected with the BII-ChPt-PLAGL2 or -GFP *Piggybac* transposons at passage 3. Enteroids were transfected with 1 μg DNA (200 ng pCMV-hyPBase, 800 ng BII-ChPt-PLAGL2, or BII-ChPt-GFP) by spinoculation as described previously (Schwank et al., 2013) using lipofectamine 2000 (Thermo Fisher Scientific). All experiments for human or mouse organoids (transgenic, knockout, or WT) were performed between passages 4 and 12.

RNA Isolation and RT-PCR

Cells/enteroids were homogenized in 1.0 mL TRIzol (Thermo Fisher Scientific) for 30–45 s using a BeadBug homogenizer (Benchmark Scientific), and total RNA isolated per the manufacturer's specifications. RT reactions were performed with oligo(dT) primers using 1–4 μg RNA and Superscript III (Thermo Fisher Scientific). qPCR was achieved with Bullseye EvaGreen qPCR MasterMix (MIDSCI) using primers in Table S2.

Colony-Forming Assays

For human organoid colony-forming assays, organoids were dissociated in TrypLE Express (Thermo Fisher Scientific) containing 250 U/mL DNase I and 10 μM Y27632 for 20 min at 37°C. Single cells were plated in quadruplicate at 5,000 cells/well on a 24-well plate. Images were taken and quantified at day 0 and day 7 (LIN28B organoids) or day 10 (PLAGL2 organoids) using the BioTek Cytation 3 Imaging Platform. Mouse enteroids were also dissociated with TrypLE, for 5 min, and plated/imaged as above.

Mutagenesis of Plagl2 in Mouse Enteroids

For generating knockouts of mouse *Plagl2* C57BL/6 jejunum and ileum enteroids (from 6- to 8-week-old mice, each at passage no. 1) were expanded for transfection as described above. Enteroids were dissociated into single cells with TrypLE (Thermo Fisher Scientific) and transfected with Cas9 plasmids, pCMV-hyPBase, and BII-ChPtG. Transfectants were selected with 2 μg/mL puromycin and picked after 7 days.

(I and J) TOP-tdT enteroids were treated 6 days with 2 μM IWP-2 and then 14 days in complete ENR medium (I), followed by microscopic imaging (J).

(K) Quantification of enteroids from (J), relative to the original numbers of enteroid colonies present prior to IWP-2 treatment.

Scale bars, 50 μm. Student's one-tailed t tests were performed to evaluate significance between means of replicates, where *$p < 0.05$ and **$p < 0.01$.

