# Horizontal Transfer of Histone H3 by Mammalian Cells

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**ABSTRACT**

Histones are small, highly basic, nuclear proteins that serve as structural elements to condense DNA into chromatin and regulate its accessibility[1]. Although histones released from dying cells can act as extracellular signaling molecules, all intracellular histone molecules are assumed to have originated from the cell in which they reside[2-6]. Here we show that histone H3 is horizontally transferred between viable cells. Using an ER-targeted biotin ligase to detect secreted proteins[7,8], we serendipitously discovered that histone H3 is selectively secreted by autophagic cells relative to histones H2A, H2B, and H4. Specific H3 posttranslational modifications are enriched or depleted on secreted H3 relative to intracellular H3, suggesting that specific PTMs dictate H3's ability to be secreted or the information it conveys to neighboring cells. Remarkably, we found that secreted H3 can enter the nuclei of recipient cells in an autophagy-independent and cell contact-independent manner ex vivo and in vivo. These findings have implications for cell-cell communication during nutrient deprivation, hypoxia, and perhaps other forms of cellular stress, and for the ability to deliver macromolecules across cell membranes.

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**MAIN TEXT**

In hopes of identifying new secreted biomarkers controlled by the *VHL* tumor suppressor gene, we introduced the BirA biotin ligase fused to an ER localization signal (ER-BirA[7,8]), or the corresponding empty expression vector (EV), into 786-O human *VHL-/-* clear cell renal carcinoma cells (ccRCCs) that reexpress the *VHL* in the presence of Doxycycline (Dox) (Fig. 1a,b and Extended Data Fig. 1a,b). In pilot experiments we detected the secreted proteins IGFBP3, VEGF, and Clusterin in the conditioned media (CM) of the ER-BirA cells, but not the EV cells, after capture on streptavidin beads and immunoblot analysis (Fig. 1c). As expected from prior studies[9], reexpression of *VHL* suppressed IGFBP3 and VEGF, and induced Clusterin. This assay was sensitive, based on the detection of VEGF (present at <10 nM in CM[10,11]) and specific, based on the failure to detect abundant intracellular proteins such as vinculin, β-Actin, β-Tubulin, COX IV, Golgin 97, Calreticulin and Lamin A/C in the CM (Fig. 1c, d).

Surprisingly, we detected the apparent *VHL*-independent secretion of histone H3 (H3) with this assay (Fig. 1e). This was specific because histone H4 (H4), histone H2A (H2A), and histone H2B (H2B) were not detected in the CM. This was not a BirA artefact, because we likewise detected H3, but not H4, H2A, or H2B, in parental 786-O CM that was concentrated using a spin column or trichloroacetic acid (TCA) precipitation (Fig. 1f). No H3 was detected in unconditioned cell culture media (Extended Data Fig. 1c). In the experiments that follow, TCA precipitation was used to concentrate the CM unless otherwise specified. H3 secretion was confirmed using two independent H3 antibodies (Fig. 1g) and in multiple cell lines including RCC4, UMRC2, 769-P, and A498 ccRCC lines, MCF7 and MDA-MB-231 human breast carcinoma cells, U2OS human osteosarcoma cells, B16 mouse melanoma cells, and HOG human oligodendroglioma cells (Extended Data Fig. 1d-f). We also observed secretion of the oncohistone H3K27M[12,13] by SF8628 diffuse intrinsic pontine glioma cells (Extended Data Fig. 1f). The secreted H3 appeared to be monomeric, as determined by non-denaturing Native Gel Electrophoresis of spin column concentrated media (Extended Data Fig. 1g,h), and was sensitive to trypsin and proteinase K digestion, indicating that it was not encapsulated in an extracellular vesicle[14] (Extended Data Fig. 1i,j).

The specific detection of H3 without other histones (nor other abundant intracellular proteins) strongly argued against non-specific release of H3 by dying cells. Moreover, the posttranslational modifications of the secreted H3 were not representative of the total pool of H3,

with a clear bias toward histone hypermethylation and against acetylation (Fig. 1h,i). Acetylation of H3 lysine 14 (H3K14) was an exception, as it was dramatically increased on secreted H3 relative to bulk H3 (Fig. 1h). This suggests that specific PTMs govern H3 secretion or its downstream consequences. In support of this idea, mutating H3 lysine 14 to arginine blocked secretion, whereas mutating it to glutamine to mimic lysine acetylation enhanced secretion (Fig. 1j), and mutating lysine 27 to methionine, corresponding to a canonical oncohistone mutation, increased H3 secretion (Extended Data Fig. 1k).

