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Characterizing primary and secondary senescence *in vivo*

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Abstract

There is robust evidence that senescence can be propagated *in vitro* through mechanisms including the senescence-associated secretory phenotype, resulting in the non-cell-autonomous induction of secondary senescence. However, the induction, regulation and physiological role of secondary senescence *in vivo* remains largely unclear. Here, we generated senescence-inducible mouse models expressing either the constitutively active form of MEK1 or MKK6 and mCherry, to map primary and secondary senescent cells. Our models recapitulate characteristic features of senescence and demonstrate that primary and secondary phenotypes are highly tissue- and inducer-dependent. Spatially-resolved RNA expression analyses at the single-cell level reveal that each senescence induction results in a unique transcriptional profile — even within cells of the same cell type — explaining the heterogeneity of senescent cells *in vivo*. Furthermore, we show that interleukin 1B, primarily derived from macrophages, induces secondary phenotypes. Our findings provide insight into secondary senescence *in vivo* and useful tools for understanding and manipulating senescence during aging.

Introduction

Cellular senescence — characterized by features such as stable cell-cycle arrest with accumulation of cyclin-dependent kinase inhibitors (CDKs) including *Cdkn2a* (p16) and *Cdkn1a* (p21), an increased senescence-associated β -galactosidase (SA- β -gal) activity and DNA damage responses¹⁻³ — is known to occur in response to various inducers, including oncogene activation, DNA damage and replication stresses⁴. Interestingly, although senescence acts as a tumor suppressor mechanism by preventing the propagation of damaged cells⁵ and is involved in aging-associated diseases, the senescence-like response has also been observed during embryogenesis^{6,7}, suggesting a broad functional role of *in vivo* cellular senescence.

Senescent cells secrete a mixture of factors known as senescence-associated secretory phenotype (SASP), primarily regulated by nuclear factor κ b (NF κ b) signaling^{8,9}. These factors act in both a cell-autonomous, autocrine manner to enhance senescence¹⁰ as well as a non-cell autonomous, paracrine manner to induce the senescence of surrounding cells, also known as secondary senescence^{11,12}. SASP has also been linked to various other biological functions including enhanced cellular plasticity, increased stemness¹³, acceleration of tissue repair modulation¹⁴, wound healing¹⁵ and precancerous cells surveillance by immune cells¹⁶.

Nevertheless, only a small fraction of cells exhibits characteristics of senescence in aged organs *in vivo*¹⁷⁻¹⁹. Furthermore, as no universal marker for senescent cells is currently available²⁰, it has been challenging to properly identify and characterize them *in vivo*. Although, the landmark study by Baker et al.¹⁸, using an elegant transgenic mouse model to eliminate senescent cells expressing p16^{Ink4a} from aged mice, showed amelioration of aging-associated disorders and even promotes longevity, as p16^{Ink4a} is not expressed in all senescent cells and not always necessary for the induction of senescence, the overall effects of senescence *in vivo* have yet to be fully clarified. Thus, elucidating the relationship between senescence and aging-related physiological and pathological features has been difficult²¹. Additionally, considering that *in vivo* secondary senescence has been observed in histological datasets²², this has further complicated the distinction of primary and secondary senescent cells in tissue microenvironments. Therefore, improved methods for the characterization of senescence *in vivo* are necessary to establish

a comprehensive understanding of senescence and to develop better therapeutic strategies for aging-related diseases.

Here, we have established genetically tractable systems for inducing oncogene-induced senescence (OIS) and stress-induced senescence (SIS) using mouse models that can be regulated by ERK and p38 MAPK pathway activation, respectively. Taking advantage of double-colored chimeric mice and single-cell RNA expression analyses, we reveal distinct transcriptional features of primary and secondary senescence *in vivo*. Moreover, senescence *in vivo* propagates to the surrounding hepatocytes through IL-1 β , primarily macrophage-derived and Notch signaling in primary senescent cells. Notably, the artificially induced senescence signatures resembled natural aging features in humans and mice. These results show that our mouse models carry significant implications for the physiological role of cellular senescence in governing individual aging and hold promise as effective systems for understanding and intervening with *in vivo* senescent cells during the organismal aging process.

Results

Senescence-inducible mouse models expressing caMEK1 or caMKK6

The MEK/ERK and MKK6/p38 signaling pathways have been described to have roles in OIS and SIS *in vitro*, respectively^{5,9,11}. To determine whether these signaling pathways *in vivo* have a similar role, we generated inducible mouse lines expressing the constitutive active form of MEK1 (caMEK1; for ERK MAPK activation) or MKK6 (caMKK6; for p38 MAPK activation) under the control of doxycycline (Dox), followed by an *IRES-mCherry* to visualize the expression with mCherry signals (Fig. 1a). ERK and p38 MAPK activation as well as mCherry expression were confirmed in mouse embryonic fibroblasts (MEFs) isolated from these mouse lines after Dox treatment (Extended Data Fig. 1a, b). Notably, p38 was not activated in caMEK1 while ERK was not activated in caMKK6 ten days after Dox treatment (Extended Data Fig. 1b), demonstrating that the two senescence-inducing pathways are mutually exclusive, thereby allowing us to analyze each pathway independently. We observed cell growth arrest, increased SA- β -gal activity, upregulated expression of *Cdkn2a* and *Cdkn1a*, and induction of several SASP factors, all of which are hallmarks of senescence, in both caMEK1 and caMKK6 (Extended Data Fig. 1c-f). Collectively, these findings confirmed that Dox treatment successfully induces senescence *in vitro* in both caMEK1 and caMMK6 MEFs, consistent with previous reports^{9,11}.

caMEK1 or caMKK6 induces senescence features *in vivo*

Considering that the systemic induction of either caMEK1 or caMKK6 resulted in early lethal phenotype within several days, we used chimeric mice in which caMEK1 or caMKK6 could only be expressed in a subset of cells within tissues to investigate the primary and secondary effects *in vivo* (Fig. 1a). We focused on the liver and colon, since mCherry expression was constantly observed in chimeric mice after Dox treatment. Immunohistochemical analyses showed that mCherry expression overlapped with the activation of direct downstream targets in both the liver and colon: p-ERK for caMEK1 and p-p38 for caMKK6. caMEK1 did not phosphorylate p38 while caMKK6 did not phosphorylate ERK in tissue sections at 7 days after Dox treatment (Fig. 1b, c and Extended Data Fig. 2a, b). Notably, the p21/*Cdkn1a* expression was increased in both mCherry-positive cells and mCherry-negative cells, indicating that artificial induction of

senescence can result in surrounding cells to express p21/*Cdkn1a* (Fig. 1d, e and Extended Data Fig. 2c-g). Dox administration had no effect on ERK and p38 activation as well as p21 induction (Extended Data Fig. 2h-j). BrdU-positive colonic epithelial cells were significantly reduced in mCherry-positive cells expressing p21 and, to a lesser extent, in mCherry-negative cells expressing p21 (Fig. 1f and Extended Data Fig. 2k). Similarly, the number of Ki67-positive hepatocytes significantly decreased in both mCherry-positive and -negative hepatocytes (Extended Data Fig. 2l). In the colon, significant increase in DNA damage response marker γ H2AX was observed in mCherry-positive cells expressing p21 and to a slightly lesser extent, in mCherry-negative cells expressing p21 (Fig. 1g). In the liver, however, increase in γ H2AX-positive cells were found broadly throughout the tissue but were notably absent in hepatocytes (Extended Data Fig. 2m), suggesting that the susceptibility to DNA damage response may vary depending on the cell type. Increased SA- β -gal activity (Fig. 1h) and nuclear NF κ B staining (Extended Data Fig. 3a, b) were also observed in both caMEK1 and caMKK6. Additionally, the expression of *Cdkn2a*, *Cdkn1a* and several SASP factors were also upregulated (Extended Data Fig. 3c).

We generated tissue-specific senescence-inducible mice by introducing a *loxP-stop-loxP* (LSL) -*rtTA3* allele²³ and a tissue-specific *Cre* allele into the caMEK1 and caMKK6. Combining the *LSL-rtTA3* allele with hydrodynamic tail vein injection of the *Cre* plasmid and the *Villin-Cre* allele, we induced caMEK1 or caMKK6 specifically in hepatocytes and intestinal epithelial cells, respectively. We detected phenotypes similar to chimeric mice even when senescence inducers were expressed only in specific cell types (Extended Data Fig. 3d-j). Although *Cdkn2a* mRNA was upregulated, we were unable to detect the expression of p16 protein, a representative senescence marker, using immunostaining, which was presumably due to low expression levels of p16 protein. Altogether, we concluded that caMEK1 and caMKK6 sufficiently induced senescence features *in vivo*.

Senescent mosaic mice show heterogeneous transcriptome

By combining our mosaic mouse models with multi-color labelling, we next investigated the effects of primary and secondary senescence on the global transcriptome. mCherry-linked senescence-inducible embryonic stem cells (ESCs) were injected into GFP-

labelled blastocysts to generate chimeric mice (Fig. 2a). We sorted mCherry-positive and GFP-positive hepatocytes and colonic epithelial cells (Extended Data Fig. 4a) and performed RNA-seq analyses. Global transcriptional changes upon primary senescence significantly differed across cell types (Fig. 2b) and senescent inducers (Fig. 2c, Extended Data Fig. 4b and Supplementary Table 1). Notably, global transcriptional profiles in GFP-positive cells were significantly altered depending on cell types and inducers as well as mCherry-positive cells (Fig. 2b, c and Extended Data Fig. 4b). Although GFP-positive cells were supposed to consist of a mixture of secondary senescent and non-senescent cells, these results were also largely supported by our subsequent single-cell analyses of GFP-positive *Cdkn1a*-high cells (refer to the following sections for details). Consistent with a previous *in vitro* study that showed primary and secondary senescence displaying distinct molecular features²⁴, mCherry- and GFP-positive cells had different transcriptional responses. A subset of altered genes overlapped between the mCherry and GFP-positive responsive genes, indicating a partial sharing of the regulatory mechanisms (Fig. 2d). Next, we focused on secreted factors (Supplementary Table 1)²⁵⁻²⁷. Many secreted genes were upregulated in not only mCherry-positive cells but also GFP-positive cells (Fig. 2e), suggesting potentially that SASP factors can alter the secretory profiles in GFP-positive cells. The transcriptional profiles of secreted genes varied depending on cell types and inducers.

Gene Ontology (GO) analyses revealed that the upregulated genes in mCherry-positive hepatocytes are mostly associated with senescence processes, but gene clusters induced in each pathway were quite different, in that the genes involved in acute phase response²⁸ and response to cytokine were induced for caMEK1, whereas cellular response to hypoxia and heme metabolic process, both related to oxidative stress, were induced for caMKK6 (Extended Data Fig. 4c and Supplementary Table 2). In GFP-positive hepatocytes, genes associated with acute phase responses (*Orm1*, *Orm2*, *Saa1* and *Saa2*), immune system processes¹⁶ and blood coagulation²⁹ (*Fga*, *Fgb* and *Fgg*) were enriched in the upregulated genes in both caMEK1 and caMKK6. Notably, genes associated with xenobiotic metabolic process, metabolism processes, including lipid, amino acid and glucose homeostasis and bile acid biosynthesis, all of which are key functions in the liver, were significantly downregulated in both mCherry- and GFP-positive hepatocytes in both caMEK1 and caMKK6. This indicates that liver function may be impaired in not only

primary senescent cells but also in surrounding cells. Similarly, GO analyses in the colonic epithelial cells suggested that the different inducers activate different signaling pathways in primary senescent cells, with the inflammatory response and I-kappa B kinase/NF-kappa B signaling for caMEK1 and transcriptional response to oxidative stress for caMKK6.

Thus, transcriptomic alterations in primary senescent cells and surrounding cells are highly heterogeneous depending on cell types and inducing signaling.

Upregulation of uPAR and Serpine1 during senescence *in vivo*

To investigate a common gene sets in senescent cells, we compared upregulated genes in mCherry-positive cells across conditions and identified 17 genes (Extended Data Fig. 4d). Additionally, 6 of 17 genes (35.3%) were also reported to exhibit increased expression during senescence in a previous report³⁰ (Extended Data Fig. 4e). Among them, plasminogen activator urokinase receptor (*Plaur*), encoding the urokinase-type plasminogen activator receptor (uPAR), was previously identified as the target of senolytic CAR T cells³⁰. We confirmed the increased expression of uPAR by immunostaining, and one of the ligands of uPAR, plasminogen activator inhibitor-1 (*Serpine1*)³¹ was also upregulated (Extended Data Fig. 4f, g), suggesting *Serpine1*-uPAR axis is linked to the reinforcement of cell-autonomous senescence. However, when secondary senescence *in vivo* and senescence *in vitro* (MEF) were included, no common genes were found, suggesting that senescence *in vivo* is highly diverse and no general marker exists. In fact, RNA *in situ* analysis revealed high levels of *Plaur* expression in primary cells, but this was less frequent in mCherry-negative cells, even in those with high expression of *Cdkn1a* (Extended Data Fig. 4h).

Senescence marker heterogeneity at single cell level

We next examined the simultaneous expression of multiple genes, in addition to *Cdkn1a*, on the same liver tissue sections. We selected *Serpine1* and *Cd9* as candidate markers because their expression levels were increased in caMEK1- and caMKK6-mCherry and GFP positive cells in the liver (Fig. 2e and Extended Data Fig. 4g, i). *Plaur*, *Serpine1*, and *Cd9* are also part of the recently identified set of 119 senescence genes, “SenMayo”³². *Serpine1* is a well-known SASP factor, a marker and mediator of senescence³¹, while *Cd9*

is upregulated in senescent endothelial cells and atherosclerotic plaques and has been utilized as a cell surface biomarker for therapeutic target³³.

RNA *in situ* analysis reappeared *Serpine1* and *Cd9* expression levels were increased in both *mCherry*-positive and -negative hepatocytes in both caMEK1 and caMKK6 (Fig. 2f, g). Interestingly, although nearly all *mCherry*-positive cells expressed at least one of these marker genes (caMEK1, 87.9%; caMKK6, 94.1%), their expression patterns varied among individual *Cdkn1a*-high cells (Fig. 2h). Furthermore, although the percentage was lower than in *mCherry*-positive cells, the majority of *mCherry*-negative cells were positive for one of these markers (caMEK1, 79.6%; caMKK6, 43.0%), suggesting that senescent cells might induce secondary senescence state in subset of the surrounding cells.

These results indicate that primary and secondary senescent cells were heterogeneous at single-cell level, highlighting the complexity of fully characterizing senescence cells.

Single-cell RNA sequencing of caMEK1 and caMKK6 hepatocytes

To further dissect this heterogeneity of the global landscape among individual cells, we performed single-cell RNA-sequencing (scRNA-seq) using hepatocytes isolated from control, caMEK1 or caMKK6 chimeric mice (Extended Data Fig. 5a).