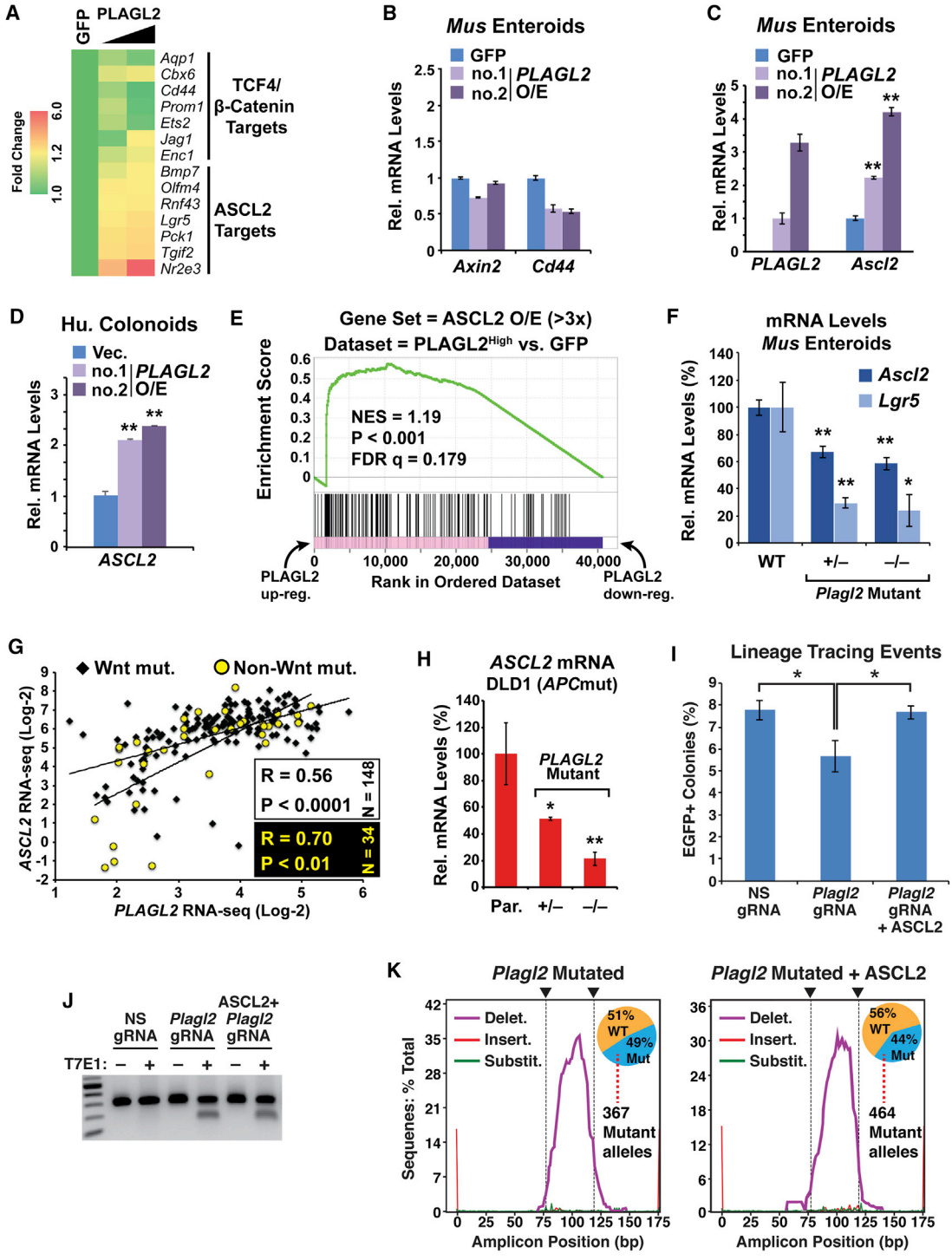

Figure 5. PLAGL2 Enhances *ASCL2* Expression, Independent of Canonical Wnt Signaling

(A–C) Heatmap reflecting RNA-seq data from clone no. 1 (PLAGL2-Low O/E, middle column) and clone no. 2 (PLAGL2-High O/E, right column) showing expression changes of TCF4/β-catenin and ASCL2 transcriptional targets relative to GFP control (A). RT-PCR was performed for canonical Wnt target genes, *Axin2* and *Cd44* (B), human *PLAGL2* and *Ascl2* (C) in mouse enteroids, clones no. 1 and 2.

(D) RT-PCR for *ASCL2* in human PLAGL2-O/E colonoids.

(E) GSEA against a gene set of mRNAs induced upon Ascl2 overexpression (Schuijers et al., 2015) revealed enrichment in the PLAGL2 RNA-seq dataset (clone no. 2).