To ask how H3 is secreted, we treated RCC4 *VHL-/-* ccRCC cells with brefeldin A, which blocks canonical protein secretion[15], or bafilomycin A1, which blocks autophagy-dependent secretion[16,17]. Brefeldin A blocked the secretion of IGFBP3, as expected, but did not block the secretion of H3 (Fig. 2a). In contrast, bafilomycin A1 blocked the secretion of H3, but not IGFB3(Fig. 2a), as did CRISPR/Cas9-based elimination of ATG7 or Beclin 1, which are required for autophagy[17-19] (Fig. 2b-e).

 Our CM experiments above were performed in synthetic, serum-free, media to reduce media complexity. Low serum promotes autophagy[19,20]. Notably, we did not detect apoptosis after 48 hours of serum starvation (Extended Data Fig. 2a-f). Conversely, apoptosis-inducing agents did not promote the selective release of H3 (Extended Data Fig. 2g). We confirmed that readdition of serum to the culture medium suppressed H3 secretion (Fig. 2f), whereas induction of autophagy by treatment with rapamycin[21] or exposure to hypoxia[22] promoted H3 secretion (Fig. 2g-i and Extended Data Fig. 2h). Therefore, H3 undergoes autophagy-dependent secretion. Prompted by this finding, we confirmed that LC3B, which is well known to undergo autophagy-dependent secretion, was likewise captured in streptavidin pulldowns of CM from BirA-ER expressing cells (Extended data Fig. 2i) and that its secretion was likewise suppressed by bafilomycin A1, but not brefeldin A (Extended Data Fig. 2j).

To monitor the fate of secreted H3, we created 786-O cells that express H3 fused to mEmerald, a brightly fluorescent monomeric variant of green fluorescent protein, or mCherry, mixed them 1:1, grew them in serum-poor media, and performed live cell FACS. Remarkably, double-positive mEmerald/mCherry cells appeared within 48 hours (Fig. 3a, b and Extended Data Fig. 3a). This was specific, because double-positive cells were virtually absent when H2A, H2B, or H4 fusion cells, were tested in parallel (Fig. 3a, b and Extended Data Fig. 3a). Similar results were observed using a Doxycycline-inducible expression system in 786-O and 293T cells

(Extended Data Fig. 3 b-f) and when autophagy was stimulated by hypoxia rather than by growth in low serum (Extended Data Fig. 4a,b).

In a complementary set of experiments, we co-cultured 293T human embryonic kidney cells expressing either H3-mCherry or H4-mCherry with RCC4 cells expressing a membrane-bound GFP (myr-GFP). Once again, we observed double-positive cells by FACS with the fluorescent H3 fusion protein cells, but not the H4 fusion protein cells (Fig. 3c,d). Using live cell fluorescence microscopy, we observed rare green cells with red nuclear inclusions that corresponded to mitotic chromosomes after Hoechst dye staining (Fig. 3c). Importantly, uptake of H3, in contrast to secretion of H3, was unaffected by loss of ATG7 or Beclin 1 (Extended Data Fig. 5a-d).

The apparent transfer of H3 did not require contact between donor and recipient cells based on transfer experiments in which filtered donor-conditioned media was added to separately plated recipient cells (Extended Data Fig. 5a-d) and based on transwell experiments in which recipient and donor cells were separated by a 3 μm filter (Fig. 3e,f). To complement our fluorescence-based assays, we harvested the CM from 786-O cells expressing FLAG epitope-tagged H3 or H4 and added it to 786-O cells expressing BirA (Turbo-ID Variant[7]) fused to a nuclear localization signal (NLS) or the corresponding EV. 48 hours later we lysed the recipient cells, captured proteins on streptavidin beads, and did immunoblot assays. We detected Flag-H3, but not Flag-H4 in the streptavidin pulldowns from BirA-NLS extracts, but not EV extracts (Fig. 3g,h). As additional controls, we confirmed that abundant non-nuclear recipient cell proteins were not biotinylated in this assay (Fig. 3h).