After stringent filtering, we obtained 7,678 cells in total for further analyses (Supplementary Table 3). Clustering analysis combined with marker gene expression patterns identified non-parenchymal cells (NPCs, 155 cells (2.02% in total)), including liver sinusoidal endothelial cells (LSECs), tissue-resident macrophage Kupffer cells and hepatic stellate cells (HSCs) (Fig. 2i, Cluster 7 and Extended Data Fig. 5b). Hepatocytes from the control, caMEK1 or caMKK6 mice displayed distinct expression profiles, while NPCs from all conditions were within the same cluster, indicating that caMEK1 and caMKK6 induced transcriptional changes dynamically in hepatocytes (Fig. 2i and Extended Data Fig. 5c).

Next, we explored detailed transcriptional changes in hepatocytes. As expected, the control *mCherry* and GFP cells were in the same cluster (Fig. 2i, Cluster 4), suggesting that this cluster can be considered to represent control hepatocytes. We observed upregulation of *Cdkn1a* expression in almost all *mCherry*-positive cells derived from

caMEK1 or caMKK6. Additionally, the GFP-positive cell population comprised high and low *Cdkn1a*-expressed cells, which is consistent with the notion that they represent a mixture of senescent and non-senescent cells (Fig. 2j). Overall transcriptional characteristics of mCherry-positive cells and GFP-positive *Cdkn1a*-high cells reproduced the results of our bulk RNA-seq analysis (Supplementary Table 4-2, 3), and representative SASP genes were also found to be upregulated in caMEK1 and caMKK6 (Fig. 2e, j and Extended Data Fig. 4g, i).

Effects of primary senescence on liver zonation

Hepatic functions are non-uniformly distributed along the lobule radial axis, termed “liver zonation”³⁴ and recent single-cell analyses on mouse have verified that hepatocytes have diverse transcriptional signatures according to the tissue zonation, which is critical for its metabolic functions³⁵. To evaluate the effects of senescence on hepatocytes in different locations within the liver, we attempted to define cells according to the marker expression of well-known regions in our scRNA-seq data. Control cells could be classified into three subclusters, periportal (PP), pericentral (PC) and mid-zone (Mid) based on the recognized zonation markers *Cyp2f2*, *Cyp2e1* and *Hamp*, respectively (Fig. 3a, Extended Data Fig. 5d, e and Supplementary Table 4). Moreover, KEGG pathway analysis showed that genes related to complement and coagulation cascades were enriched in the PP cluster, whereas genes related to cytochrome P450 and bile acid biosynthesis were enriched in the PC cluster (Extended Data Fig. 5f and Supplementary Table 5). Altogether, consistent with the previous results³⁵, our scRNA-seq data could successfully classify hepatocytes by liver zonation.

Next, we investigated caMEK1 mCherry cells, which could be divided into five potential subclusters (Fig. 3a). We noticed that both *Cyp2f2* and *Cyp2e1* expressions were downregulated, and PP/PC scores also declined, relative to those in the control samples (Fig. 3a and 3b, see Methods). Other PC marker genes also displayed significant decrease compared to PP marker genes across all subclusters, and the overall expression profiles tended to shift towards the PP profile (Fig. 3a), indicating a zonal diminishment (Extended Data Fig. 5e). A similar phenotype was previously reported in Ha-ras hyperactivated tumours³⁵ showing an increase in gene expression associated with PP features. We further validated the zonal diminishment phenotype in a hepatocyte-specific

system with an adeno-associated viral vector (AAV8-*CAG-Cre*) together with hybridization-based *in situ* sequencing (HybISS), a spatially-resolved single-cell RNA analysis technique³⁶. Indeed, caMEK1 induction resulted in loss of liver zonation in comparison to the control condition (No Dox) (Fig. 3c and Extended Data Fig. 6a-d).

In contrast, caMKK6 mCherry cells could be classified into subpopulations mainly based on their zonation marker expressions. PP markers were upregulated in subcluster 0, while PC markers were upregulated in subclusters 1 and 2 (Fig. 3a, Extended Data Fig. 5e and Supplementary Table 4). Although zonation itself was preserved, the expression of zonation marker genes appeared to be attenuated (Fig. 3b), confirmed by HybISS analysis (Fig. 3c and Extended Data Fig. 6d).

We also explored the mechanisms underlying the loss of hepatic zonation in caMEK1 mCherry. We found no evidence that the characteristic gene expression profile of a particular subcluster was affected by the expression levels of caMEK1 or caMKK6, expected to correlate with *mCherry* expression levels (Extended Data Fig. 5g). Previous reports have suggested that Wnt signaling is a central regulator of liver zonation that drives pericentral gene programming, while the RAS/ERK pathway induces periportal gene programming^{35,37,38}. Our scRNA-seq revealed that the gradient of Wnt target genes expression along with the zonation axis was lost in caMEK1 mCherry but maintained in caMKK6 mCherry (Extended Data Fig. 6e). These results suggest that the ERK signaling pathway is closely linked to the Wnt pathway, whereas p38 signaling has limited effect on Wnt pathway.

Thus, liver zonation, which is responsible for hepatic functions, was compromised in primary senescent cells, and caMEK1 had a more significant impact on liver zonation than caMKK6.

Heterogeneity in caMEK1-induced primary senescent cells

Next, we aimed to identify DEGs in caMEK1 mCherry to understand effects beyond zonation defects. We identified subcluster-specific genes and found that the uniquely upregulated genes in each subcluster effectively characterized each population (Fig. 4a, b, Extended Data Fig. 7a and Supplementary Table 4). Alpha-fetoprotein (*Afp*), a marker for embryonic hepatic stem cells, was highly expressed in subcluster 0. *Afp*-positive cells also expressed higher levels of other hepatic stem cell markers, such as *Gpc3* and *Igf2*³⁹

(Extended Data Fig. 7b) and immunostaining confirmed AFP expression in caMEK1 liver (Extended Data Fig. 7c). These results suggest that a subpopulation of caMEK1 mCherry gains stem cell-like features, consistent with recent studies showing that SASP promotes a pro-regenerative response through the induction of cell plasticity and stemness^{13,40-42}. In subcluster 1, TGF- β signaling (*Serpine1* and *Zfp3612*), known to regulate SASP involved in paracrine senescence^{11,22}, was enriched (Fig. 4b and Extended Data Fig. 7d). The expression of *Serpine1* was also confirmed with RNA *in situ* hybridization (Fig. 2f and Extended Data Fig. 7e). In subcluster 2, reactive oxygen species (ROS) signaling and inflammatory response-associated genes (*Cd14* and *Il1rn*, TNF signaling; *Ifngr1*, interferon- γ signaling) were upregulated (Fig. 4b, Extended Data Fig. 7f and Supplementary Tables 4, 5). Interestingly, in subcluster 2, the expression of the cell cycle-related genes, *Cdkn2b* (p15) and *Ccnd1* also increased, suggesting underlying additional growth arrest mechanisms. Acute phase response genes were enriched in subcluster 3 and secreted genes related to blood coagulation and complement activation were enriched in subcluster 4 (Fig. 4b). These results indicate that caMEK1 causes a distinct senescence phenotype in each subpopulation.

Using HybISS, we investigated the spatial expression of subcluster-specific genes in AAV8-*Cre*; caMEK1 (*Afp* and *Cxcl14*, CL0; *Serpine1* and *Angptl8*, CL1; *Cd14*, *Il1rn* and *Nrg4*, CL2; *Tff3* and *Saa2*, CL3), which is challenging to analyze using conventional scRNA-seq. Interestingly, these genes were found to be expressed independently of zonation, suggesting that subpopulations of caMEK1-senescence profiles were induced stochastically or regulated by microenvironmental factors distinct from hepatic zonation (Fig. 4c). Collectively, the caMEK1-induced primary senescence displays a heterogeneous phenotype even within the same tissue at the single-cell level.

caMKK6-induced primary senescence within liver zones

We also identified DEGs in subcluster of caMKK6 mCherry (Fig. 4d and Supplementary Table 4). Among secreted factors, while many secreted genes were globally upregulated, some genes were subcluster-specific and highly zone dependent. *Hsd17b13* expression was especially upregulated in PP (CL0), while *Serpina7*, *Igfbp1* and *Serpine1* expressions were upregulated in PC (CL2) (Fig. 4e and Extended Data Fig. 7e, g). HybISS analysis in AAV8-*Cre*; caMKK6 nicely recapitulated the spatial

expression of subcluster-specific zonation-related secreted genes (*Hsd17b13*, CL0 (PP); *Serpina7*, *Igfbp1* and *Serpine1*, CL2 (PC)) (Fig. 4f). Interestingly, subcluster 3 was enriched for genes associated with acute phase response (*Saa2*, *Saa1*, *Lcn2* and *Serpina3n*), immune system process and blood coagulation, similar to the pattern of changes observed in GFP-positive cells, suggesting that cells in the subcluster were affected by SASP in an autocrine or a paracrine manner and gained secondary phenotype. The GO analysis revealed that lipid metabolic process was enriched in subcluster 0. *Hsd17b13*, a lipid droplet-associated protein, has been previously reported to be strongly associated with nonalcoholic fatty liver disease (NAFLD) in both mice and humans⁴³. On the other hand, subcluster 2 was enriched with response to oxidative stress. Thus, in sharp contrast to caMEK1-induced senescence, caMKK6-induced primary senescence demonstrates distinct responses depending on liver zonation.

Effects of cell-cell interactions on secondary senescence

The GFP-positive cells derived from both caMEK1 and caMKK6 could be further divided into subpopulations based on their zonation marker expressions (Fig. 5a, Extended Data Fig. 5e, and Supplementary Table 4). The expression of zonation marker genes appeared to be attenuated, although the degree of attenuation was less than that of mCherry-positive cells (Fig. 3b). HybISS analyses in AAV8-*Cre*; caMEK1 and AAV8-*Cre*; caMKK6 mCherry-negative cells confirmed the zonal patterns of gene expression (Fig. 3c and Extended Data Fig. 6d). HybISS also demonstrated that secondary senescence upregulated genes (*Orml1*, *Saa1*, and *Saa2*) were highly expressed in mCherry-negative *Cdkn1a*-positive cells in both AAV8-*Cre*; caMEK1 and AAV8-*Cre*; caMKK6 (Fig. 5b).

Next, to distinguish secondary senescent cells more precisely in scRNA-seq, we extracted *Cdkn1a*-high expressing cell population (*Cdkn1a*-high cells, see Methods) for further analysis (Fig. 5c, left). GO analyses in caMEK1 and caMKK6 GFP *Cdkn1a*-high cells revealed that acute phase response, immune system process and blood coagulation were upregulated in both conditions, consistent with our bulk RNA-seq data (Supplementary Table 5). We also confirmed the expression pattern of *Serpine1* and *Cd9* in GFP *Cdkn1a*-high cells, which is consistent with the results from RNA *in situ* analysis (Fig. 2h and Extended Data Fig. 7h).

Next, we examined the zonation-dependent differences in caMEK1 GFP

Cdkn1a-high cells and identified some subcluster-specific genes (Fig. 5c, upper middle and Supplementary Table 4). In subcluster 1 (PP), among secreted genes, those related to the immune system (*Fga*, *Fgb*, *Fgg*, and *Serpina10*), complement and coagulation (*C3*, *Cfh*, and *Cfi*), and acute phase response (*Serpina3n*, *Orm2*, *Il1rn*, and *Fnl1*), that were all also related to PP function, were upregulated. In subcluster 3 (PC), the expression of *Serpine1* increased, and genes related to the cellular response to reactive oxygen species were enriched (Fig. 5c, lower middle and Supplementary Table 5), suggesting that the heterogeneity of caMEK1-induced secondary senescence was shaped by the cellular properties of the receiving cell, which is dependent on zonation.

We also identified subcluster-specific genes in caMKK6 GFP *Cdkn1a*-high cells (Fig. 5c, upper right and Supplementary Table 4). Interestingly, some genes upregulated in subcluster 2 (PC) were linked to PP-related genes encoding complement and coagulation factors (*Fga*, *Fgg*, *Fgb*, *Crp*, *Fnl1*, and *C4bp*) (Fig. 5c, lower right and Supplementary Table 5). caMKK6 GFP hepatocytes in PC seemed to be more affected than those in PP, suggesting that the caMKK6 mCherry-induced SASP factors in PC, including *Serpine1*, altered the secretions of nearby secondary cells in a paracrine manner.

Altogether, these results suggest that secondary senescence is also heterogeneous and controlled by both SASP factors from primary cells and the inherent properties of the exposed cells.

To investigate the potential signaling network involved in secondary senescent cells, we first explored the link to Notch signaling, which has been described as mediating not only primary senescence but also the lateral induction of secondary senescence through a juxtacrine mechanism by upregulating the NOTCH ligand JAG1^{24,44}. Previous reports have shown that NRAS-driven senescent hepatocytes are accompanied by Notch activity, induced *Hes1* and p21 expression in neighboring hepatocytes; mediated lateral induction of Notch signaling and senescence⁴⁴. In our bulk RNA-seq data, *Hes1*, a Notch target gene, was elevated in both caMEK1- and caMKK6-mCherry and caMEK1- and caMKK6-GFP positive cells (Extended Data Fig. 8a). Focusing on Notch ligands, *Jag1* was elevated in caMEK1 mCherry and GFP whereas *Dll1* was elevated in caMKK6 mCherry and GFP (Extended Data Fig. 8a). Our scRNA-seq analysis further revealed higher *Hes1* expression in GFP *Cdkn1a*-high cells compared to GFP *Cdkn1a*-low cells in both models (Extended Data Fig. 8b).

We also examined *Cdkn1a* and *Hes1* expression on tissue sections, distinguishing between *mCherry*-negative proximate cells (close to *mCherry*-positive cells) and remote cells (distant from *mCherry*-positive cells) (Extended Data Fig. 8c). Notably, *Cdkn1a* expression was higher in proximate cells than in remote cells in both caMEK1 and caMKK6 (Extended Data Fig. 8d). Furthermore, *Hes1* was increased in *Cdkn1a*-high proximate cells in both models. These results suggest that Notch signaling may contribute to the induction of secondary senescence in both caMEK1 and caMKK6.