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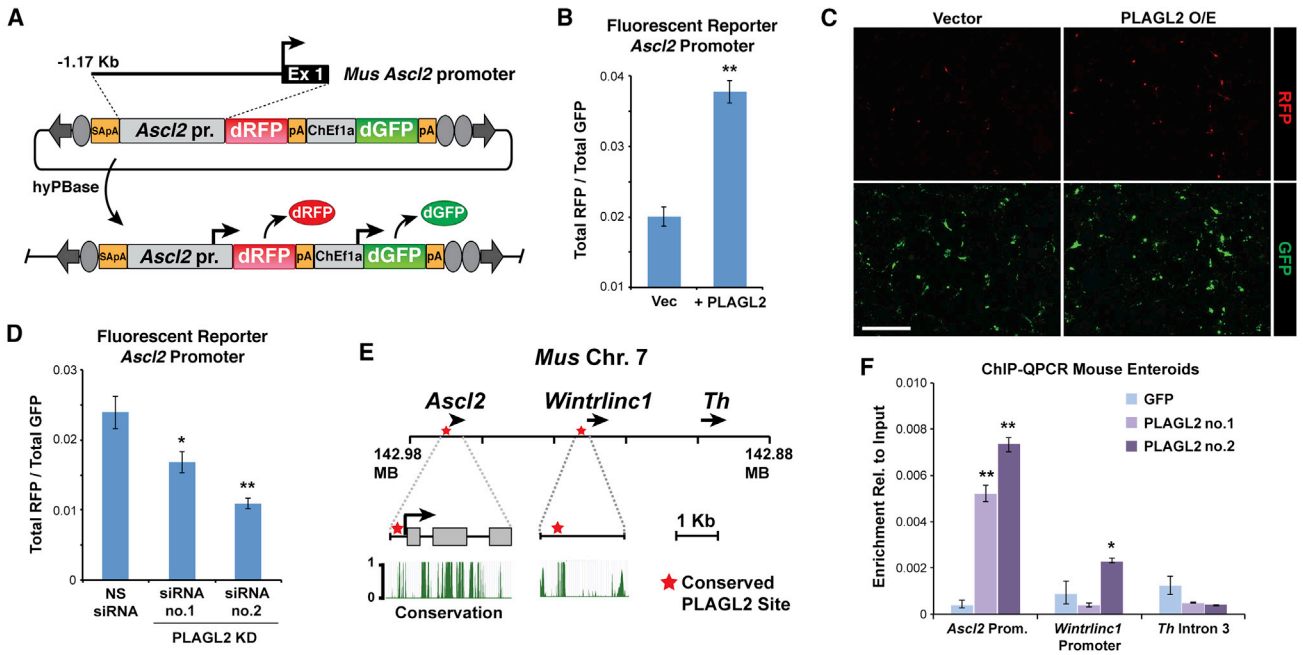

Figure 6. PLAGL2 Activates Transcription of the Mouse *Ascl2* Promoter and Directly Binds Sites near *Ascl2*

(A) Reporter transposon construct for measuring *Ascl2* promoter activity.

(B) *Ascl2* promoter activity as measured in DLD1 cells, after co-transfection with PLAGL2 expression construct or empty vector.

(C) Images of RFP and GFP fluorescent DLD1 cells from (B).

(D) siRNA knockdown of endogenous PLAGL2 in DLD1 cells following stable integration of the Ascl2 promoter reporter, pictured in (A). Reporter activity was measured 72 hr after siRNA transfection.

(E) Map of mouse chromosome 7 showing locations of *Ascl2, Wntrlinc1,* and *Th* genes and sites where two conserved PLAGL2 consensus sequences ($GRGGCN_{6\text{-}8}RGGK$) are located.

(F) Chromatin immunoprecipitation and qPCR for sequences encompassing these sites using lysates from PLAGL2 O/E (clones no. 1 and 2) and GFP-expressing mouse enteroids. Sequences within *Th* intron 3 were amplified as negative controls.

Scale bar, 500 μm. Student's one-tailed t test was performed to evaluate significance between means of replicates, where $*p < 0.05$ and $**p < 0.01$.

Transient Mutagenesis of *Plagl2* and Lineage Tracing in Mouse Enteroids

Mouse enteroids were first transfected and selected for stable expression of the Cas9 nuclease. This line of Cas9-expressing enteroids was then transfected with a *Piggybac* transposon driving constitutive expression of specific gRNAs and GFP. GFP expression and lineage-tracing events were imaged on days 4–7 after transfection, and lineage-tracing events quantified.

IWP2 Treatment of PLAGL2 Mouse Enteroids

BII-ChPt-PLAGL2 mouse enteroids were plated onto a 12-well plate (Greiner) in ENR-containing vehicle (0.1% DMSO), or the Wnt

(F) RT-PCR for *Ascl2* and *Lgr5* in mouse *Plagl2* mutant enteroids.