In analogous experiments with SF8628 "donor" cells, which endogenously express H3K27M (see above), and 786-O cells "recipient" expressing BirA-NLS, we detected H3K27M in the streptavidin pulldowns from BirA-NLS extracts, but not EV extracts (Extended Data Fig. 5e,f), indicating endogenously produced oncohistones can be transferred to non-oncohistone producing cells.

Single-molecule, live-cell imaging of 293T cells expressing of H3 or H4 fused to HaloTag confirmed secretion of H3, but not H4, after serum-starvation (Extended Data Fig. 6a,b) The low basal H3-HaloTag and H4-HaloTag detected in the media without serum starvation by this highly sensitive technique may reflect the low basal levels of amphisomal secretion of nucleosomes reported in unstressed cells[23]. We co-cultured 293T cells expressing H3-HaloTag or

H4-HaloTag with RCC4 cells expressing H2B-mEmerald in the absence of serum. 48 hours later, 15-20% of the H2B-mEmerald RCC4 cells contained H3-HaloTag, as determined by single molecule imaging, with ~80% of the protein in the cytoplasm and ~20% in the nucleus (Supplementary Movie 1, and Extended Data Fig. 6c-g). In contrast, transfer of H4 was virtually undetectable. It is possible that uptake into the nucleus would increase upon dissolution of the nuclear membrane during mitosis.

Encouraged by these results, we cocultured 786-O cells expressing H3 or H4 fused to Cre recombinase with mouse tex.loxP.EG cells[24] that conditionally express GFP after Cre-mediated excision of a Lox-Stop-Lox cassette (Fig. 3i and Extended Data Fig. 7a). In pilot experiments we first confirmed that both the H3-Cre and H4-Cre fusions could recombine a GFP-based reporter in cotransfection experiments (Extended Data Fig. 7b-c). In contrast, only H3-Cre, but not H4-Cre, induced GFP expression in the recipient cell coculture experiments, arguing that H3, but not H4, can deliver a functional payload to the nucleus (Fig. 3j,k). Similar results, albeit with a lower recombination frequency, were obtained with a "color switch reporter[25]" in which Cre-recombination switches expression from a red fluorescent protein (RFP) to GFP (Fig. 3l-n).

Consistent with the secretion results (Fig. 1j), mutating H3K14 to arginine blocked transfer, whereas mutating H3K14 to glutamine to mimic lysine acetylation enhanced transfer of both H3-Cre and fluorescently-tagged Histone 3 (Extended Data Fig. 7 and Extended Data Fig. 8).

To ask whether horizontal transfer of H3 can take place in vivo, we mixed 786-O cells expressing H3-Cre or H4-Cre 1:1 with 786-O cells with the color switch reporter and grew them subcutaneously in nude mice (Fig. 4a). Notably, the Cre expressing cells grew more slowly than the color switch cells, presumably because targeting Cre recombinase to chromatin impairs cellular fitness. Five weeks later, we removed palpable tumors, dissociated the tumor cells, and monitored recombination by FACS and immunoblot analysis for GFP-positive cells. The H3-Cre, but not H4-Cre, cells promoted recombination of the color switch reporter (Fig. 4b-d, Extended Data Fig. 9a). Remarkably, H3-Cre-dependent recombination was also detectable when the Cre donor cells and color switch recipient cells were grown as separate tumors on opposite flanks (Fig. 4e-h, Extended Data Fig. 9b). Finally, we detected circulating H3, but not circulating H4, in non-tumor bearing mice that were fasted to promote autophagy[26,27] (Fig. 4i,j).

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Histones released by dying cells can elicit danger signals by interacting with Toll-like receptors on cell surfaces and contribute to the pathogenesis of diseases such as septic shock[2,5]. Our findings indicate that autophagy causes the specific secretion of H3 in a form that can cross cell membranes and enter the nuclei of other cells. Our preliminary data indicate that certain PTMs are enriched or depleted on secreted H3 compared to the bulk H3 population, suggesting that specific PTMs influence H3 secretion, uptake, or the information conveyed to recipient cells. H3K14 acetylation, for example, appears to promote H3 secretion. Whether the transfer of H3 from donor to recipient cells influences cell behavior, perhaps through incorporation into chromatin or by a non-canonical function, remains to be determined. Transfer of histone H3 might reflect an ancient signaling mechanism that predated the emergence of cytokines, growth factors and hormones.

