SUPPLEMENTAL INFORMATION

MATERIALS AND METHODS

EXPERIMENTAL MODELS AND SUBJECT DETAILS

hTERT immortalization

The pBABE-hygro-hTERT plasmid (Addgene #1773) was a gift from Dr. Sidong Huang (McGill University). Phoenix Ampho cells were transfected and used to harvest retrovirus. Primary GCT lines were transduced with hTERT retrovirus, and selected with 100 μg/ml hygromycin for 4 days.

CRISPR/Cas9 guide and ssODN sequences

H3F3A G34W sgRNA: 5'-TTCTTCACCCATCCAGTAG-3'

H3F3A WT ssODN: 5'-
ACCCAGGAAGCAACTGGCTACAAAAGCCGCTCGCAAGAGTGCGCCATCTACTGGAG
GGGTGAAGAAACCTCATCGTTACAGGTATTAAAAAACAGGAAAAAAA-3'

Re-introduction of H3.3G34W in edited GCT cell lines

Cells were infected and stably transduced with recombinant lentiviruses produced using pCDH-EF1a-MCS-Puro expression vector containing histone H3.3G34W and H3.3WT transgenes as described previously (1).

H3.3G34W Immunoblotting

Total histones were extracted using the EpiQuick Total Histone Extraction Kit (Epigentek). 1-3 μg of total histones were prepared according to NuPAGE Bis-Tris Mini Gel Protocol (Thermo Fisher Scientific) and separated on NuPAGE Bis-Tris Gel. Wet gel electrotransfer to a PVDF membrane (GE Healthcare) was performed. Blots were blocked for 2 hours in Tris buffered saline (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4) (0.1% TBS-T) containing 5% skim milk and incubated overnight at 4°C in primary antibody solutions in 2% BSA with anti-histone H3 antibody (1:1000 dilution, Abcam 1791) or H3.3G34W-specific antibody (1:500 dilution, RevMAb 31-1145-00) (2). Blots were washed in 0.1% TBS-T and incubated for 1 hour in 0.1%

TBS-T containing 5% skim milk supplemented with ECL anti-rabbit IgG-conjugated HRP (1:5000 dilution; GE Healthcare). Membranes were washed in 0.1% TBS-T and the signal was resolved with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and imaged on ChemiDoc MP Imaging System (Bio-Rad).

Myogenic Differentiation - Immunofluorescence

For immunofluorescence, cells were fixed with 4% paraformaldehyde and 15% sucrose in PBS solution for 20 minutes at 4°C, permeabilized with 0.1% Triton X-100 for 3 mins on ice, followed by incubation in 5% goat serum blocking buffer for 1h at room temperature. Cells were incubated o/n at 4°C with primary antibodies anti-α-smooth muscle actin (1:200, Abcam ab5694) and anti-calponin 1 (1:200, Abcam ab46794) in 1% BSA/PBS solution. Incubation with secondary anti-rabbit Alexa Fluor 488 or 594 antibodies (ThermoFisher Scientific) was performed at 1:1000 dilution in PBS under light protection for 90 minutes. ProLong Diamond Antifade Mountant with blue DNA DAPI stain (ThermoFisher Scientific) was applied and fluorescent signal captured the following day.

Histone post-translational modification quantification with nLC/MS

Briefly, cell pellets (~1×10^6 cells) were lysed on ice in nuclear isolation buffer supplemented with 0.3% NP-40 alternative. Isolated nuclei were incubated with 0.4 N H2SO4 for 3h at 4 °C with agitation. 100% trichloroacetic acid (w/v) was added to the acid extract to a final concentration of 20% and samples were incubated on ice overnight to precipitate histones. The resulting histone pellets were rinsed with ice cold acetone + 0.1% HCl and then with ice cold acetone before resuspension in water and protein estimation by Bradford assay.

Approximately 20µg of histone extract was then resuspended in 100 mM ammonium bicarbonate and derivatized with propionic anhydride. 1µg of trypsin was added and samples were incubated overnight at 37 °C. After tryptic digestion, a cocktail of isotopically labeled synthetic histone peptides was spiked in at a final concentration of 250 fmol/µg and propionic anhydride derivatization was performed for second time.