(G) RNA-seq data of one TCGA CRC patient cohort (N = 182), with tumors stratified according to activating Wnt pathway mutations (missense mutations in *CTNNB1* or truncating mutations in *APC* or *AXIN2*).

(H) RT-PCR for *ASCL2* in DLD1 CRC cells with *PLAGL2* heterozygous or homozygous null mutations.

(I) Quantification of lineage-tracing events in stable $Cas9^{D10A}$-expressing mouse enteroids 7 days after transfection with a transposon expressing gRNAs against *Plagl2*, and co-transfected for stable constitutive expression of ASCL2 (conferring blasticidin resistance).

(J) T7E1 assay of PCR products from the targeted (exon 3) region of *Plagl2* from blasticidin-resistant enteroids from (I).

(K) Illumina sequencing of targeted region of *Plagl2* from enteroids in (I) confirms mutagenesis of *Plagl2*, with vast majority of mutations consisting of insertions or deletions (indels). Targeted region in *Plagl2* amplicon by each unique gRNA (for paired nickase with $Cas9^{D10A}$) is indicated with arrowheads.

Student's one- or two-tailed t test was performed to evaluate significance between means of replicates, where $*p < 0.05$ and $**p < 0.01$. See also Figures S1 and S2.

inhibitor, IWP-2 (Selleckchem) at 1 or 2 μM. Medium was changed every 48 hr. Enteroids were imaged daily for 7 days using a BioTek Cytation 3 Imaging Platform with Gen5 software to monitor enteroid viability.

Transfection with siRNA (for Knockdown) or miRNA Mimics

Transient transfection of DLD1 cells with the Let-7b mimic (IDT) was performed as follows. A total of 5×10^5 cells was plated in a six-well plate and transfected the next day with 1.25 μL of 20 μM miRNAs using the Lipofectamine RNAiMAX (Thermo Fisher Scientific) transfection reagent according to the manufacturer's instructions at a final concentration of 10 nM. After 72 hr, cells were homogenized in TRIzol and RNA isolated for RT-PCR.

mRNA-Seq

Data from these mRNA-seq experiments are available at the GEO at the NIH under accession number GEO: GSE115532.

Vertebrate Animals

Mouse models used in this study conform to standards of care and ethical treatment as determined by the Washington University Institutional Animal Care and Use Committee. The investigators sought and received approval for animal protocols in this study.

Statistical Methods

Statistical considerations are given for a parallel study of two groups, for pairwise comparison of two conditions, genotypes, or treatments. For comparison of two groups in a parallel design, performed in triplicate, we have a 90% likelihood of detecting a significant difference ($p < 0.05$) between groups if the true difference is 2.95 times the SD. This power calculation uses the non-central t-function distribution. Pairwise comparisons use Student's t test, either one- or two-tailed, depending on the initial hypothesis. Assays were performed three times for each experiment, in triplicate or quadruplicate. Data are represented as the mean, with error bars depicting the SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.06.009.

AUTHOR CONTRIBUTIONS

B.B.M. designed all DNA constructs, which were generated with assistance from T.P. A.M.S. and D.A.V.P. performed organoid cultures, including maintenance, colony-forming assays, and other manipulations. B.B.M. generated PLAGL2-overexpressing mouse enteroids, *Plagl2* knockout enteroids, TOP-tdT enteroids, and LIN28B-overexpressing human organoids, with assistance from A.M.S. and D.A.V.P. A.M.S. generated PLAGL2-overexpressing human organoids. B.B.M. performed all experiments in Figures 1 and 6, with assistance from L.D. for Let-7 reporter construction. B.B.M. prepared RNA for mRNA-seq and performed GSEA and all analysis of TCGA data. T.Z. generated PLAGL2-null DLD1 cell lines. P.O.B. and J.A.J.F. performed confocal microscopy analysis of TOP-tdT-expressing organoids, with assistance from D.A.V.P.

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