Solid tumors invariably contain hypoxic cells and are therefore likely to secrete H3. It will be important to ask if circulating H3 contributes to any of the systemic manifestations of cancer, such as cachexia and hypercoagulability, and whether they can serve as diagnostic or prognostic biomarkers. Our finding that an oncohistone can cross cell membranes also suggests that certain oncohistones play paracrine roles in transformation.

It has been known since the 1960s that exogenous histones can cross cell membranes under certain experimental conditions and can enhance the transfection efficiency of DNA, although the physiological relevance of these findings was previously unclear[28-33]. Delivering macromolecules across cell membranes has been a holy grail in drug discovery, although previous protein transduction methods based on positively charged peptides and proteins such as the Antennapedia protein homeodomain (penetratin), HIV TAT, and histones, have not proven robust[34]. Our finding that the H3 secreted by autophagic cells can be used to deliver a protein cargo suggests that histone-based protein transduction can be optimized further by understanding the structure-function relationships underlying this phenomenon, including the importance of specific H3 PTMs, together perhaps with directed evolution.
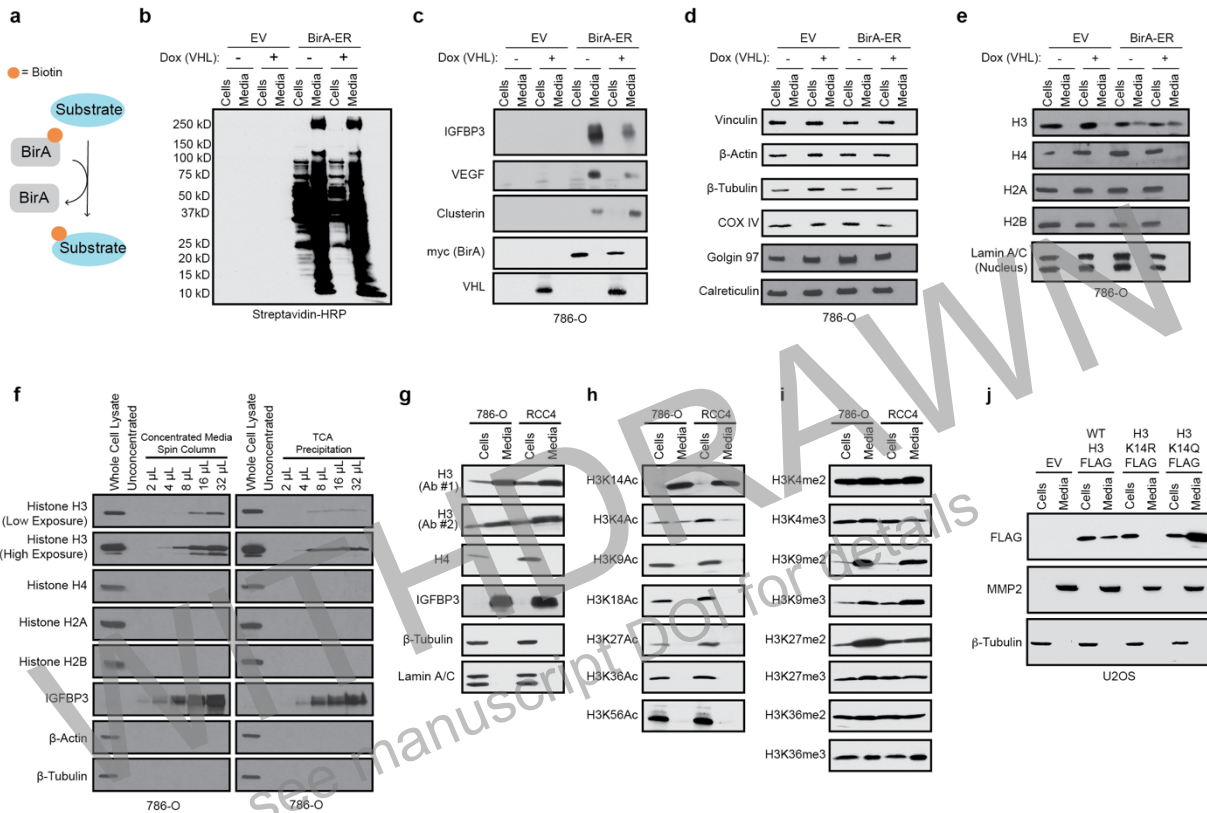
**Fig. 1. Specific Secretion of Modified Histone H3**