The resulting histone peptides were desalted using C18 Stage Tips, dried using a centrifugal evaporator, and reconstituted using 0.1% formic acid in preparation for nanoLC-MS analysis.nanoLC was performed using a Thermo ScientificTM Easy nLCTM 1000 equipped with a 75µm × 20 cm in-house packed column using Reprosil-Pur C18-AQ (3µm; Dr. MaischGmbH, Germany). Buffer A was 0.1% formic acid and Buffer B was 0.1% formic acid in 80% acetonitrile. Peptides were resolved using a two-step linear gradient from 5 to 33% B over 45 min, then from 33 to 90% B over 10 min at a flow rate of 300nL/ min. The HPLC was coupled online to an Orbitrap Elite mass spectrometer operating in the positive mode using a Nanospray FlexTM Ion Source (Thermo Scientific) at 2.3 kV. Two full MS scans (m/z 300–1100) were acquired in the orbitrap mass analyzer with a resolution of 120,000 (at 200 m/z) every 8 DIA MS/MS events using isolation windows of 50 m/z each (e.g., 300–350, 350–400, ...,650–700).

MS/MS spectra were acquired in the ion trap operating in normal mode. Fragmentation was performed using collision-induced dissociation (CID) in the ion trap mass analyzer with a normalized collision energy of 35. AGC target and maximum injection time were 10e6 and 50ms for the full MS scan, and 10e4 and 150ms for the MS/MS can, respectively. Raw files were analyzed using EpiProfile.

Secreted proteome - Golgi Apparatus purification and Mass Spectrometry

We performed mass spectrometry on the Golgi apparatus of GCT cells instead of tissue culture supernatant because highly abundant proteins in serum-containing media (i.e. fetal bovine serum) can significantly confound detection of less-abundant secreted factors in the supernatant, while culturing mesenchymal cells in serum-free media can reduce the total amount of protein that cells secrete (3,4). GCT cells were lysed in 2 ml of homogenization buffer (250 mM sucrose, 10 mM Tris-HCl pH 7.4) using a 7 ml Dounce stainless tissue grinder (Wheaton). For each replicate, optimal number of strokes (between 3-10) was determined to obtain ~80% cell lysis. 4 ml of 2M sucrose solution was added to lysates, mixed and transferred into ultra-clear SW 41 centrifugation tubes (Beckman). On top of the sucrose layer containing lysates, 3.5 ml of 1.2M sucrose and 2 ml of 0.8M sucrose were overlaid. Samples were centrifuged at 25400 rpm (110,000g) for 120 minutes at 4^0C. The enriched Golgi membranes fractions located at the 0.8M to 1.2M sucrose

interface were collected. Protein concentration was determined using Bradford assay, and Golgi membranes were stored at -80^{0}C.

Samples were reconstituted in 200 mM HEPES buffer at pH 8.2 with 10 mM TCEP [Tris(2-carboxyethyl) phosphine hydrochloride; Thermo Fisher Scientific], and vortexed for 1 h at 37°C. Chloroacetamide (Sigma-Aldrich) was added for alkylation to a final concentration of 55 mM. Samples were vortexed for another hour at 37°C. 1µg of trypsin (Promega, Madison, WI) was added, and digestion performed for 8 h at 37°C. Each TMT label from a TMT10-plex (Thermofisher Scientific) was reconstituted in 40µl of anhydrous acetonitrile. Samples were mixed with label solution and the reaction proceeded for an hour at room temperature.

Samples were desalted on a C18 stage-tip (The Nest Group, Southborough MA), dried down and solubilized in 5% ACN-0.2% formic acid (FA). Samples were loaded on a 1.5µl pre-column (Optimize Technologies, Oregon City, OR). Peptides were separated on a home-made reversed-phase column (150-µm i.d. by 200 mm) with a 230-min gradient from 10 to 30% ACN-0.2% FA and a 600-nl/min flow rate on an Easy nLC-1000 connected to an Orbitrap Fusion (Thermo Fisher Scientific, San Jose, CA) equipped with the FAIMS Pro interface. FAIMS was operated in standard resolution with default parameters. Compensation voltage values were stepped from -37V to -93V. Dispersion voltage was set at -5000V with 3Mhz frequency. Each full MS spectrum acquired at a resolution of 120,000 was followed by tandem-MS (MS-MS) spectra acquisition on the most abundant multiply charged precursor ions for a maximum of 3s. Tandem-MS experiments were performed using higher energy collision dissociation (HCD) at a collision energy of 35%. Data were processed using PEAKS X (Bioinformatics Solutions, Waterloo, ON) and Uniprot human database (20349 entries). Mass tolerances on precursor and fragment ions were 10 ppm and 0.01 Da, respectively. Fixed modification was carbamidomethyl (C) and TMT. Variable selected posttranslational modifications were oxidation (M), deamidation (NQ), phosphorylation (STY). Data were processed with Perseus 1.6.10.45.