IL1 signaling regulates secondary senescence in the liver

Regardless of zonation, there was a common set of genes expressed in all GFP-positive *Cdkn1a*-high cells induced by caMEK1 and caMKK6 (Fig. 5c, upper middle and right, and Supplementary Table 4). As the juxacrine mechanisms are limited to the immediate surrounding cells, to investigate the broader paracrine signaling network acting in secondary senescent cells, we performed upstream regulator analysis implemented in Ingenuity Pathway Analysis (IPA) software for shared genes among caMEK1 GFP and caMKK6 GFP *Cdkn1a*-high cells. The analyses identified IL6 and IL1B as candidate upstream regulators in both caMEK1- and caMKK6-induced secondary senescent cells (Fig. 6a and Supplementary Table 6). *Il1r1* was significantly upregulated in *Cdkn1a*-high cells compared to *Cdkn1a*-low cells, among GFP-positive cells from the scRNA-seq analysis (Fig. 6b), as well as within the *mCherry*-negative population from the RNA *in situ* analysis (Fig. 6c). Our bulk RNA-seq data also shown that IL6 and IL1B were candidate upstream regulators in caMEK1 GFP and caMKK6 GFP cells and *Il1r1* was upregulated in GFP-positive cells (Extended Data Fig. 8e, f and Supplementary Table 6). Consistent with previous *in vitro* findings^{10,45}, these results raised the possibility that IL1 signaling is an upstream regulator of secondary senescence *in vivo*.

IL-1 contributes to secondary senescence *in vivo*

Next, we aimed to directly assess the physiological roles of IL-1 signaling in secondary senescence *in vivo*. Kupffer cells are known as the main source of IL1B, and recent studies have revealed that *Il1b*-expressing Kupffer cells increased in aged mice¹⁹. In fact, our scRNA-seq data showed that *Il1b* expression almost overlapped with *Clec4f*, a Kupffer cell marker gene, while it was hardly expressed in hepatocytes. (Extended Data Fig. 8g,

h). Moreover, *in situ* RNA expression analyses confirmed that *Il1b* expression significantly increased in *Clec4f*-positive Kupffer cells (Fig. 6d). *Il1b* expression was also slightly detected in some *Clec4f*-negative NPCs, but was rarely observed in hepatocytes.

Then, we employed hepatocyte-specific models (Fig. 6e) and confirmed the expression patterns of *Cdkn1a*, *Il1r1* and *Il1b*, which were consistent with those observed in the caMEK1 and caMKK6 chimeric mice (Fig. 6f, g and Extended Data Fig. 9a-c). Next, to determine whether macrophages are the source of IL1B, we administered clodronate liposomes, which deplete systemic macrophages (Fig. 6e). *Il1b* expression was significantly downregulated, suggesting that macrophages were the primary source of IL1B (Extended Data Fig. 9b). Furthermore, Anakinra, a clinical available recombinant human IL1R1 antagonist⁴⁶, as well as clodronate liposome reduced *Cdkn1a* expression in primary and secondary senescent cells (Fig. 6e-g) and tended to decrease SA- β -gal activity (Extended Data Fig. 9d). These findings demonstrate that IL1 signaling, mediated by IL1B primarily produced by macrophages, contributes to the paracrine induction of secondary senescence.

Senescence *in vivo* shapes aging-associated transcriptome traits

To further explore the relevance of caMEK1 and caMKK6 mouse models to physiological and pathological features in the natural aging process, we compared caMEK1 and caMKK6 mCherry transcriptomic data with recently reported senescence-associated signatures. Genes upregulated and downregulated in the caMEK1 mCherry positive hepatocytes and colonic epithelial cells significantly correlated with those in NRAS-induced OIS in the liver³⁰ and BRAF-induced OIS in the intestine⁴⁷, respectively (Fig. 7a and Extended Data Fig. 10a, b). Moreover, the caMEK1 mCherry colon signature was also closely associated with that of human organoids from sessile serrated adenoma/polyps (SSAP), together with a high frequency of BRAF mutation and enrichment in senescent cells (Extended Data Fig. 10c)⁴⁸. As previous reports have suggested that BRAF mutations cause stem cell loss⁴⁷, we found that the expression of *Lgr5* was lower in caMEK1- mCherry and GFP (Extended Data Fig. 10d). RNA *in situ* analyses also showed that the prevalence of *Lgr5*-positive intestinal stem cells decreased in caMEK1 (Extended Data Fig. 10e). These results strongly suggest that caMEK1 mouse

model closely resembles the previous RAS/RAF mutated OIS model and human SSAP at the transcriptional level.

When we compared caMKK6 mCherry with aged individuals using Tabula Muris Consortium data^{19,49,50} as well as aged mice liver data^{51,52} and human liver proteomic data⁵³, these models shared similar signatures with the natural aging process particularly in downregulated genes (Fig. 7b, c and Extended Data Fig. 10f-h). Unexpectedly, the caMEK1 mCherry signature also displayed aging features. We speculated that caMEK1 also shares commonalities with aging signatures, as oncogenes mutations increase with age even in non-cancer tissues⁵⁴. Given the critical role of zonation in liver function, we further compared scRNA-seq data of caMKK6 with recently published spatial transcriptomics data of aged mice liver⁵¹. Transcriptional changes in both PP and PC were similar between caMKK6 mCherry and aged hepatocytes, particularly among downregulated genes (Fig. 7d). GO analysis of spatially specific downregulated genes suggested that key zonation-dependent liver functions were impaired. Genes associated with the cholesterol metabolism, fatty acid beta-oxidation and glucose metabolism were downregulated in PP, whereas genes involved in bile acid biosynthesis and xenobiotic metabolism were downregulated in PC (Fig. 7e and Supplementary Table 5). Furthermore, FXR/RXR activation, involved in the metabolism of lipid and bile acid, as well as xenobiotic metabolism⁵⁵ tended to be impaired in both of our models, but also in the Tabula Muris Consortium data^{19,50} and human aging data extracted from the GTEx portal⁵⁶ (Fig. 7f). This suggests that the decline in liver function typically observed during physiological aging was also observable in caMEK1 and caMKK6. We also compared with aging- and senescence-related disease models. The chronic liver damage model, in which fibrosis was induced by CCl₄⁵⁷, displayed transcriptional similarities with both caMEK1 and caMKK6 (Fig. 7g). In contrast, when compared with a diet-induced NAFLD model⁵⁸, caMKK6 exhibited shared transcriptional signatures, whereas caMEK1 did not (Fig. 7h), consistent with the established association between MKK6/p38 pathway and NAFLD^{59,60}. Furthermore, altered expression of aged human plasma proteome⁶¹ was also similarly observed at the transcriptional level in both caMEK1 and caMKK6 (Fig. 7i, j, Extended Data Fig. 10i and Supplementary Table 7). Furthermore, mTORC1-p38MAPK-p53 pathway drives intestinal aging, leading to a decrease in stem cells and an increase in enteroendocrine cells in the intestine^{62,63}. The

expression of *Lgr5* (Extended Data Fig. 10d) and the prevalence of *Lgr5*-positive intestinal stem cells (Extended Data Fig. 10e) decreased in caMKK6. Meanwhile, the proportion of ChgA-positive enteroendocrine cells increased in caMKK6 (Extended Data Fig. 10j), thereby recapturing the aged mouse intestine phenotypes. Together, our results suggest that caMKK6 recapitulate characteristic features of the senescence in natural aging process.

Discussion

In this study, we successfully generated an *in vivo* senescence-inducible system, by expressing either caMEK1 or caMKK6 together with double-color labelling, to trace and characterize primary and secondary senescent cells in mice. Our findings demonstrated that *in vivo* senescent cells exhibit diverse transcriptome and secretome profiles and are closely associated with individual aging.

The RAS/MEK/ERK signaling pathway is known to be altered at high frequencies across many different human cancers^{64,65} and OIS triggered by RAS or RAF has been extensively studied both *in vitro* and *in vivo*^{5,10,11,16}. Notably, the caMEK1-induced senescence features *in vivo* shares transcriptomic signatures in OIS models *in vivo*. Conversely, although stress-induced p38 MAPK activation is necessary and sufficient to initiate growth arrest and SASP events *in vitro*⁹, it has remained uncertain *in vivo*. The MKK6/p38 pathway has been linked to several aspects of human aging and pathologies⁶⁶ including in aged organs such as intestine⁶², islet, liver⁶⁷, lung and brain⁶⁸ as well as aging- and senescence-related diseases⁶⁶, such as Alzheimer's disease⁶⁹, atherosclerosis⁷⁰, chronic obstructive pulmonary disease⁷¹, NAFLD⁵⁹ and inflammatory bowel disease⁷². Here we demonstrated that caMKK6 exhibit several phenotypes and transcriptomic signatures related to naturally occurring senescence and aging.

Although several studies have attempted to identify common markers for senescent cells *in vitro* using unbiased transcriptomic analysis, senescence phenotypes are highly heterogeneous, depending on the cell type and pro-senescence stimulus⁷³⁻⁷⁵, even at the single-cells level^{24,76}. Since most previous studies have focused on *in vitro* senescent cells, information about diversity of the individual cell *in vivo*⁷⁷ has been largely unclear. Our analyses have shown that primary and secondary senescent cells *in vivo* are heterogeneous cell population. Indeed, the transcriptional profiles of the senescence-induced cells in MEFs, liver and colon were substantially different from each other. Moreover, we showed that intercellular communication between distinct cell types is crucial for the induction of secondary senescence. These findings highlight the importance of *in vivo* studies to understand the dynamics and characteristics of senescence. Although a recent single-cell study elucidated cell type-specific heterogeneity in the same tissues during *in vivo* senescence⁷⁷, our analyses of hepatocytes revealed considerable cell-to-cell variation even within the same cell type. In this manner,

we demonstrated heterogeneous nature of *in vivo* senescence at multiple levels: primary/secondary senescence, triggers, cell types, cellular microenvironment in the same cell type. These findings indicate that cellular senescence *in vivo* is a highly complex phenotype and cannot be defined by a single gene expression profile or hallmark.

Recent studies have uncovered that SASP factors, IL1, TGF- β , and Notch, can induce secondary senescence through paracrine or juxtacrine mechanisms^{11,22,24,44}. While IL-1 β is known to promote secondary senescence in the OIS setting *in vitro*¹¹, its role in OIS and other types of senescence *in vivo* has been unclear. We found that IL-1 β , primarily expressed by macrophages regulates, at least in part, secondary senescence in the liver of both caMEK1 and caMKK6. Notably, IL1 signaling inhibition effectively reduces primary and secondary senescence in both models, suggesting the presence of the same regulatory mechanism regardless of the heterogeneity in senescence. The recombinant human IL1R antagonist, Anakinra, clinically available for the treatment of rheumatoid arthritis⁷⁸, may serve as a potential compound to prevent and/or treat senescence-associated diseases.

Although we established a unique and powerful tool for analyzing the senescence dynamics *in vivo*, further research is necessary. First, since we analyzed only two organs, examinations of other organs using organ-specific models will certainly provide additional valuable insights. Second, among cell types in the liver, we focused on the hepatocytes because hepatocytes were most affected by ERK and p38 activation. Nonetheless, we demonstrated that IL-1 β , which is predominantly produced by macrophages, plays a critical role in secondary senescence, and therefore, investigating further intercellular communication between different cell types will also be important. Finally, because senescent cell state changes over time⁴⁴, more detailed temporal analyses would be required to understand dynamics of *in vivo* senescence.

Decreased liver function is observed in the physiological aging process. Our bulk RNA-seq analysis revealed that crucial liver functions are impaired in the caMEK1 and caMKK6 liver. Additionally, in scRNA-seq analysis, almost all caMEK1 mCherry hepatocytes exhibited PP-like transcriptional profiles. Given that the spatial organization of hepatocytes plays a critical role in the liver, the zonal diminishment may be associated with the functional impairment of the liver. Furthermore, analyses of PP and PC hepatocytes in caMKK6 mCherry suggested zonation-dependent hepatic dysfunction.

Recent studies have reported that p38 MAPK activation causes NAFLD in mice after four weeks^{59,60}. Although caMKK6 mice did not exhibit lipid accumulation, likely due to the short duration of signaling activation, transcriptomic similarities suggest that p38-mediated transcriptional alterations may contribute to the NAFLD development. The fact that the global transcriptomic features of caMEK1 and caMKK6 resembled, at least in part, the aging signatures may underscore a shared mechanism in OIS, SIS and organismal aging. Having developed the caMEK1 and caMKK6 mouse models, it will now be possible to dissect causal aspects of senescence during the aging process *in vivo*.

Methods

Mice

All animal experiments were conducted in accordance with the Regulation on Animal Experimentation at Kyoto University and approved by the Animal Experimentation Committee of Kyoto University, under protocol 18-103 and 24-103. All mice were housed in a specific pathogen-free animal facility under a 12 h light/12 h dark cycle with a temperature of 21-25 °C and a humidity of 45-55%, with food and water provided ad libitum. *Rosa LSL-rtTA3* mice²³, *Villin-Cre* mice (JAX stock #004586)⁷⁹ and C57BL/6-Tg (CAG-EGFP) (JAX stock #003291) mice⁸⁰ have been described previously. Mouse genotyping was performed as previously described²³. Genotyping primers are shown in Supplementary Table 8.

Vector construction

pBS31 vectors for the KH2 system: The cDNA fragments with the Kozak sequence of *Xenopus MEK1SDSE* (1194 bp), human *MKK6EE* (1011 bp) and *mCherry* (717 bp) were generated using KOD-Plus-neo (TOYOBO). The primers used for cloning are shown in Supplementary Table 8. Each fragment was inserted into pCR8-GW-TOPO using Topo cloning technology (Invitrogen) and transferred into the *pBS31* vector.

pCMV-Cre vector: *HindIII-Cre-KpnI* fragments (1082 bp) were generated using KAPA HiFi HotStart ReadyMix (KAPA Biosystems). The cDNA fragments and pcDNA3.1(+) vector (Invitrogen) were digested with *HindIII* and *KpnI* restriction enzymes (New England Biolabs) and ligated using Ligation high ver.2 (TOYOBO).

Adeno-associated virus vectors: The *GFP* fragment in the pAAV-CAG-GFP vector (Addgene, #37825) was replaced with the *Cre* fragment by ligation into the *KpnI* and *EcoRI* cloning sites using Ligation high Ver.2 (Takara Bio).

Preparation of adeno-associated viruses

HEK293 cells in 15 cm dishes were transfected using PEI MAX with the pAAV2/8 plasmid (Addgene, #112864), pAAV-CAG-Cre plasmid and pHelper plasmid from the AAVpro Helper Free System kit (Takara Bio). At 48 hours post transfection, cells were collected, and AAV vector particles were concentrated using an AAVpro Purification Kit (Takara Bio). AAV titer was determined through quantitative PCR using the AAVpro

Titration Kit (Takara Bio).

Establishment of MEFs

Embryos were harvested at E13.5, and MEFs were derived from the eviscerated and decapitated embryos with 0.25% trypsin. MEFs were maintained in high glucose DMEM (Nacalai Tesque) supplemented with 2 mM L-glutamine (Nacalai Tesque), 1× NEAA (Nacalai Tesque), 100 U/mL penicillin, 100 µg/mL streptomycin (Nacalai Tesque), 10% foetal bovine serum (Gibco), and 0.11 mM mercaptoethanol.