(**a**) Schema for protein biotinylation by BirA. (**b-e**). 786-O *VHL-/-* renal carcinoma cells expressing BirA-ER or the corresponding empty vector (EV) were grown, where indicated, in the presence of 2 μg/mL doxycycline (Dox) for 4 days to induce exogenous VHL expression prior to the addition of 50 μM biotin. Cell lysates (Cells) and secreted proteins captured on streptavidin beads (Media) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with horseradish peroxidase-conjugated streptavidin (**b**) or with the indicated antibodies (**c-e**). (**f**) Immunoblot analysis of increasing amounts of 786-O cell conditioned media after concentration with a spin-column or by TCA precipitation compared to whole cell extract and unconcentrated media. (**g-j**) Immunoblot analysis of 786-O (**g-i**), RCC4 *VHL-/-* renal carcinoma (**g-i**), and U2OS *VHL+/+* osteosarcoma cell (**j**) extracts and TCA-precipitated conditioned media. In (**j**) the cells expressed the indicated FLAG-tagged histone H3 point-mutants.
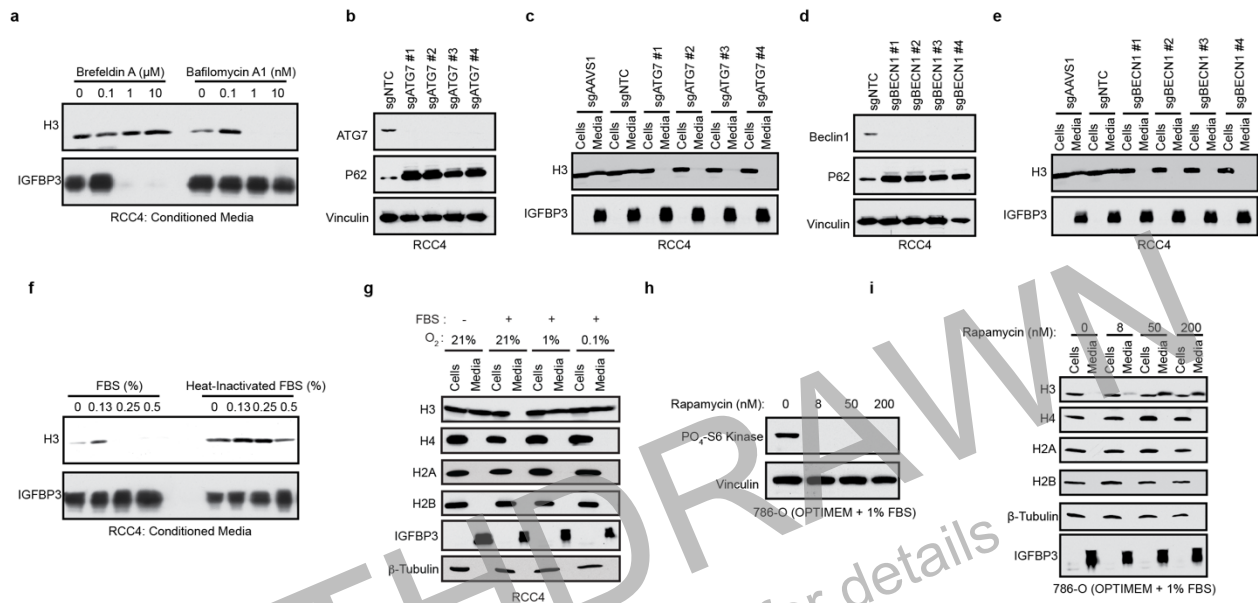
**Figure 2**