NEXT-GENERATION SEQUENCING

RNA-seq Library Preparation and Sequencing

Total RNA was extracted from cell pellets using Aurum Total RNA Mini Kit (Bio-Rad). Library preparation was performed with ribosomal RNA depletion according to instructions from the manufacturer (Epicentre) to achieve greater coverage of mRNA and other long non-coding transcripts. Paired-end sequencing was performed on Illumina HiSeq 4000 (RRID: SCR_016386) and Illumina NovaSeq 6000 S4 (RRID: SCR_016387) platforms.

Chromatin Immunoprecipitation Library Preparation and Sequencing, and ChIP-qPCR

Cells were fixed with 1% formaldehyde (Sigma). Fixed cell preparations were washed, pelleted and stored at −80 °C. Sonication of lysed nuclei (in buffer containing 1% SDS) was performed on a BioRuptor UCD-300 for 60 cycles, 10s on, 20s off. Samples were checked for sonication efficiency using the criteria of 150–500 bp by gel electrophoresis. After sonication, chromatin was diluted to reduce SDS level to 0.1% and before ChIP reaction, 2% of sonicated Drosophila S2 cell chromatin was spiked-in the samples.

ChIP reaction for histone modifications was performed on a Diagenode SX-8G IP-Star Compact using Diagenode automated Ideal ChIP-seq Kit. 25µL Protein A beads (Invitrogen) were washed and incubated with antibodies (anti-H3K27me3 (1:40, CST 9733), (anti-H3K27ac (1:80, Diagenode C15410196)), (anti-H3K36me3 (1:100, Active Motif 61021)), (anti-H3.3 (1:66, Millipore 09–838)), (anti-H3.3G34W (1:66, RevMAb 31-1145-00)), (anti-H3K36me2 (1:50, CST 2901)), (anti-H3K9me3 (1:66, Abcam 8898)), and 2 million cells of sonicated cell lysate combined with protease inhibitors for 10 h, followed by 20 min wash cycle with provided wash buffers.

SUZ12 ChIP reaction was performed by conjugating 40µl protein A beads with anti-SUZ12 (1:150, CST 3737) antibody at 4°C for 6 h, then chromatin from 4 million cells was added to RIPA buffer, incubated at 4°C o/n, washed using buffers from Ideal ChIP-seq Kit (one wash with each buffer, corresponding to RIPA, RIPA + 500 mM NaCl, LiCl, TE), eluted from beads by incubating with Elution buffer for 30 min. at room temperature.

Reverse cross linking took place on a heat block at 65°C for 4 h. ChIP samples were then treated with 2µl RNase Cocktail at 65°C for 30 min followed by 2µl Proteinase K at 65°C for 30 min. Samples were purified with QIAGEN MiniElute PCR purification kit. In parallel, input samples (chromatin from 50,000 cells) were reverse crosslinked and DNA isolated following the same protocol.

Library preparation was carried out using Kapa HTP Illumina library preparation reagents, following manufacturer's instructions. Single end sequencing was performed on the Illumina HiSeq 2500/4000 (RRID: SCR_016383, RRID: SCR_016386) and Illumina NovaSeq 6000 S4 (RRID: SCR_016387) platforms.

H3K27ac ChIP-seq results were validated by qPCR comparing ChIP to input DNA using Bio-Rad SsoFast EvaGreen Supermix on a Roche LightCycler 96 instrument, with the following primers:

SOX9 enhancer – For: GCTTATGGTCAGGAGTTCCCTA
SOX9 enhancer – Rev: TGGTGATCAAATGACAAGTGG
BMP2 promoter – For: CTAGGAGAGCGAGGGGAGAG
BMP2 promoter – Rev: CACTCTGCCTTACTCCAGTGC
GAPDH promoter – For: TGAGCAGTCCGGTGTCACTA
GAPDH promoter – Rev: ACGACTGAGATGGGGAATTG
chr2q14 – For: CGGTTGGTGGGAGAAATAAA
chr2q14 – Rev: TCCTGCACCAAATAACTCTCG