Cell growth assay

MEFs were plated into 12-well culture plates at a density of 2×10^4 cells/well. Dox was added at a concentration of 2 µg/mL. Passages were performed every three or four days for MEFs not treated with Dox. The experiment was performed in triplicate, and each sample was measured twice. The number of cells was determined by an automatic cell counter TC10™ (Bio-Rad).

Establishment of embryonic stem cells (ESCs)

ESCs were cultured in ESC medium (Knockout DMEM (Gibco) supplemented with 2 mM L-glutamine (Nacalai Tesque), 1× nonessential amino acids (Nacalai Tesque), 100 U/ml penicillin and 100 µg/ml streptomycin (Nacalai Tesque), 15% foetal bovine serum (Gibco), 0.11 mM mercaptoethanol (Gibco) and 1,000 U/ml human leukaemia inhibitory factor (Wako)) on mitomycin-C-treated mouse embryonic fibroblasts.

To generate mice in which *MEK1SDSE*, *MKK6EE* and *mCherry* could be induced, the Flip-in recombination system was used to introduce the pBS31 vector containing the *tetO-MEK1SDSE-IRES-mCherry*, *tetO-MKK6EE-IRES-mCherry* and *tetO-mCherry-IRES-mCherry* alleles into the *Colla1* locus of KH2 ESCs ((C57BL/6J × 129S4/SvJae) F1) with a *Rosa26-M2rtTA* allele⁸¹.

Fifty micrograms of each targeting vector and 25 µg of pFlapase were mixed, purified and collected by ethanol precipitation. The pellets were dissolved in 100 µL of high-glucose DMEM (Nacalai Tesque) containing 25 mM HEPES buffer (Gibco). ESCs were dissociated with 0.25% trypsin (Nacalai Tesque) and dissolved in 500 µL of high glucose DMEM (Nacalai Tesque) containing 25 mM HEPES. A mixture of the targeting

vector and ESCs (2.0×10^7) was transferred to a Gene Pulser Cuvette (BIO-RAD), and electroporation was performed with a Gene Pulser Xcell (BIO-RAD). After electroporation, 1.0×10^7 ESCs were seeded over MEFs in a 6-cm dish (total 2 dishes) and cultured at 37 °C with 5% CO₂ in ESC medium. After 24 h of electroporation, we started the selection with 150 µg/mL hygromycin B (Roche). After one week of antibiotic selection, the surviving ESC colonies were picked and expanded to establish ESC lines.

Generation of chimeric mice

Eight-week-old ICR female mice (Japan SLC) received 7.5 IU of serotropin (ASKA Animal Health) by intraperitoneal injection. Forty-eight hours after serotropin treatment, mice were injected with 7.5 IU of gonadotropin (ASKA Pharmaceutical). These mice were then mated with ICR male mice (Japan SLC). For EGFP-positive blastocysts, eight-week-old B6D2F1 female mice (Japan SLC) and C57BL/6-Tg (CAG-EGFP) male mice were mated instead. Plug checks were performed on the following morning. Two days later, these female mice were sacrificed by cervical dislocation, and their oviducts were harvested. Two-cell-stage fertilized eggs were collected by perfusion with M2 medium (Sigma) and maintained in KSOM medium. Two days later, the blastocysts were subjected to microinjection. For this purpose, ES cells were treated with trypsin and pipetted up and down 15 times to dissociate into single cells. The ES cells and MEFs were incubated in a gelatine-coated 10-cm dish with 10 mL ESC medium for 30 min to allow only MEFs to attach onto the dish. Three mL of supernatant containing ES cells were collected. Three to five ES cells were injected into each blastocyst under an OLYMPUS IX71 microscope. In all, 20–25 injected blastocysts were transplanted into the uterus of each pseudopregnant ICR female mouse (Japan SLC). For germline transmission, the male chimeric mice were mated with C57BL/6J female mice (Japan SLC). All chimeric mice were generated by injecting male ES cells with a C57BL/6J × 129S4/SvJae F1 genetic background into blastocysts of ICR or B6D2F1 mice mated with C57BL/6-Tg (CAG-EGFP) mice.

Mouse experiments

Doxycycline (Sigma) was dissolved in drinking water at 0.2 mg/mL for caMEK1 mice and 2 mg/ml for caMKK6 and control mice; the drinking water was also supplemented

with 10 mg/mL sucrose (Nacalai Tesque). For single-cell RNA-seq of the liver and AAV8-*CAG-Cre* injection experiments, mice were additionally given intraperitoneal injection of Dox-containing PBS twice a day before sample collection to induce uniform transgene expression (0.4 mg/injection for caMEK1 and 1 mg/injection for caMKK6 and control).

Intrahepatic delivery of the plasmids via hydrodynamic tail vein injection was achieved as previously described¹⁶. For transient intrahepatic expression of *Cre* recombinase, 50 µg of *pCMV-Cre* vector was suspended in 2 mL of saline solution and injected via the tail vein within 10 sec.

AAV8 recombination was performed as previously described²². Briefly, viral particles [5×10^9 GC per mouse] of AAV8-*CAG-Cre* were injected intraperitoneally in 200 µl PBS. AAV8 exerts higher affinity to hepatocytes and transduces more than 90–95% of hepatocytes in sufficient doses²². By using a mosaic dosage of AAV8-*CAG-Cre*, we observed approximately 20-40% *mCherry*-positive hepatocytes.

BrdU (Sigma) was administered two hours before tissue harvest. Clodronate liposomes (12.5 mg/kg in PBS) (Hygieia Bioscience) or control PBS (200 µl) were injected intraperitoneally 2 days after Dox treatment. The Interleukin-1 receptor (IL-1R) antagonist Raleukin (anakinra) (MedChemExpress) was given at 25 mg/kg/daily by intrageneration injection⁴⁶.

Mouse strain, number, age and drug treatment period were as indicated in each figure. Experiments were carried out with male and female animals, except for transcriptomic analysis (RNA-seq and scRNA-seq), for which only males were used. Since we used chimeric mice to evaluate pathological data, we excluded mice with no or extremely low chimeric contribution. In AAV8-*CAG-Cre* experiments, samples with an unexpectedly extremely low percentage of *mCherry*-positive cells were excluded. In bulk RNA-seq analysis, samples with extremely low *mCherry* expression levels or high levels of *mCherry* and EGFP cross-contamination were excluded.

Paraffin-embedded specimen preparation

Dissected tissue samples were fixed in 4% PFA (Wako) overnight at room temperature. The next day, the samples were dehydrated through an ethanol series (70% ethanol for several hours, followed by 80, 90, and 100% ethanol) and Histo-Clear (Wako). After dehydration, the samples were embedded in paraffin using a spin tissue processor

(STP120, Thermo Scientific) or CT-Pro20 (NIPPON Genetics). Sections were sliced into 3-4 μm thick sections and stained with haematoxylin and eosin (Muto Pure Chemicals). Serial sections were used for immunohistochemical analyses.

Immunostaining

Samples were soaked in Histo-Clear three times for 5 min to remove paraffin and then 100% ethanol for 5 min three times to rehydrate. After washing with water for several minutes, the samples were soaked in epitope retrieval buffer (Nichirei Bioscience) and microwaved at 100 W for 10 min. The samples were then soaked in 1 $\times$ PBS for several minutes and incubated with 150 μL of primary antibody in PBS with 2% bovine serum albumin (BSA) (MP Biomedicals) at 4 $^{\circ}\text{C}$ overnight. The primary antibodies used were goat polyclonal anti-AFP (Santa Cruz Biotechnology, #sc8108, dilution 1/300), rat monoclonal anti-BrdU (Abcam, #ab6326, dilution 1/200), rabbit polyclonal anti-Chromogranin A (Abcam, #ab15160, dilution 1/800), rabbit monoclonal anti-NF- κB p65 (Cell Signaling Technology, #8242, dilution 1/800), rabbit polyclonal anti-mCherry (Abcam, #ab167453, dilution 1/500), rabbit monoclonal anti-p16 (Abcam, #ab211542, dilution 1/200), mouse monoclonal anti-p21 (BD, #556431, dilution 1/50 and Santa Cruz Biotechnology, #sc-6246, dilution 1/500), rabbit monoclonal anti-phospho-p44/42 MAPK (Erk1/2) (Cell Signaling Technology, #4376, dilution 1/400), rabbit monoclonal anti-phospho-p38 MAPK (Cell Signaling Technology, #4511, dilution 1/1,600), and goat polyclonal anti-uPAR (R&D, # AF534, dilution 1/50). The sections were incubated with HRP-conjugated secondary antibodies: Histofine Simple Stain Mouse MAX PO (M) (Nichirei Bioscience, #414321, undiluted), Histofine Simple Stain Mouse MAX PO (R) (Nichirei Bioscience, #414341, undiluted), Histofine Simple Stain Mouse MAX PO (G) (Nichirei Bioscience, #414351, undiluted), Histofine Simple Stain Mouse MAX PO (Rat) (Nichirei Bioscience, #414311, undiluted) for 30 min at room temperature, and the chromogen was developed using DAB (Nichirei Bioscience). The stained slides were counterstained with Meyer haematoxylin. After washing with water for 10 min, samples were placed in 100% ethanol three times and then placed in Histo-Clear three times. Finally, the samples were evaluated under a microscope (Olympus BX51).

Immunofluorescent staining

The same procedure as immunostaining described in manuscript was performed until epitope retrieval treatment. The samples were incubated with blocking buffer (Blocking One Histo, Nacalai Tesque) for 10 min at room temperature. Then, the samples were incubated with 150 μ L of primary antibody in 20 $\times$ Blocking One Histo (Nacalai Tesque) at 4 °C overnight. The primary antibodies used were rabbit polyclonal anti-BrdU (Abcam, #ab152095, dilution 1/200), rabbit polyclonal anti-Ki67 (Abcam, #ab15580, dilution 1/200), mouse monoclonal anti-mCherry (Abcam, #ab125096, dilution 1/200, 1/500), rat monoclonal anti-p21 (Abcam, #ab107099, dilution 1/100), and rabbit monoclonal anti-Phospho-Histone H2A.X (Cell Signaling Technology, #9718, dilution 1/100). The sections were incubated with fluorescence-labeled secondary antibodies for 30 min at room temperature: Goat anti-mouse Alexa Fluor 488 (Invitrogen, #A11001, dilution 1/500), Goat anti-rabbit Alexa Fluor 555 (Invitrogen, #A21428, dilution 1/500). If needed, quench endogenous peroxidase activity with 3% H₂O₂/MeOH (Nacalai Tesque) for 15 min at room temperature and then the sections were incubated with HRP-conjugated secondary antibodies: Histofine Simple Stain Mouse MAX PO (Rat) (Nichirei Bioscience, #414311, undiluted) for 30 min at room temperature followed by TSA puls Cyanine 5 (Akoya Bioscience, NEL745001KT, dilution 1/500) for 5 at room temperature. After washing in PBST for 5 min three times, the samples were counterstained using DAPI and evaluated with an Eclipse Ti2 microscope (Nikon).

Quantification of histological findings

To assess p21-, BrdU-, γ H2AX- and NF κ B-positive rates in the colon and p21- and Ki67-positive rate in the liver, we randomly photographed each mouse at 20 $\times$ magnification three to five times on a BX51 (Olympus) or Eclipse Ti2 microscope (Nikon) and then divided the number of positive nuclei by the number of total epithelial cell or hepatocyte nuclei. To assess Ki67-, γ H2AX- and NF κ B-positive cells in the liver and *Lgr5* and ChgA in the colon, we randomly photographed each mouse at 20 $\times$ magnification three to five times and the number of positive nuclei were counted. The results were evaluated with GraphPad Prism 8 Software (MDF).

Senescence-associated β -gal staining

SA- β -gal staining was performed with a Senescence β -Galactosidase Staining Kit

(#9860S, Cell Signaling) according to the manufacturer's protocol. Mice under anaesthesia were perfused through the vascular system with 10 mL of PBS to clear the blood. Then, perfusion was performed with 10 mL of 4% PFA to obtain adequate fixation. The dissected samples were collected and fixed with ice-cold 4% PFA for 1 h. The tissues were poured into PBS containing 15% sucrose (Nacalai Tesque) for 1 h and then placed in PBS containing 30% sucrose for 2 h at 4 °C. After fixation, the samples were embedded in Tissue-Tek O.C.T. compound (Sakura). Frozen tissues were cut serially into 10 µm thick sections and stained with the procedure described above. Counterstaining was performed using Nuclear Fast Red (Merck Millipore). Staining was performed as previously described at pH = 5.5 for mouse. We randomly photographed each mouse at 20× magnification three times on a BX51 (Olympus). SA-β-Gal tissue staining was quantified using ImageJ Software (NIH) by measuring the percentage of stained area in each section and multiplying it by its mean intensity value⁸².

RNA *in situ* hybridization

RNA-ISH was performed on paraffin-embedded tissues fixed in 4% PFA for 24 h at room temperature following the procedure described above for paraffin-embedded specimen preparation. All probes, RNAscope 2.5 HD assay-Duplex (Advanced Cell Diagnostics, 322500) and Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics, 323110) were used, and ISH was performed according to the manufacturer's protocol. After sections were pretreated with Pretreatments 1–3, probes were hybridized in the HybEZ oven for 2 h at 40 °C, and then the slides were processed for signal amplification. For the Duplex kit, Green-HRP and Fast Red-ALP were used for signal detection, and the slides were counterstained using haematoxylin. For the Multiplex kit, signals were detected by using Opal 520 (Akoya Bioscience, FP1487001KT), Opal 570 (Akoya Bioscience, FP1488001KT), Opal 650 (Akoya Bioscience, FP1496001KT) and Opal Polaris 780 (Akoya Bioscience, FP1501001KT), and the slides were counterstained using DAPI. Probes for *Cd9* (#430631-C3), *Cdkn1a* (#408551), *Cdkn2a-tv2* (#447491), *Clec4f* (#480421-C2), *Hes1* (#417701-C4), *Il1b* (#316891), *Il1r1* (#413211, #413211-C3), *mCherry* (#431201-C2), *Plaur* (#448731-C3) and *Serpine1* (#402501, 402501-C4) were purchased from Advanced Cell Diagnostics.