**Fig. 2. Secretion of Histone H3 is Autophagy-Dependent**

(**a**, **f** and **g**). Immunoblot analysis of RCC4 cell extract and TCA precipitated conditioned media after the indicated treatments for 24 h. (**b-e**) Immunoblot analysis of RCC4 cells after CRISPR/Cas9-based editing with the indicated sgRNAs (NTC is Nontargeting Control). Cells = whole cell extracts. Media = TCA precipitated conditioned media. (**h and i**) Immunoblot analysis of 786-O cells grown in Opti-MEM synthetic media supplemented with 1% fetal bovine serum and the indicated amount of rapamycin for 24 h, or grown under the indicated oxygen concentration. Cells = whole cell extracts. Media = TCA precipitated conditioned media.
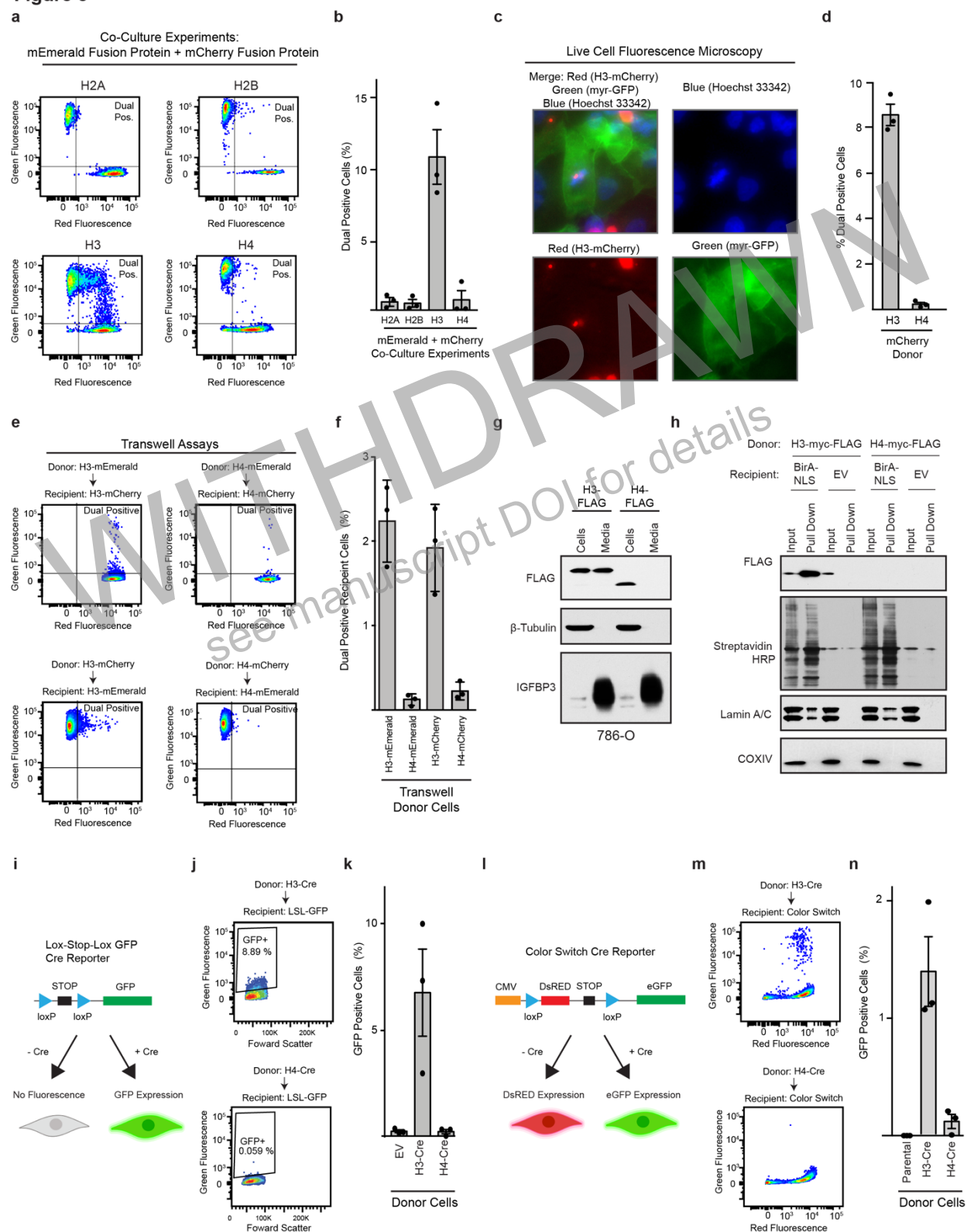
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# Figure 3