Single-cell RNA-seq Library Preparation and Sequencing

Fresh tumours collected after surgery were dissociated in Collagenase-Dispase (Sigma-Aldrich). Red blood cells were lysed by ammonium chloride treatment for 5 min on ice. Cell viability was assessed with Trypan Blue. For samples with low viability (<60%), dissociated cells were enriched for live cells using Dead Cell Removal kit (Miltenyi Biotech). 10,000 dissociated cells per sample were loaded on the 10X Genomics Chromium controller. The Chromium Single Cell 3′ (10X Genomics, Version 3) protocol was strictly followed to prepare libraries. The 10X libraries were sequenced on Illumina HiSeq 4000 (RRID: SCR_016386) sequencing platform and Illumina NovaSeq 6000 S4 platforms (RRID: SCR_016387).

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of ChIP-seq data

Sample selection

We profiled G34W and edited lines derived from Im-GCT-4072 and Im-GCT-3504 by bulk ChIP-seq. We focused primarily on Im-GCT-4072 (H3.3, H3.3G34W, H3K27ac, H3K27me3, H3K36me2, H3K36me3, H3K9me3, SUZ12) because of its tumor forming ability *in vivo* and the high quality of ChIP-seq experiments obtained using this model (**Table S1)**. Im-GCT-3504 (H3.3, H3K27ac, H3K36me3, H3K27me3), which did not have tumor forming ability *in vivo*, was used to confirm findings where possible.

Read processing and alignment

The ChIP-seq pipeline from C3G's GenPipes toolset (RRID: SCR_016376) (5) (v3.1.0) was used to process ChIP-seq data. Duplicate reads and reads mapping to random, mitochondrial or sex chromosomes, or low-mappability regions in ENCODE's blacklist (6) were discarded. We used the "bamCoverage" functionality of deeptools (RRID: SCR_016366) (v3.1.3) to produce RPKM-normalized BigWig tracks and visualized them using the Integrative Genomic Viewer (IGV; RRID:SCR_011793) (7). Alignment quality control summaries can be found in **Table S1**.

H3.3 peak enrichment analysis

Peak calls for H3.3 were performed using the "callpeak" functionality of MACS2 (RRID: SCR_013291) (8) (v2.1.2) using IP samples as treatment and input samples as control. Only significant peaks (adjusted $P<0.05$) were considered. Differential peaks were called with DiffBind (RRID: SCR_012918) (v2.5.1) in DESeq2 mode ("method"=DBA_DESeq2), with size factors corresponding to unit-normalized library depth (i.e. total # of non-duplicate mapped reads), no tagwise estimation of dispersion under small sample conditions ("bTagWise"=FALSE), and ignoring duplicate reads ("bRemoveDuplicates"=TRUE).

To identify H3.3-occupied loci recapitulating the *in cis* patterns of G34W observed by histone mass spectrometry (i.e. loss of H3K36me3 and gain of H3K27me3), we first produced consensus H3.3 peaks. We then characterized changes in H3K36me3 and H3K27me3 at these peaks by calculating differential enrichment using DiffBind (RRID: SCR_012918) in DESeq2 mode with size factors corresponding to the unit-normalized library depth of the respective marks.

Comparison of H3.3 and H3.3G34W deposition patterns

We characterized co-localization of H3.3 and H3.3G34W in parental lines of Im-GCT-4072 at three levels of resolution: (1) at gene TSS and TES, (2) at H3.3 peaks, and (3) genome-wide (10kb bins). First, we partitioned genes into four quartiles of expression and computed H3.3 and H3.3G34W abundance (in RPKM) in the ±5kb window around the TSS and TES in each quartile using the "computeMatrix reference-point" functionality of deeptools (RRID: SCR_016366) (v3.1.3). The average abundance of H3.3 and H3.3G34W at each quartile were plotted using "plotProfile" functionality of deeptools. The same procedure was used to characterize H3.3G34W deposition in the ±10kb window around H3.3 peaks partitioned into four quartiles of H3.3 abundance. Finally, we assessed similarity between H3.3 and H3.3G34W deposition genome-wide over 10kb genomic bins.

Data visualization

Genome-wide changes in deposition patterns were assessed using principal component analysis (PCA) over RPKM-normalized read counts for all marks except H3.3, where DiffBind-normalized (RRID:SCR_012918) read counts consensus peaks were used instead.

Analysis of bulk RNA-seq data

Sample selection

We profiled G34W and edited lines derived from the three isogenic models: Im-GCT-4072, Im-GCT-6176 and Im-GCT-3054.