Quantification of ISH

To assess expression, we took 4 images at 20× magnification or 20 images at 40× magnification for each mouse on an Eclipse Ti2 microscope (Nikon). To measure the ratio of *Il1b*- and *Clec4f*-positive areas, images were processed and analyzed using ImageJ Software (NIH). To analyze the expression levels of *Cdkn1a*, *Cd9*, *Hes1*, *Il1r1*, *Serpine1*, *Plaur* and *mCherry*, images were processed using ImageJ after nucleus segmentation with Cellpose⁸³ and determining cell borders by enlarging the nucleus with Cellprofiler⁸⁴. Then, we computed the number of RNAscope puncta or intensity per cell with in-house developed Python code and then classified according to expression as follows: *mCherry*-positive, cells whose mean intensity was more than 70 (20× magnification) or 20 (40× magnification), or cells in which three or more gene spots were detected (x20 magnification) in the 8-bit *mCherry* staining image; *mCherry*-negative, non-*mCherry*-positive cells; Proximate cells (Prox), *mCherry*-negative cells having a nuclear center of gravity less than 75 pixels (20× magnification) or 120 pixels (40× magnification) from the nuclear center of gravity of *mCherry*-positive cells; Remote cells, *mCherry* negative cells other than Prox. The results were evaluated with Graphpad Prism 8 Software (MDF).

Hybridization-based *in situ* sequencing (HybISS)

Mice under anaesthesia were perfused through the vascular system with 10 mL of PBS to clear the blood. Then, perfusion was performed with 10 mL of 4% PFA to obtain adequate fixation. The dissected samples were collected and fixed with 4% PFA for 24 h at 4 °C. The tissues were poured into PBS containing 15% sucrose (Nacalai Tesque) for 2 h and then placed in PBS containing 30% sucrose for 22 h at 4 °C. After fixation, the samples were embedded in Tissue-Tek O.C.T. compound (Sakura). Embedded tissue pieces were stored at -80 °C until cryosectioning. Tissues were cryosectioned at 8 µm thickness and collected on glass slides (MAS-01, MATSUNAMI).

HybISS was performed as described previously³⁶ with slight modifications. Briefly, slides were fixed with 3% formaldehyde for 5 minutes, washed with PBS twice, permeabilized with 0.1 M HCl for 5 minutes and washed with PBS twice. After passing through 70% and 100% ethanol for dehydration, gaskets (SecureSeal Hybridization chambers, Grace Bio-Labs) were glued onto the sides enclosing the tissue. The mRNA was reverse-transcribed *in situ* to complementary DNA through the following steps:

Tissues were treated with random decamers (5 μ M, obtained from IDT) for 5 minutes at 65 °C and cooled on ice for 5 minutes. Then, tissues were incubated with *in situ* reverse transcription mix including superscript IV (20 U/ μ l, Thermo Fisher Scientific) and random decamers (5 μ M) for 10 minutes at RT and then overnight at 42 °C. After reverse transcription, tissues were fixed again with 3% formaldehyde for 40 minutes at RT, followed by degradation of the mRNA with RNaseH (0.4 U/ μ l, NEB) for 30 minutes at 37 °C, hybridization of padlock probes (PLPs) to the remaining cDNA (20 nM for each PLP in 20% formamide), and ligation of padlock probes with Tth ligase (0.5 U/ μ l, BLIRT) for 90 minutes at 45 °C. For the padlock probe design, 3 to 10 target sequences per gene were selected using the Python padlock design software package (https://github.com/Moldia/multi_padlock_design) with the following parameters: arm length, 15 (if targets were not found, 18); Tm, low 65, high 75; space between targets, 15. Every set of padlock probes for a given gene carries a unique 20 nucleotide (nt) ID sequence.

After padlock probe hybridization and ligation, the cDNA was digested with exonuclease I (0.4 U/ μ l, Thermo Fisher Scientific) for 3 hours at 37 °C. Then, rolling circle amplification (RCA) was performed with phi29 polymerase (1 U/ μ l, Monserate) overnight at 30 °C. The resulting RCA products (RCPs) were subjected to sequencing by hybridization. Each round of sequencing included bridge probe hybridization (0.2 μ M each) in 1X hybridization buffer (2XSSC, 20% formamide), detection probe hybridization (0.2 μ M each) with Hoechst staining (1 μ g/mL) in 1X hybridization buffer, imaging, and stripping with stripping solution (2XSSC, 65% formamide). The sequences of PLPs, bridge probes, and detection probes used in this study are shown in Supplementary Table 8. A set of genes for HybISS analyses was selected based on scRNA-seq data containing subcluster-specific genes and hepatocyte zonation genes (Glul, Cyp2e1, Hamp, Igfbp2, Cyp2f2 and Sds) as markers to identify the portal-central trajectory.

Imaging and sequencing by hybridization

Imaging was performed using a standard epifluorescence microscope (Nikon Ti2-E) connected to an LED light source (Lumencor SPECTRA X light engine). Images were obtained with a CMOS camera (ORCA-Flash4.0V3, Hamamatsu) with a CFI Plan

Apochromat Lambda 40x objective (1.3 NA, oil). Filter cubes for wavelength separation were as follows: Chroma 89402X (Hoechst, Cy5), Chroma 89403X (AlexaFluor750), Semrock GFP-A-Basic (AlexaFluor488), Semrock Cy3-4040C (Cy3), and Semrock CFP-2432C (Atto425).

For multiplex analyses, we assigned a unique 4 (cycle 1) or 3 (cycle 2) digit code to each gene, in which each digit corresponds to one of four different fluorophore-conjugated detection probes (1: AlexaFluor750, 2: AlexaFluor488, 3: Cy5). Every digit code was reconstituted through 4 (cycle 1) or 3 (cycle 2) rounds of hybridization and imaging *in situ*. *Eef1a1*, *mCherry*, *Saa1*, *Saa2*, *Apoe*, *Lcn2*, and *Ptpcr* were imaged separately to avoid optical crowding. Multispectral images were obtained for multiple cycles. Each image consists of multiple tiles that together cover the tissue section (15% overlap), and each field of view consists of Z-stacks of 0.8 μm steps through the entire tissue thickness. The tiles were stitched together, and the Z-stacks were merged to maximum-intensity projections (NIS-Elements). An 8-bit TIF image of each channel from each round was exported for data analyses.

HyBISS data analysis

Analyses of HyBISS data and subsequent quantification were performed with in-house developed Python code, Cellpose, and Fiji. For decoding gene spots, first, images were roughly aligned to the first round of images using Hoechst staining. Then, the images were top-hat filtered and split into multiple smaller images, hereafter referred to as tiles. For each tile in each round, composite images of the four detection probe channels were created, aligned to the first-round composite images, and split into separate channels again. Then, the tiles were stitched together to create the whole image. Gene spots were detected using the Laplacian of Gaussian filter on the first-round image, and the signal intensity of each channel at the spots was calculated in the remaining rounds.

The spot intensity was normalized by dividing the intensity by the 99th percentile value in the channel. Subsequently, spots were removed from the analysis due to low quality if the maximum intensity in the set of channels was less than 0.15 or the maximum intensity in the set of channels divided by the sum of all of the channels was less than 0.5. Spots that passed through the quality control were assigned to the gene according to their reconstituted digit code. For cell segmentation and classification, samples were stained

with anti-pan-Cadherin (Abcam, #ab16505, dilution 1/1000), anti-mCherry (Abcam, #ab125096, dilution 1/500), anti-rabbit Alexa Fluor 555 (Invitrogen, #A11034, dilution 1/500) and anti-mouse Alexa Fluor 647 (Invitrogen, #A32728, dilution 1/500) antibodies after HybISS imaging. Cells were segmented based on the cadherin images with cellpose⁸³ and then classified according to mCherry expression as follows: mCherry-positive, cells whose mean intensity was more than 75 in the 8-bit mCherry staining image; mCherry-negative, non-mCherry-positive cells; *Cdkn1a*-positive, cells in which one or more gene spots were detected; *Cdkn1a*-negative, cells with no detectable gene spots. For cell type classification by zonation, the field was manually segmented based on the *Glu1*, *Cyp2e1* and *Cyp2f2* gene spots from the HybISS data. After cell type classification, a count matrix was created that included the coordinates of each gene spot and the cell type of its parental cells and was used for graph representation. All the python code used is available upon request from the corresponding author.

Capillary-based immunodetection

Total protein was extracted using CytoBuster® Protein Extraction Reagent (Novagen) supplemented with Protease Inhibitor cocktail (Roche) and Phosphatase Inhibitor Cocktail (Nacalai Tesque, #07575-51). The protein was quantified on a Qubit (Thermo Fisher Scientific). Western blot analysis using a capillary-based technology, the Wes system (Protein Simple), was performed according to the manufacturer's instructions and using Compass for SW software (v6.1.0). The following reagents were used: 12–230 kDa Wes Separation Module (Protein Simple SM-W004A), Anti-Rabbit Detection Module (Protein Simple DM-001A), and Anti-Mouse Detection Module (Protein Simple DM-002A). The following antibodies were used: mouse monoclonal anti-p44/42 MAPK (Erk1/2) (Cell Signaling Technology, #4696, dilution 1/50), rabbit monoclonal anti-phospho-p44/42 MAPK (Erk1/2) (Cell Signaling Technology, #4376, dilution 1/50), rabbit monoclonal anti-p38 MAPK (Cell Signaling Technology, #8690, dilution 1/50), and rabbit monoclonal anti-phospho-p38 MAPK (Cell Signaling Technology, #4511, dilution 1/50).

Hepatocyte isolation

Hepatocytes were isolated by a previously described two-step collagenase perfusion

method⁸⁵, with modifications. Mice were anaesthetized, and the inferior vena cava was cannulated under aseptic conditions. The liver was perfused with EGTA solution (calcium-free Hank's balanced salt solution (HBSS) (Wako) containing 0.5 mM EGTA (Sigma) and 10 mM HEPES (Nacalai Tesque)), followed by a second perfusion with HBSS containing collagenase D (SIGMA) for bulk RNA-seq or Liberase TM Research Grade (Roche) for scRNA-seq and 10 mM HEPES. After passage through a 70 μ m cell strainer, the cells were centrifuged at 50 g for 2 minutes three times to enrich for hepatocytes. Cell viability was confirmed by trypan blue staining.

Crypt Isolation

Crypts of the colon were isolated by a previously described method⁸⁶, with modifications. The colon was dissected, cut open longitudinally and rinsed with ice-cold HBSS three times. Chimeric contribution was evaluated using mCherry signal under a fluorescence stereomicroscope (LED530-3WRF, OptoCode). For qPCR analysis of the chimeric mice, segments of the mucosa with high contributions of mCherry signal were further resected. For RNA-seq samples, the total colon without the caecum was used. The tissue was incubated for 30 min at 37 °C in isolation buffer (HBSS containing 10 mM HEPES (Nacalai Tesque), 5 mM EDTA (GIBCO) and 1 mM DTT (Sigma)) and then shaken vigorously for 30 sec to separate the crypt from the stromal tissues. After passage through a 70 μ m cell strainer, the crypt suspension was centrifuged at 50 g for 3 min. For RNA-seq samples, the supernatant was decanted, and the crypt pellet was subsequently resuspended in 0.25% trypsin/1 mM EDTA and incubated for 15 min at 37 °C. The cell suspension was centrifuged at 800 g for 5 min.

Flow cytometry

Dissociated hepatocytes and crypt cells were passed through a 40 μ m cell strainer and sorted with a FACS Aria II (BD Bioscience) machine using a 130 μ m nozzle. Dead cells were gated out by DAPI incorporation (100 ng/mL for crypt cells and 500 ng/mL for hepatocytes). To enrich for hepatocytes, we used 2 FSC-neutral density (ND) filters and 10-SSC ND filters. The gating strategies are shown in Extended Data Fig. 3b. Double-negative cells were observed in both the liver and the colon presumably due to the non-ubiquitous expression and/or silencing of mCherry and GFP. For bulk RNA-seq, cells

were sorted into RNAprotect Cell Reagent (QIAGEN) and stored at -80 °C until RNA extraction. For scRNA-seq, cells were sorted into low-glucose DMEM (Nacalai Tesque) with 10% foetal bovine serum (Gibco) and 10 mM HEPES and proceeded to the 10X pipeline. Data were analyzed using FlowJo (BD Biosciences).

RNA extraction

Total RNA was extracted using the RNeasy plus Mini or Micro Kit (QIAGEN) according to the manufacturer's protocol. RNA was quantified with a NanoDrop instrument (Thermo Scientific).

cDNA synthesis and quantitative RT-PCR

Five hundred nanograms of RNA reverse transcribed to cDNA by using the PrimeScript RT Reagent Kit (Takara Bio). Real-time qPCR was performed by using Go Taq qPCR Master Mix and CXR Reference Dye (Promega) on a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). Transcript levels were normalized to *Actb*. Experiments were performed in biological triplicates. The PCR primers are shown in Supplementary Table 8.

Library preparation for RNA sequencing

Total RNA (*in vitro* sample; 100 ng, FACS-sorted samples; 50 ng) was prepared for library construction. High-quality RNA samples (RNA Integrity Number value ≥ 8 , as determined by Bioanalyzer) were used for library construction. RNA-seq libraries were generated using the NEBNext Ultra II Directional RNA Library Prep kit for Illumina (NEB). The number of PCR cycles was minimized to avoid skewing the representation of the libraries. RNA-seq libraries were sequenced on a NextSeq 500 (Illumina) as 76-bp single-end reads.

RNA-seq data analysis

The sequenced reads were mapped to the mm10 mouse reference genome and reporter sequences (mCherry, EGFP, Xenopus MEK1SDSE and human MKK6EE) using STAR v.2. 6.0.c with the GENCODE vM25 annotation gtf file after trimming adaptor sequences and low-quality bases with cutadapt-1.12. The uniquely mapped reads were summarized

at the gene level using HTSeqcount v.0.11.2, with the GENCODE vM25 annotation file, and expression values were calculated as transcripts per million by using DESeq2 v. 1.22.2. Principal component analysis (PCA) was performed using the R software package (version 3.5.1) using the genes showing FPKM > 1 in at least one sample. Differential expression analysis was performed by DESeq2 using the Wald test. The *P* values were corrected for multiple testing using the Benjamini and Hochberg method and represented as the false discovery rate (FDR). The significantly differentially expressed genes were determined as those with an FDR less than 0.05. We manually curated the mouse secreted gene list collected from several available datasets (Supplementary Table 2)²⁵⁻²⁷.