**Fig. 3. Horizontal Transfer of Histone H3 Ex Vivo**

(**a** and **b**). Raw data (**a**) and quantification (**b**) of 2 color FACS after co-culturing 786-O cells expressing the indicated histone fused to either mEmerald or mCherry (1:1 mix) in serum-free Opti-MEM for 2 days. (**c** and **d**). Representative live cell fluorescence micrographs (**c**) and quantification (**d**) after coculturing 293T embryonic kidney cells expressing either H3-mCherry or H4-mCherry with RCC4 cells expressing Myr-GFP in serum-free media for 2 days. (**e** and **f**). Raw data (**e**) and quantification (**f**) of 2 color FACS of recipient cells after growing the indicated donor and recipient 786-O cells in transwell plates in serum-free media for 2 days. (**g**) Immunoblot analysis of cell extract and TCA precipitated media from 786-O cells expressing either H3-FLAG or H4-FLAG. (**h**) 786-O cells expressing either BirA-NLS or EV were treated with conditioned media from 786-O cells expressing either H3-FLAG or H4-FLAG for 2 days. Cell lysates before (input) and after (pull down) capture on streptavidin beads were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with horseradish peroxidase-conjugated streptavidin or with the indicated antibodies (**i**). Schematic for Cre recombinase reporter in Lox-Stop-Lox (L-S-L) GFP mouse T cells. (**j-k**) Raw data (**j**) and quantification (**k**) after coculture of H3-Cre or H4-Cre 786-O cells with L-S-L-GFP cells (**l**). Schematic for Color Switch Cre recombinase reporter in 786-O cells. (**m-n**) Raw data (**m**) and quantification (**n**) after coculture of H3-Cre or H4-Cre 786-O cells with Color Switch reporter cells. For b,d,f,k,and n, data are mean ± s.e.m. from n = 3 biological replicates.
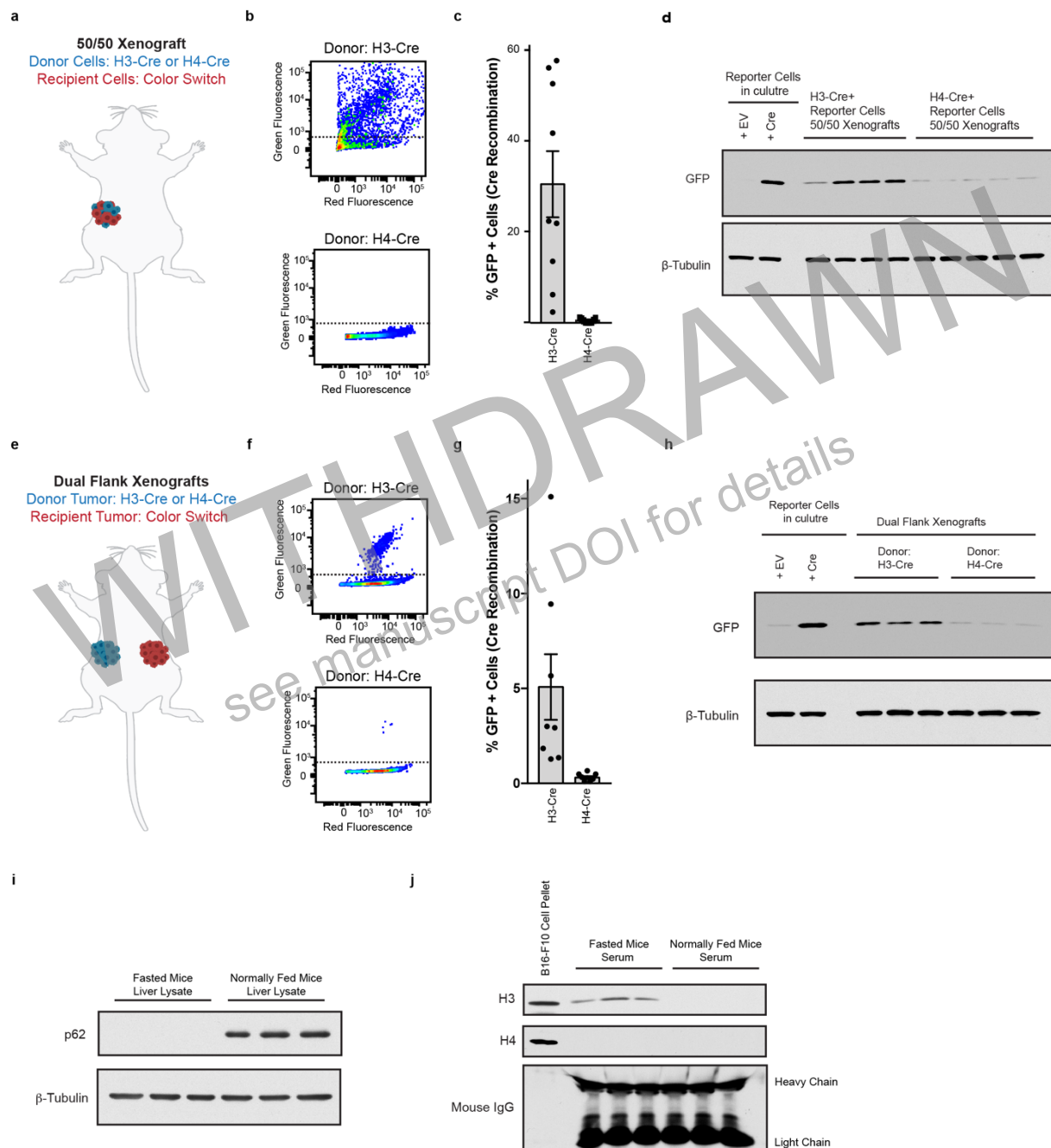
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**Fig 4. Horizontal Transfer of Histone H3 In Vivo**