Read processing and alignment

Trimmomatic (RRID: SCR_011848) (9) (v0.32) was used to remove adaptor sequences as well as the first four nucleotides of each read. Up to three additional nucleotides were clipped from the start or end of each read if found to be of low quality (phred33<30). Reads were truncated when the average quality (phred33) of a 4-nucleotide sliding window fell below 30. Short reads (<30bp) were discarded. The remaining read sets were aligned to the reference genome build hg19 (GRCh37) using the default parameters of STAR (RRID: SCR_015899) (10) (v2.3.0e). Primary alignments of reads mapping to 9 or fewer locations in the genome were retained, and the rest was discarded. Multiple quality control metrics were obtained using FASTQC (RRID: SCR_014583) (v0.11.2), samtools (RRID: SCR_002105) (11) (v0.1.19), BEDtools (RRID: SCR_006646) (12) (v2.17.0) and custom scripts. Alignment quality control summaries can be found in **Table S1**. Bigwig tracks from BAM files were generated and normalized to library depth using BEDtools (12) (v2.17.0) and UCSC tools and visualized using IGV (RRID: SCR_011793) (7).

Gene expression analysis

We quantified gene expression by counting the number of uniquely mapping reads (reads with MAPQ>3 that were also flagged as primary alignments) falling into exons described in Ensembl's ensGene "exon" reference using featureCounts (RRID: SCR_012919) (13) (v1.4.4). Exonic read counts were then collapsed at the gene-level (GRCh37 n=60234 genes). Mean-of-ratios normalization and variance-stabilized transformations of gene read counts were calculated using DESeq2 (RRID: SCR_015687) (14) (v1.18.1). Principal component analysis (PCA) over normalized and variance-stabilized transformed read counts were used to assess global changes in gene expression between samples.

Differential gene expression analysis

Differential gene expression analysis was performed using DESeq2 (RRID: SCR_015687) (14) (v1.18.1). Unless otherwise stated, all reported P values have been adjusted for multiple testing

using the Benjamini-Hochberg procedure. Genes with (1) sufficient coverage (baseMean>50), (2) LFC exceeding ±1 (corresponding to a 2-fold change), and (3) $P<0.05$, were considered as significantly differentially expressed. All reported *P* values have been adjusted for multiple testing using the Benjamini-Hochberg procedure. Boxplots illustrating changes in gene expression between G34W and edited lines report them as median-of-ratios-normalized read counts.

Pathway enrichment analysis

Pathway enrichment analysis was performed on significantly differentially expressed genes between G34W and edited lines using the "gost" functionality of gprofiler2, an R package that implements g:Profiler (RRID: SCR_01819) (15) (v0.1.8). To avoid redundant pathways and control for multiple testing, we restricted terms to those provided by Gene Ontology (GO; RRID: SCR_002811) and adjusted *P* values for multiple testing using the default g:SCS (Set Counts and Sizes). The analysis was repeated separately for all significantly expressed genes, upregulated genes, downregulated genes, and those with consistent epigenetic changes (concurrent upregulation, H3K36me3 gain and H3K27me3 loss; or concurrent downregulation, H3K36me3 loss and H3K27me3 gain).

Analysis of single-cell RNA-seq data

Read processing, alignment and counting

Cell Ranger was used to demultiplex and align sequencing reads to the reference human genome hg19 v1.2.0 and to calculate gene expression counts over the Ensembl ensGene annotation using CellRanger's count function (v3.0.2). In the case of xenograft snRNA-seq samples, samples were aligned to a combined human and mouse reference and intronic counts were included (16). Barcodes with more than 5% of reads mapping to the mouse genome were removed from downstream analysis. Alignment quality control summaries can be found in **Table S1.**

Sample inclusion criteria

Only high-quality samples were used for downstream analysis. Quality was determined using summaries generated by the 10X alignment pipeline. The patient tumor P-6027 and mouse xenograft M103 were excluded on the basis of "Low Fraction Reads in Cells" (< 70%), indicative of high levels of ambient RNA.

Clustering and visualization

Linear dimensionality reduction was performed using principal component analysis (PCA) in Seurat. Statistically significant principle components (PCs) were selected using elbow and jackstraw plots. Cell clusters were identified using Seurat's shared-nearest neighbor algorithm following modularity optimization using the Louvain algorithm with multilevel refinement. The clusters were visualized using embeddings determined by the uniform manifold approximation and projection (UMAP) algorithm (17). Clustering analysis was performed with and without regressing cell cycle scores (18) to confirm that cell types identified were not driven solely by cell cycle signal. Only results without regression were reported for brevity.