Preparation of a single-cell RNA-seq library

For scRNA-seq, cells isolated from three mice were loaded into a 10X Genomics Chromium system. scRNA-seq libraries were prepared using Single Cell 3' Reagent Kits (v.3.1, 10X Genomics). The libraries were then sequenced on a HiSeq 4000 (Illumina) using paired-end sequencing.

scRNA-seq data analysis

scRNA-seq data were mapped against the mm10 mouse reference genome and reporter sequences (mCherry, EGFP, Xenopus MEK1SDSE and human MKK6EE) and quantified using Cell Ranger pipelines (v. 4.0.0 10X Genomics). The raw unique molecular identifier count data were imported into the Seurat package (v. 3.2.0). The UMAP plots, violin plots, feature plots, dot plots and heatmaps were generated using the functions in the Seurat package with R-3.5.1. A total of 7,678 cells with nFeature > 500 or 1,000, nCount between 1,000 or 2,000 and 80,000 or 100,000, and low mitochondrial gene expression (< 20%) were further analyzed. The filter conditions for each condition are shown in Supplementary Table 3. To strictly separate mCherry-positive cells from GFP-positive cells, we used the numbers of sequenced reads (mCherry and EGFP transcripts) and SNPs in selected mRNA sequences. GFP-positive blastocyst-derived cells (C57BL/6J × (C57BL/6J × DBA/2) F1) were distinguished from ESCs ((C57BL/6J × 129S4/SvJae) F1) by using the following five spots: apolipoprotein C2 (Apoc2) (chr1, location 171,225,758 and 171,225,795) and apolipoprotein C3 (Apoc3) (chr9, location 46,233,156, 46,233,203 and 46,233,210) (Supplementary Table 3). The SNP data for C57BL/6J,

DBA/2 and 129/Sv mice were obtained from the Mouse Genome Database (MGI) (<http://www.informatics.jax.org/snp>).

The raw counts were normalized using the log-normalization method in the Seurat package. The Scale Data function with vars.to.regress option in the Seurat package was used to remove sources of variation that were not of interest (total unique molecular identifier counts and percentage of mitochondrial transcripts). UMAP analysis and clustering were performed using the Seurat RunUMAP function, with default parameters (dims = 1:50) and the FindClusters function, with a resolution of 0.4. The cell type was defined according to the expression of well-known markers. Differential expression analysis was performed by the Seurat FindMarkers function (default setting) using the Wilcoxon rank-sum test. The *P* values were corrected for multiple testing using the Bonferroni method and represented as adjusted *P* values. Adjusted *P* values < 0.05 were considered significant. PC and PP scores were calculated by the Seurat AddModuleScore functions (default setting) using gene sets obtained from control mouse data (37 PP genes and 43 PC genes). *Cdkn1a*-high expressing cells in GFP-positive cells were defined as cells with expression level higher than one taking into account the expression values and rate of control and mCherry positive cells. By the above definition, the *Cdkn1a*-high rates were determined as follows (Control, 8.4%; caMEK1 mCherry, 78.5%; caMKK6 mCherry, 94.7%; caMEK1 GFP, 39.4%; caMKK6 GFP, 29.0%). *Cdkn1a*-low expressing cells in GFP-positive cells were defined as cells with expression level lower than 0.5.

Statistics and reproducibility

Statistical parameters, including sample size, statistical comparisons and statistical significance, are described in the figures or figure legends. Statistical analyses were performed using GraphPad Prism 8 Software (MDF). Data are presented as the means $\pm$ SD. Statistical comparisons of two groups were performed using the two-sided unpaired t test, and comparisons of more than three groups were performed by one-way ANOVA or Kruskal–Wallis test. A value of *P* < 0.05 was considered significant. The reproducibility of representative images was confirmed in a minimum of three biologically independent samples. No statistical methods were used to predetermine the sample size. No experiments were randomized. Investigators were not blinded to allocation during the experiments.

Data availability

Bulk RNA-seq and scRNA-seq data were deposited in the Gene Expression Omnibus (GEO) under accession number GSE242709 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=kzgbiskkrhkdhwv&acc=GSE242709>). The publicly available datasets used in this study are: mm10 mouse reference genome (<https://www.10xgenomics.com/support/jp/software/cell-ranger/latest/release-notes/cr-reference-release-notes#2020-a>), GSE145642 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145642>) (Amor et al., 2020), GSE132040 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132040>) (Tabula Muris senis bulk RNA-seq data, 2020), GSE148080 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148080>) (Esmaili et al., 2021), GSE106330 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106330>) (Tong et al., 2017), GSE74843 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74843>) (Fujii et al., 2016), and GSE149590 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149590>) (Tabula Muris senis single cell data, 2020). All other data are available from the corresponding authors upon request.

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Author Contributions Statement

Y.S., Y.Y. and T.Y. designed and conceived the study and wrote the paper. Hiroshi S supervised the experiments. Y.S., Hirofumi S. and M.H. generated vectors, cell lines and mice, performed experiments. M.N-K. and E.N. provided technical instruction for experiments. K.S. performed HybISS. K.W. and E.N. provided vectors. A.T. performed blastocyst injections. K.M. analyzed FACS data. Y.S. and K.S analyzed RNA *in situ* hybridization and HybISS data. Y.S., M.K. and T.Y. analyzed RNA-seq data.

Competing Interests Statement

The authors declare no competing interests.

Supplementary Table 1. caMEK1- and caMKK6-induced DEGs identified by bulk RNA-seq and list of secreted genes.

Supplementary Table 2. GO analysis of caMEK1- and caMKK6-induced DEGs in bulk RNA-seq.

Supplementary Table 3. Filter conditions for scRNA-seq.

Supplementary Table 4. caMEK1- and caMKK6-induced DEGs and subcluster-specific genes identified by scRNA-seq.

Supplementary Table 5. KEGG analysis of controls and GO analysis of caMEK1- and caMKK6-induced subcluster-specific DEGs in scRNA-seq.

Supplementary Table 6. Upstream regulator prediction analysis.

Supplementary Table 7. Genes overlapping with proteins in aging human plasma.

Supplementary Table 8. Primer and probe lists.

Figure Legends

Figure 1. Establishment of mouse models of inducible senescence.

a, Schematic illustration of the genomic construct for caMEK1 and caMKK6. A protocol for the systemic induction in chimeric mice is shown below. The Dox concentration was 0.2 mg/ml for caMEK1 and 2.0 mg/ml for caMKK6.

b, c Representative histological images and immunostaining for mCherry, p-ERK and p-p38 in the caMEK1 (**b**) and caMKK6 (**c**) liver and colon. Scale bars: 100 μ m.

d, Upper: RNA *in situ* analysis of *mCherry* and *Cdkn1a* in the liver. Arrow indicates *mCherry*-positive *Cdkn1a*-high hepatocytes and arrowhead indicates *mCherry*-negative *Cdkn1a*-high hepatocytes. Scale bars, 50 μ m. Lower left: Quantification of *Cdkn1a* expression in No Dox, Dox on *mCherry*-positive and *mCherry*-negative cells. The center, box and whiskers represent the median, upper and lower quartiles and 1.5 $\times$ interquartile range (IQR), respectively. Kruskal–Wallis with Dunn’s multiple comparisons test, two sided. Lower right: Quantification of the *Cdkn1a*-high ratio. *Cdkn1a*-high, $\log_2(Cdkn1a \text{ dots/cell} + 1) > 3$.

e, Left: Immunofluorescence for mCherry and p21 in the colon. Scale bars: 50 μ m. Right: Quantification of the p21-positive rate in mCherry-positive/negative cells.

f, Upper: Immunofluorescence for mCherry, p21 and BrdU in the colon. Arrows indicate p21-positive BrdU-negative cells. Scale bars: 20 μ m. Lower: Quantification of the BrdU-positive rate in mCherry-positive/negative and p21-positive/negative epithelial cells.

g, Upper: Immunofluorescence for mCherry, p21 and γ H2AX in the colon. Arrows indicate p21-positive γ H2AX-positive cells. Scale bars: 20 μ m. Lower: Quantification of the γ H2AX-positive rate in mCherry-positive/negative and p21-positive/negative epithelial cells.

h, Upper: SA- β -gal staining in the liver. Scale bars: 100 μ m. Lower: Quantification of the SA- β -gal index (caMEK1: No Dox, n = 5; Dox 7 d, n = 4; caMKK6: No Dox, n = 3; Dox, n = 5).

Experiments were performed with n = 3 mice per group (**b-g**). Insets are magnified pictures of the dashed areas (**d, e**). Data are presented as the means $\pm$ SDs of biologically independent samples (**d-h**). *P* values were calculated using ordinary one-way ANOVA with Tukey’s (**d, e**) or Dunnett’s (**f, g**) multiple comparisons test, two sided or Unpaired *t* test, two sided (**h**).

Figure 2. Bulk and single-cell RNA sequencing analysis of senescence-induced cells and surrounding cells.

a, Schematic illustration of the bulk RNA sequence experiment. mCherry-positive senescence-induced cells and GFP-positive surrounding cells were sorted. Liver; Control Dox, n = 2; caMEK1 Dox, n = 3; caMKK6 Dox, n = 2. Colon; Control Dox, n = 2; caMEK1 Dox, n = 2; caMKK6 Dox, n = 3. No Dox, n = 3 for control, caMEK1 and caMKK6. The Dox concentration was 0.2 mg/ml for caMEK1 and 2.0 mg/ml for caMKK6 and the control.

b, PCA for bulk RNA sequencing of MEFs, liver and colon showing the log₂ fold change (FC) compared to control. Data of MEFs 2, 4 and 7 days after Dox treatment are compared with those from No Dox samples (n = 3 each group). Liver and colon data from caMEK1 mCherry, caMKK6 mCherry and caMEK1 GFP, caMKK6 GFP are compared with control mCherry and control GFP, respectively.

c, d, Venn diagram showing the number of upregulated/downregulated genes (FC > 2 or < -2, FDR < 0.05 and average FPKM > 1) in caMEK1 and caMKK6 in mCherry and GFP of the liver and colon.

e, Heatmap showing the relative expression of upregulated secreted genes (FC > 2, FDR < 0.05 and average FPKM > 1) in mCherry (mC) and GFP (G) in the liver (left) and colon (right). The color range is shown using a log₂ scale.

f, RNA *in situ* analysis of *mCherry*, *Cdkn1a*, *Serpine1* and *Cd9* gene expression in the liver. Insets are magnified pictures of the dashed areas. *Cdkn1a* high-expressing cells double-positive for *Serpine1* and *Cd9* appear in both *mCherry*-positive and *mCherry*-negative cells. Scale bars, 50 μm.

g, Quantification of *Serpine1* and *Cd9* expressions in No Dox, Dox on *mCherry*-positive and *mCherry*-negative cells in (f). Kruskal–Wallis with Dunn’s multiple comparisons test, two sided.

h, The proportion of *Serpine1* and/or *Cd9* high expressing cells in *Cdkn1a*-high cells in (f). Data are presented as the means ± SDs of biologically independent samples. *Cdkn1a*-high, log₂ (*Cdkn1a* dots/cell+1) > 3; *Serpine1*-high, log₂ (*Serpine1* intensity/cell+1) > 1; *Cd9*-high, log₂ (*Cd9* dots/cell+1) > 3.5.

i, UMAP visualization of 7,678 hepatocytes and NPCs (control mCherry, n = 732; control

GFP, n = 145; caMEK1 mCherry, n = 1,252; caMEK1 GFP, n = 1,577; caMKK6 mCherry, n = 3,270; caMKK6 GFP, n = 702). The distribution of control, caMEK1 and caMKK6 cells (upper). Cell-type clusters (Hep, hepatocytes; NPC, nonparenchymal cells) (lower). **j**, Violin plot of *Cdkn1a* and SASP genes (*Serpine1* and *Cd9*) in hepatocytes of mCherry (m) and GFP (G) in control, caMEK1 and caMKK6. *Adjusted $P < 0.0001$. Experiments were performed with n = 3 mice per group (**f-h**). The center, box and whiskers represent the median, upper and lower quartiles and 1.5× interquartile range (IQR), respectively (**g, h**).

Figure 3. Liver zonation changes in primary senescence.

a, UMAP plot of subcluster, relative PP/PC zonation score and expression levels of representative zonation marker genes in control, caMEK1 mCherry and caMKK6 mCherry cells. PP, periportal; PC, pericentral; Mid, mid zone.
b, Upper: violin plot of *Cyp2f2* and *Cyp2e1* in hepatocytes of mCherry (m) and GFP (G) in control, caMEK1 and caMKK6 cells and NPCs. Lower: violin plot of PP and PC zonation scores.
c, HybISS showing the expression of the indicated zonation marker transcripts (*Glul*, *Cyp2e1*, *Hamp*, *Igfbp2*, *Cyp2f2* and *Sds*) and immunostaining for cadherin and mCherry. Zonation gene expression was markedly diminished in mCherry-positive cells. In mCherry-negative cells, zonation gene expression was also diminished, but zonal differentiation was maintained. Magnified areas (white box) are shown below. Scale bars, 200 μ m. AAV8-*Cre*; caMEK1 No Dox, n = 1; Dox, n = 1; AAV8-*Cre*; caMKK6 No Dox, n = 1; Dox, n = 1.

Figure 4. scRNA-seq analysis reveals the cell-to-cell heterogeneity of primary senescence in the liver.

a, The number of upregulated and downregulated genes compared with the control in the caMEK1 mCherry subcluster.
b, Right: Heatmap showing relative expression levels of subcluster-specific genes in caMEK1 mCherry upregulated compared with control. The color range indicates the z score. Dot plots showing relative expression of caMEK1 mCherry compared with control. Dot size denotes the percentage of cells expressing the gene, and color denotes the logFC

of average expression across all cells of that type. Left: representative GO terms of each subcluster are shown. DAVID Bioinformatics Resources (v6.8) were used for the analysis.

c, Quantified profiles for upregulated mRNA in AAV8-*Cre*; caMEK1 from HybISS in the No Dox and Dox mCherry-positive groups. The lines show the number of amplicons/1000 μm^2 .

d, The number of genes upregulated and downregulated compared with the control in the caMKK6 mCherry subcluster.

e, Heatmap showing relative expression levels of secreted genes upregulated in caMKK6 mCherry compared with control. The color range indicates the z score. Dot plots showing relative expression in caMKK6 mCherry compared with control. The dot size and color are the same as in (b).

f, Quantified profiles for upregulated mRNA in AAV8-*Cre*; caMKK6 from HybISS in No Dox and Dox mCherry-positive groups. The lines show the number of amplicons/1000 μm^2 .

Figure 5. scRNA-seq analysis of secondary senescence.

a, UMAP plot of subcluster, relative PP/PC zonation score and expression levels of representative zonation marker genes in caMEK1 GFP and caMKK6 GFP. PP, periportal; PC, pericentral; Mid, mid zone.

b, Left: filtering of Dox mCherry-negative *Cdkn1a*-positive cells in HybISS. Right: quantified profiles for upregulated mRNA in AAV8-*Cre*; caMEK1 and AAV8-*Cre*; caMKK6 from HybISS in the No Dox, Dox mCherry-negative *Cdkn1a*-positive and Dox mCherry-negative *Cdkn1a*-negative groups. The lines show the number of amplicons/1000 μm^2 .

c, Left: Filtering of GFP *Cdkn1a*-high cells in scRNA-seq. Upper middle and right: The number of genes upregulated and downregulated compared with the control in the subclusters of caMEK1 GFP *Cdkn1a*-high cells (middle) and caMKK6 GFP *Cdkn1a*-high cells (right). Lower: Heatmap showing relative expression levels of secreted genes upregulated in caMEK1 GFP *Cdkn1a*-high (middle) and caMKK6 GFP *Cdkn1a*-high (right) compared with the control. The color range shows the z score. Dot plots showing relative expression in caMEK1 GFP *Cdkn1a*-high and caMKK6 GFP *Cdkn1a*-high compared with control cells. The dot size denotes the percentage of cells expressing the

gene, and the color denotes the logFC of average expression across all cells of that type.