(**a** and **e**). Schemas for in vivo transfer experiments in tumor-bearing nude mice**.** Raw data (**b**) and quantification (**c**) of 2 color FACS of dissociated tumors formed 5 weeks after implanting 786-O cells expressing either H3-Cre or H4 Cre mixed 1:1 with RCC4 color switch reporter cells. (**d**) Immunoblot analysis of tumors from (**a-c**). 786-O color switch reporter cells infected

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ex vivo with a Cre expressing lentivirus (+Cre) or the empty vector (EV) were included as controls. (**f** and **g**) Raw data (**f**) and quantification (**g**) of 2 color FACS of tumors formed 786-O color switch reporter cells 5 weeks after implanting them on one flank and 786-O cells expressing either H3-Cre or H4 Cre on the opposite flank. (**h**) Immunoblot analysis of tumors from (**e-g**). 786-O color switch reporter cells infected ex vivo with a Cre expressing lentivirus (+Cre) or the empty vector (EV) were included as controls. (**i-j**) Immunoblot analysis of liver lysates (**i**) and serum (**j**) from c57/Bl6 mice that were or were not fasted for 24 h. Each lane contains the same from a different mouse. For **c** and **g,** each dot represents an independent tumor, bars are mean ± s.e.m.

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## Acknowledgments

## Author contributions

P.L.S. designed and performed experiments and contributed to all aspects of the study. H.E.N. performed experiments cloning and developing BirA*-G3-ER system in 786-O cells. M.Z.L. performed experiments and contributed to data analysis and compiling of the manuscript. N.P. provided reagents and introduced the BirA system to the Kaelin Laboratory. Z.L. designed, performed, and analyzed the Single-molecule imaging studies, and contributed to compiling of the manuscript. W.G.K. conceptualized and supervised the study and interpreted the data. W.G.K., and P.L.S. wrote the manuscript.

## Competing interests

W.G.K., and P.L.S. are inventors on the U.S. patent application submitted by and assigned to Dana-Farber Cancer Institute, which covers Recombinant Histone Polypeptides and Uses Thereof. W.G.K. has financial interests in Circle Pharma, LifeMine Therapeutics, Lilly Pharmaceuticals, Fibrogen, Cedilla Therapeutics, Nextech Invest, Tango Therapeutics.

## Data Availability

All data generated or analysed during this study are included in this published article (and its supplementary information files) There are no restrictions on data availability. All unique biological materials will be available. Please contact W.G.K., the corresponding author.

## Code Availability Statement

There was no custom code used in this study.

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