Identification of cell types

Stromal cells were classified based on canonical marker expression (**Fig. S5C-D**), high average expression of G34W DEGs from bulk isogenic RNA-seq samples (**Fig. 5A**), enriched detection of G34W mutation (**Fig. S5E**), and enrichment for pathways similar to isogenic G34W stromal cells (**Fig. S5F**). Osteoclasts were confidently assigned based on canonical marker gene expression (**Fig. S7B**), increased UMI count per cell (due to multinucleated giant cells), and enrichment for pathways such as: "osteoclast differentiation", "bone resorption", "bone remodelling" (**Fig. S7C**).

Identification of the* H3F3A *G34W mutation

To identify stromal cells based on detection of the *H3F3A* G34W mutation (G->T substitution at chr1:226,252,155), we employed a method similar to a previously described pipeline for variant calling in 10X data (19). Briefly, the CellRanger-produced BAM file for each GCT sample was individually run through the following steps using GATK v4.0.1.2 (20). Duplicate reads were identified and marked using MarkDuplicates (parameters

VALIDATION_STRINGENCY=SILENT). Reads were then reformatted using SplitNCigarReads. SNPs were called using HaplotypeCaller (parameters ip=100, dont-trim-active-regions=true, dont-use-soft-clipped-bases=true, standard-min-confidence-threshold-for-calling=20.0) and SelectVariants (select-type=SNP) and filtered using VariantFiltration (FS>30.0, QD<2.0, MQ<40.0, ReadPosRankSum<-8.0). To identify barcodes with mutant reads, we used cb_sniffer, as previously described (19). Since detection depends on gene expression, we defined a normalized frequency of G34W in a given cell type as the ratio of the number of cells with ≥1 reads supporting the mutation over the total number of cells where ≥1 reads were observed for *H3F3A*. We failed to detect G34W in cells from P-5071, confirmed as G34W positive by other methods.

Cell-to-cell interaction analysis

To predict cell-to-cell interactions between stromal cells and myeloid cells in the GCT tumor microenvironment, we used a method similar to that of Ximerakis et al. 2019 (21). Briefly, a curated database of ligand-receptor interactions (https://baderlab.org/CellCellInteractions) was accessed using the R package CCInx v0.4 (https://github.com/BaderLab/CCInx). The scaled expression level of each ligand or receptor was calculated using the function BuildGeneStatList in the R package scClustViz (v1.2.8) (22). To determine which interactions were specific to each pair of cell types, the gene sets for each cluster were filtered to retain only cell-type specific markers, as previously determined. The interactions were visualized as a bipartite graph where nodes represent the receptor or ligand, and edges represent the predicted interaction. Node colour represents the magnitude of gene expression in the given cell type, and edge colour represents the sum of the scaled gene expression magnitude.

Analysis of Golgi secretome mass spectrometry data

Data processing

We derived *n*=4 technical replicates for mutant lines and *n*=4 for edited lines from Im-GCT-4072 (**Table S1**). The abundances of proteins enriched in the Golgi Apparatus of isogenic lines were

quantified as intensities using the PEAKS proteomics software program. The intensities were then normalized to the median intensities of the TMT ion reporters to adjust for the amount of material.

Protein enrichment analysis

Principal component analysis (PCA) over $\log_2(1+x)$-normalized intensities were used to assess global changes in protein abundances in the isogenic context. Differential enrichment analysis of proteins was performed using an unpaired two-tailed *t*-test on the $\log_2(1+x)$-normalized intensities of proteins. *P* values were adjusted for multiple-testing using the Benjamini-Hochberg procedure.

Identification of secreted factors by stromal cells

We obtained candidate secreted factors upregulated by G34W by considering, (i) markers specific to the neoplastic stromal clusters (in single cell transcriptomics), and (ii) whose corresponding proteins were significantly enriched in the secretome (by mass spectrometry). We focused on proteins upregulated in G34W rather than downregulated ones because there were no non-neoplastic stromal cells in single cell transcriptomic data against which to reliably compare the neoplastic stromal cells.

Model Figures

Schematic and model figures (**Fig. 6D, 7A-B**) were created with the aid of BioRender software.

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