Figure 6. IL1 mediates secondary senescence.

a, Upstream analysis of genes commonly upregulated in all subclusters of caMEK1 GFP *Cdkn1a*-high and caMKK6 GFP *Cdkn1a*-high according to scRNA-seq. Representative upstream regulator and *P* values are shown (Fisher's exact test, one-sided.). IPA software was used for analysis.

b, Violin plot of *Il1r1* expression in scRNA-seq in control, caMEK1 and caMKK6 mCherry, GFP *Cdkn1a*-high and GFP *Cdkn1a*-low cells. *Cdkn1a*-high, Expression Level > 1; *Cdkn1a*-low, Expression Level < 0.5.

c, Right: RNA *in situ* analysis of *mCherry*, *Cdkn1a* and *Il1r1* gene expression in caMEK1 and caMKK6 chimeric mice. Insets are magnified pictures of the dashed areas. Arrows indicate *mCherry*-negative *Cdkn1a*-high cells with elevated *Il1r1* expression and arrowheads indicate *mCherry*-negative *Cdkn1a*-low cells without elevated *Il1r1* expression. Scale bars, 50 μ m. Left: Quantification of *Il1r1* expression in No Dox, Dox on *mCherry*-positive, *mCherry*-negative *Cdkn1a*-high and *mCherry*-negative *Cdkn1a*-low cells (n = 3 mice per group). *Cdkn1a*-high, $\log_2$ (*Cdkn1a* dots/cell+1) > 3; *Cdkn1a*-low, $\log_2$ (*Cdkn1a* dots/cell+1) $\leq$ 1.

d, Left: RNA *in situ* analysis of *Il1b* and *Clec4f* gene expression in caMEK1 and caMKK6 chimeric mice. Insets are magnified pictures of the dashed areas. Scale bars, 50 μ m. Right: Quantification of the *Il1b*- and *Clec4f*-positive area (caMEK1 No Dox, n = 3; Dox, n = 8; caMKK6 No Dox, n = 3; Dox, n = 6). Data are presented as the means $\pm$ SDs of biologically independent samples. Unpaired t test, two sided.

e, Schematic illustration of the experiments. The Dox concentration was 0.2 mg/ml for AAV8-*Cre*; caMEK1 and 2.0 mg/ml for AAV8-*Cre*; caMKK6.

f, RNA *in situ* analysis of *Cdkn1a* and *mCherry* gene expression in AAV8-*Cre*; caMEK1 and AAV8-*Cre*; caMKK6 after 7 days of Dox treatment without/with IL1R1 inhibitor and clodronate liposomes. Scale bars, 50 μ m.

g, Quantification of *Cdkn1a* expression in No Dox, Dox on *mCherry*-positive, *mCherry*-negative proximate (Prox) and remote cells. AAV8-*Cre*; caMEK1 No Dox, n = 5; Dox, n = 4; Dox + IL1R1 inhibitor, n = 5; Dox + clodronate liposome, n = 4; AAV8-*Cre*; caMKK6 No Dox, n = 5; Dox, n = 5; Dox + IL1R1 inhibitor, n = 3; Dox + clodronate

liposome, n = 5.

The center, box and whiskers represent the median, upper and lower quartiles and 1.5× interquartile range (IQR), respectively. Kruskal–Wallis with Dunn’s multiple comparisons test, two sided. * $P < 0.0001$. (c, g)

Figure 7. caMEK1 and caMKK6 transcriptome are associated with aging.

a, Gene expression patterns of caMEK1 mCherry liver were compared with Nras-induced OIS data of mouse liver (GSE145642)³⁰. NC, 1000; up, top 100; down, top 100.

b, Gene expression patterns of caMEK1 mCherry and caMKK6 mCherry in the liver were compared with aged mouse liver data (GSE132040)⁵⁰. NC, 1000; up, top 100; down, top 100.

c, Gene expression patterns of caMEK1 mCherry and caMKK6 mCherry in the liver were compared with aged human liver proteome data⁵³ (up, 436; down, 283 (FDR < 0.05); NC, 2,344 from 3,063 proteins).

d, Gene expression patterns of caMKK6 mCherry PP and PC in the liver were compared with aged mouse liver spatial transcriptomic data⁵¹. NC, 1,000; up, 243 (PP) or 215 (PC), down, 301 (PP) or 214 (PC).

e, Gene Ontology (GO) term enrichment of genes downregulated in caMKK6 mCherry PP (upper) and PC (lower) compared with those of control mice (fold change < -1.5, FDR < 0.05). Representative GO terms and P values are shown (Fisher’s exact test, one-sided.). DAVID Bioinformatics Resources (v6.8) were used for the analysis.

f, Heatmap showing the relative expression of FXR/RXR and xenobiotic metabolism genes in caMEK1 and caMKK6 mCherry liver, aged mouse liver^{19,50} and aged human liver⁵⁶ data. The color range indicates the log₂ scale.

g, Gene expression patterns of caMEK1 mCherry and caMKK6 mCherry in the liver were compared with mouse chronic liver disease data, in which fibrosis was induced by CCl₄⁵⁷. NC, 1,000; up, 1,625; down, 574.

h, Gene expression patterns of caMEK1 mCherry and caMKK6 mCherry in the liver were compared with mouse dietary induced NAFLD data (GSE148080)⁵⁸. NC, 1,000 ; up, 117, down, 62.

i, Venn diagram showing the overlap of expression signatures from aged human plasma proteins⁶¹ (FDR < 0.05 from 2,925 genes) and caMEK1 (upper) and caMKK6 (lower)

mCherry liver.

j, Expression trends with age for representative proteins are shown. These proteins were analyzed in a large proteomic dataset derived from 4,263 human patients aged 18-95 years⁶¹. A Loess regression line is shown for each protein. RFU = relative fluorescence unit.

Box plots show the relative expression levels of NC, unchanged; up, upregulated; down, downregulated genes. The center, box and whiskers represent the median, upper and lower quartiles and $1.5\times$ interquartile range (IQR), respectively. Kruskal–Wallis with Dunn’s multiple comparisons test, two sided (**a-d**, **g**, **h**).

Extended Data Fig. 1. Senescence induction in caMEK1 and caMKK6 MEFs.

- a. Representative images of caMEK1 and caMKK6 MEFs derived from germline transmission in mice without Dox treatment and after 7 days of 2.0 $\mu\text{g/ml}$ Dox treatment. Scale bars: 100 μm .
- b. Western blot analysis for p-ERK, ERK, p-p38 and p38 in caMEK1 and caMKK6 MEFs at 3, 7 and 10 days after 2.0 $\mu\text{g/ml}$ Dox treatment. ERK was specifically phosphorylated in caMEK1 MEFs, and p38 was specifically phosphorylated in caMKK6 MEFs.
- c. caMEK1 and caMKK6 MEFs cease proliferation after 2.0 $\mu\text{g/ml}$ Dox treatment. Data are presented as the means $\pm$ SDs of biological triplicates. Paired t test, two sided.
- d. Representative images of SA- β -gal staining of caMEK1 and caMKK6 MEFs with/without Dox treatment (2 $\mu\text{g/ml}$, 7 days). Insets are magnified pictures of the dashed areas. Scale bars: 50 μm .
- e. The SA- β -gal-positive ratios of caMEK1 and caMKK6 MEFs without and with Dox treatment are shown. Data are presented as the means $\pm$ SDs of biological triplicates. Ordinary one-way ANOVA test, two sided.
- f. qRT-PCR analyses of the expression of senescence markers in caMEK1 and caMKK6 MEFs at 2, 4 and 7 days after 2 $\mu\text{g/ml}$ Dox treatment. Data are presented as the means $\pm$ SDs of biological triplicates. Expression levels relative to those of MEFs without Dox treatment are shown. One-way repeated-measures ANOVA test, two sided.

Extended Data Fig. 2. caMEK1 and caMKK6 induced senescence *in vivo*.

- a. Representative histological images and immunostaining for mCherry and p-ERK in caMEK1 liver and colon without Dox treatment. Scale bars: 100 μm .
- b. Representative histological images and immunostaining for mCherry and p-p38 in caMKK6 liver and colon without Dox treatment. Scale bars: 100 μm .
- c. RNA *in situ* analysis of *mCherry* and *Cdkn1a* gene expression in caMEK1 and caMKK6 liver without Dox treatment. Scale bars, 50 μm .
- d. Immunofluorescence for mCherry and p21 in caMEK1 and caMKK6 colon without Dox treatment. p21-positive cells were rarely detected in the absence of Dox treatment. Scale bars: 50 μm .

- e. Immunostaining for p21 in caMEK1 and caMKK6 liver without Dox treatment. p21-positive cells were rarely detected in the absence of Dox treatment. Scale bars: 100 μ m.
- f. Immunostaining for mCherry and p21 in caMEK1 and caMKK6 liver serial sections after 7 days of Dox treatment. Black asterisks indicate mCherry-positive/p21-positive hepatocytes, and white asterisks indicate mCherry-negative/p21-positive hepatocytes. Arrows indicate p21-negative hepatocytes for comparison. Scale bars: 50 μ m.
- g. Quantification of the p21-positive ratio in mCherry-positive and mCherry-negative hepatocytes in caMEK1 and caMKK6 livers (n = 3 per group).
- h. Schematic illustration of the genomic construct to activate mCherry as control.
- i. Immunostaining for mCherry, p-ERK and p-p38 in control liver and colon after 7 days of Dox treatment. Scale bars: 100 μ m.
- j. Immunostaining for mCherry and p21 in control liver and colon serial sections after 7 days of Dox treatment. Scale bars: 100 μ m.
- k. Left: Immunostaining for BrdU in the caMEK1 and caMKK6 colon after 3 and 7 days of Dox treatment. Scale bars: 100 μ m. Right: Quantification of the BrdU-positive rate in mCherry-positive crypts of the caMEK1 and caMKK6 colon after 3 and 7 days of Dox treatment (n = 3 per group). Data are presented as the means $\pm$ SDs of biologically independent samples. Ordinary one-way ANOVA test, two sided.
- l. Left: Immunofluorescence for mCherry and Ki67 in caMEK1 and caMKK6 liver after 7 days of Dox treatment. Arrowheads indicate Ki67-positive hepatocytes and arrow indicates Ki67-positive non-parenchymal cells (NPCs). Scale bars: 50 μ m. Upper right: Quantification of the Ki67-positive rate in mCherry-positive and mCherry-negative hepatocytes in caMEK1 and caMKK6 liver (n = 3 per group). Lower right: Quantification of the number of Ki67-positive NPCs per field in caMEK1 and caMKK6 liver (n = 3 per group). Data are presented as the means $\pm$ SDs of biologically independent samples.
- m. Left: Immunofluorescence for mCherry and γ H2AX in caMEK1 and caMKK6 liver after 7 days of Dox treatment. Insets are magnified pictures of the dashed areas. Arrows indicate γ H2AX-positive NPCs. Scale bars: 100 μ m. Right: Quantification of the number of γ H2AX-positive hepatocytes (upper) and NPCs (lower) per field in caMEK1 and caMKK6 liver (n = 3 per group). Data are presented as the means $\pm$ SDs

of biologically independent samples. Unpaired t test, two sided.

Insets are magnified pictures of the dashed areas. Data are presented as the means $\pm$ SDs of biologically independent samples. Ordinary one-way ANOVA test, two sided. Unpaired t test, two sided.

Extended Data Fig. 3. caMEK1 and caMKK6 induced senescence *in vivo*.

- a. Left: Immunostaining for NF κ B in the caMEK1 and caMKK6 liver after 7 days of Dox treatment. Scale bars: 100 μ m. Right: Quantification of NF κ B-positive cells in caMEK1 and caMKK6 after 7 days Dox treatment (n = 3 per group).
- b. Left: Immunostaining for NF κ B in the caMEK1 and caMKK6 colon after 7 days of Dox treatment. Scale bars: 100 μ m. Right: Quantification of NF κ B-positive ratio in caMEK1 and caMKK6 after 3 and 7 days of Dox treatment (n = 3 per group, except caMKK6 day3 (n=2)). Data are presented as the means $\pm$ SDs of biologically independent samples.
- c. qRT-PCR analyses of the expression of senescence markers in the liver and colon of caMEK1 and caMKK6 chimeric mice at 3 and 7 days after Dox treatment (caMEK1 No Dox, n = 6; caMEK1 Dox 3 d, n = 7; caMEK1 Dox 7 d, n = 7; caMKK6 No Dox, n = 7; caMKK6 Dox 3 d, n = 6; caMKK6 Dox 7 d, n = 8). Expression levels relative to those in the liver or colon in the absence of Dox treatment are shown. Data are presented as the means $\pm$ SDs of biologically independent samples. Ordinary one-way ANOVA test, two sided.
- d. Schematic illustration of the genetic constructs for HDTV1; caMEK1 and HDTV1; caMKK6 for tissue-specific expression in the liver (upper panel). The lower panel shows the experimental protocol.
- e. RNA *in situ* analysis of *Cdkn1a* and *mCherry* gene expression in the HDTV1; caMEK1 and caMKK6 liver. Scale bars, 50 μ m. n = 3 per group of mice examined.
- f. Quantification of the SA- β -gal index in HDTV1; caMEK1 and HDTV1; caMKK6 liver (HDTV1; caMEK1 No Dox, n = 5; HDTV1; caMEK1 Dox 7 d, n = 6; HDTV1; caMKK6 No Dox, n = 4; HDTV1; caMKK6 Dox, n = 6). Data are presented as the means $\pm$ SDs of biologically independent samples. Unpaired t test, two sided.
- g. Schematic illustration of the genetic constructs *Villin-Cre*; caMEK1 and *Villin-Cre*; caMKK6 for tissue-specific expression in the colon (upper panel). The lower panel shows the experimental protocol.

- h. Immunostaining for p21 and BrdU in *Villin-Cre*; caMEK1 and *Villin-Cre*; caMKK6 colon serial sections after 7 days of Dox treatment. Scale bars: 100 μ m.
 - i. Quantification of the p21- and BrdU-positive cells in *Villin-Cre*; caMEK1 and *Villin-Cre*; caMKK6 colons at 3 and 7 days after Dox treatment (*Villin-Cre*; caMEK1 No Dox, n = 3; *Villin-Cre*; caMEK1 Dox 3 d, n = 3; *Villin-Cre*; caMEK1 Dox 7 d, n = 4; *Villin-Cre*; caMKK6 No Dox, n = 3; *Villin-Cre*; caMKK6 Dox 3 d, n = 3; *Villin-Cre*; caMKK6 Dox 7 d, n = 4). Data are presented as the means $\pm$ SDs of biologically independent samples. Ordinary one-way ANOVA test, two sided.
 - j. qRT-PCR analyses of the expression of *Cdkn2a* and *Cdkn1a* in *Villin-Cre*; caMEK1 and *Villin-Cre*; caMKK6 colons at 3 and 7 days after Dox treatment (*Villin-Cre*; caMEK1 No Dox, n = 3; *Villin-Cre*; caMEK1 Dox 3 d, n = 3; *Villin-Cre*; caMEK1 Dox 7 d, n = 4; *Villin-Cre*; caMKK6 No Dox, n = 3; *Villin-Cre*; caMKK6 Dox 3 d, n = 3; *Villin-Cre*; caMKK6 Dox 7 d, n = 4). Expression levels relative to those in the colon in the 3 days Dox treatment for *Cdkn2a* or the absence of Dox treatment for *Cdkn1a* are shown. Data are presented as the means $\pm$ SDs of biologically independent samples. Ordinary one-way ANOVA test, two sided.
- Data are presented as the means $\pm$ SDs of biologically independent samples. Unpaired t test, two sided. Ordinary one-way ANOVA test, two sided.

Extended Data Fig. 4. RNA-seq analysis of caMEK1 and caMKK6 mosaic mice.

- a. FACS sorting strategy to isolate hepatocytes (upper) and colonic epithelial cells (lower).
- b. PCA for bulk RNA sequencing of the liver (upper) and colon (lower).
- c. Gene Ontology (GO) term enrichment of genes upregulated and downregulated in the liver and colon of caMEK1- and caMKK6-treated mice compared with those of control mice (fold change > 2 or < -2, FDR < 0.05 and FPKM > 50). Representative GO terms and p values are shown (Fisher's exact test, one-sided.). DAVID Bioinformatics Resources (v6.8) were used for the analysis.
- d. Heatmap showing the relative expression of 17 commonly upregulated genes in all of caMEK1 mCherry and caMKK6 mCherry of the liver and the colon. The color range shows the log₂ scale.
- e. Heatmap showing the relative expression of genes shared with previously reported senescence models³⁰ (listed in Extended Data Fig. 4d). Bolded genes indicate 6 genes

increased in both our study and the previous study. TIS; therapy-induced senescence in mouse lung adenocarcinoma *Kras*^{G12D}; *p53*^{-/-} cells that are triggered to become senescent by a combination of MEK inhibition and CDK4 and CDK6 inhibition, OIS; oncogene-induced senescence in mouse hepatocytes, mediated by the *in vivo* delivery of *Nras*^{G12V} through hydrodynamic tail vein injection, RIS; replication-induced senescence in mouse hepatic stellate cells. RNA-seq data were obtained from GSE145642. The color range shows the log₂ scale.

- f. Immunostaining for uPAR in the liver (left) and colon (right) of caMEK1 and caMKK6 chimeric mice after 7 days of Dox treatment. Insets are magnified images of the dashed areas. Scale bars: 100 μ m.
- g. Expression levels of *Plaur* and *Serpine1* in mCherry (m) and GFP (G) cells in the liver and colon of control, caMEK1 and caMKK6 mice. Data are presented as the means $\pm$ SDs of biologically independent samples.
- h. Left: RNA *in situ* analysis of *mCherry*, *Cdkn1a* and *Plaur* gene expression in caMEK1 and caMKK6 liver after 7 days of Dox treatment. Insets are magnified pictures of the dashed areas. Arrows indicate *mCherry*-positive *Plaur*-high hepatocytes. Scale bars, 50 μ m. Upper right: Quantification of *Plaur* expression in No Dox, Dox on *mCherry*-positive and Dox on *mCherry*-negative cells. *Cdkn1a*-high, log₂ (*Cdkn1a* dots/cell+1) > 3; *Plaur*-high, log₂ (*Plaur* dots/cell+1) > 2. The black dots in each box indicate the median. The bottom and top of the boxes are the lower and upper quartiles, respectively. Whiskers indicate the $\pm$ 1.5 interquartile range (IQR). Kruskal–Wallis test, two sided. Lower right: Quantification of the *Plaur*-high ratio in *Cdkn1a*-high cells (n = 3 per group). Data are presented as the means $\pm$ SDs of biologically independent samples. Ordinary one-way ANOVA test, two sided.

Expression levels of *Cd9* in mCherry (m) and GFP (G) cells in the liver of control, caMEK1 and caMKK6 mice. Data are presented as the means $\pm$ SDs of biologically independent samples.

Extended Data Fig. 5. scRNA-seq analysis, subclustering and zonation of the caMEK1 and caMKK6 liver.

- a. The scRNA-seq experimental protocol is shown. The Dox concentration was 0.2 mg/ml for caMEK1 and 2.0 mg/ml for caMKK6 and the control.

- b. Expression levels of representative marker genes for each cell type in the UMAP visualization.
- c. The proportion of NPCs (Cluster 7) in each condition. m, mCherry; G, GFP.
- d. The cell number and proportion of subclusters in control mCherry and control GFP.
- e. Heatmap showing the relative expression of subcluster-specific genes in the control, caMEK1 mCherry, caMKK6 mCherry, caMEK1 GFP and caMKK6 GFP. The color range shows the z score.
- f. KEGG pathway analysis of subcluster-specific genes in the control. DAVID Bioinformatics Resources (v6.8) were used for the analysis.
- g. Violin plot of *mCherry* in caMEK1 mCherry and caMKK6 mCherry.

Extended Data Fig. 6. HybISS analysis of zonation and secreted transcripts.

- a. Schematic illustration of the genetic constructs AAV8-*Cre*; caMEK1 and AAV8-*Cre*; caMKK6 in a hepatocyte-specific manner. When using a mosaic dosage of AAV8-*CAG-Cre*, we observed approximately 20% *mCherry*-positive hepatocytes. The lower panel shows the experimental protocol for HybISS experiment. AAV8-*Cre*; caMEK1 No Dox, n = 1; Dox, n = 1; AAV8-*Cre*; caMKK6 No Dox, n = 1; Dox, n = 1. The Dox concentration used was 0.2 mg/ml for AAV8-*Cre*; caMEK1 and 2.0 mg/ml for AAV8-*Cre*; caMKK6.
- b. These coordinates were used to reconstruct the spatial zonation profiles of liver genes. The fields were manually segmented based on the *Glul*, *Cyp2e1* and *Cyp2f2* gene spots.
- c. Zonation segmentation map in HybISS. mCherry-positive PC- and PP-hepatocytes were distinguished with reference to the concentric zonation gene expression of mCherry-negative hepatocytes. Scale bars, 200 μ m. PC, pericentral; PP, periportal.
- d. Quantified profiles for zonation marker transcripts from HybISS in No Dox, Dox mCherry-positive and Dox mCherry-negative in AAV8-*Cre*; caMEK1 and AAV8-*Cre*; caMKK6. The lines show the number of amplicons/1000 μ m².
- e. Violin plot of Wnt target genes in control, caMEK1 mCherry and caMKK6 mCherry. PC, pericentral; PP, periportal.

Extended Data Fig. 7. caMEK1 and caMKK6 liver demonstrated heterogeneity at the single-cell level.

- a. Heatmap showing the relative expression levels of secreted genes upregulated in caMEK1 mCherry compared with the control. The color range shows the z score. Dot plots showing relative expression in caMEK1 mCherry compared with the control. Dot size denotes the percentage of cells expressing the gene, and color denotes the logFC of average expression across all cells of that type.
- b. Expression levels of hepatic stem cell marker genes in caMEK1 mCherry visualized by UMAP.
- c. Immunostaining for AFP in caMEK1 chimeric mouse livers after 7 days of Dox treatment. Scale bars, 100 μ m. n = 3 mice per group were examined.
- d. Violin plot of *Serpine1* and *Zfp3612* in caMEK1 mCherry.
- e. RNA *in situ* analysis of *Serpine1* and *mCherry* gene expression in caMEK1 and caMKK6 chimeric mice. Scale bars, 200 μ m. n = 3 mice per group were examined. CV, central vein; PN, portal node.
- f. Violin plot of cell cycle-related genes (*Cdkn2b* and *Ccnd1*) and inflammatory response-associated genes (*Cd14*, *Il1rn* and *Ifngr1*) in caMEK1 mCherry.
- g. Expression levels of representative subcluster-specific genes (subcluster 0 (PP), *Hsd17b13*; subcluster 2 (PC), *Serpina7* and *Serpine1*) in caMKK6 mCherry visualized by UMAP.
- h. The proportion of *Serpine1* and/or *Cd9* high expressing cells in *Cdkn1a*-high cells in in scRNA-seq. *Cdkn1a*-high, Expression Level > 1; *Serpine1*-high, Expression Level > 0; *Cd9*-high, Expression Level > 0.5.

Extended Data Fig. 8. IL1 and Notch signaling pathway in caMEK1 and caMKK6.

- a. Expression levels of *Hes1*, *Jag1* and *Dll1* in bulk RNA-seq analysis in mCherry (m) and GFP (G) cells in the liver of control, caMEK1 and caMKK6 mice. Data are presented as the means $\pm$ SDs of biologically independent samples.
- b. Violin plot of *Hes1* expression in scRNA-seq in control, caMEK1 and caMKK6 mCherry, GFP *Cdkn1a*-high and GFP *Cdkn1a*-low cells. *Cdkn1a*-high, Expression Level > 1; *Cdkn1a*-low, Expression Level < 0.5.
- c. Schematic illustration of RNA *in situ* quantification analysis. *mCherry*-negative Proximate cells having a nuclear center of gravity less than 75 pixels from the nuclear center of gravity of *mCherry*-positive cells.

- d. Left: RNA *in situ* analysis of *mCherry*, *Cdkn1a* and *Hes1* gene expression in caMEK1 and caMKK6 chimeric mice after 7 days of Dox treatment. Insets are magnified pictures of the dashed areas. Arrows indicate *Cdkn1a*-high proximate hepatocytes with elevated *Hes1* expression and arrowheads indicate *Cdkn1a*-low remote hepatocytes without elevated *Hes1* expression. Scale bars, 50 μ m. Right: Quantification of *Hes1* expression in No Dox, Dox on *mCherry*-positive and Dox on *mCherry*-negative Proximate (Prox) and Remote, *Cdkn1a*-high and low cells. *Cdkn1a*-high, $\log_2(\text{Cdkn1a dots/cell}+1) > 3$; *Cdkn1a*-low, $\log_2(\text{Cdkn1a dots/cell}+1) \leq 1$. The black dots in each box indicate the median. The bottom and top of the boxes are the lower and upper quartiles, respectively. Whiskers indicate the ± 1.5 interquartile range (IQR). Kruskal–Wallis test, two sided. n = 3 mice per group were examined. The tables show the percentages of *Cdkn1a* high- and low-expressing cells in proximal and remote cells. Fisher’s exact test, two sided.
- e. Upstream analysis of genes upregulated in caMEK1 GFP and caMKK6 GFP in bulk RNA-seq analysis.
- f. Expression levels of *Il1r1* in bulk RNA-seq analysis in mCherry (m) and GFP (G) of control, caMEK1 and caMKK6 in the liver. Data are presented as the means $\pm$ SDs of biologically independent samples.
- g. Expression levels and distribution of *Il1b* (left) and *Clec4f* (right).
- h. Left: Quantification of the *Il1b*-positive cell ratio in hepatocytes (Hep, n = 7,523 cells) and nonparenchymal cells (NPC, n = 155 cells). Middle: Expression level of *Il1b* in *Clec4f*-negative (n = 131 cells) and *Clec4f*-positive cells (n = 24 cells) in Cluster 7. Right: Expression level of *Il1b* in *Clec4f*-positive cells in mCherry-positive (n = 5 cells) and GFP-positive cells (n = 19 cells). Unpaired t test, two sided.

Extended Data Fig. 9. IL1 mediates secondary senescence.

- a. Left: RNA *in situ* analysis of *Il1r1* and *mCherry* gene expression in AAV8-*Cre*; caMEK1 and AAV8-*Cre*; caMKK6 after 7 days of Dox treatment. Insets are magnified pictures of the dashed areas. Arrows indicate *mCherry*-negative *Il1r1*-high hepatocytes. Scale bars, 50 μ m. Right: Quantification of *Il1r1* in No Dox, Dox *mCherry*-positive, Dox *mCherry*-negative proximate (Prox) and Dox *mCherry*-negative remote cells (AAV8-*Cre*; caMEK1 No Dox, n = 5; Dox, n = 4; AAV8-*Cre*;

- caMKK6 No Dox, n = 5; Dox, n = 5). The black dots in each box indicate the median. The bottom and top of the boxes are the lower and upper quartiles, respectively. Whiskers indicate the ± 1.5 interquartile range (IQR). Kruskal–Wallis test, two sided.
- Left: RNA *in situ* analysis of *Il1b* and *Clec4f* gene expression in AAV8-*Cre*; caMEK1 and AAV8-*Cre*; caMKK6 after 7 days of Dox treatment without/with clodronate liposomes. Insets are magnified pictures of the dashed areas. Scale bars, 50 μ m. Right: Quantification of *Il1b*- and *Clec4f*-positive areas. Data are presented as the means $\pm$ SDs of biologically independent samples. Ordinary one-way ANOVA test, two sided.
 - Quantification of mCherry-positive cells. Approximately 30-40% mCherry-positive hepatocytes were observed after Dox treatment. Ordinary one-way ANOVA test, two sided.
 - Quantification of the SA- β -gal index in AAV8-*Cre*; caMEK1 and AAV8-*Cre*; caMKK6 after 7 days of Dox treatment without/with IL1R1 inhibitor and clodronate liposomes. Ordinary one-way ANOVA test, two sided.

Extended Data Fig. 10. caMEK1 and caMKK6 are associated with the aging.

- Gene expression patterns of caMKK6 mCherry liver were compared with previously reported Nras-induced OIS data of mouse liver³⁰. Box plots show the relative expression levels of the 1,000 unchanged genes, top 100 upregulated genes, and top 100 downregulated genes in each senescence-associated condition. RNA-seq data were obtained from GSE145642.
- Gene expression patterns of caMEK1 mCherry and caMKK6 mCherry colonic epithelial cells were compared with previously reported data from mouse intestines with Braf-induced OIS⁴⁷. Box plots for relative expression levels of 1,000 unchanged genes, the top 100 upregulated genes, and the top 100 downregulated genes in each senescence-associated condition. RNA-seq data were obtained from GSE106330.
- The gene expression patterns of the caMEK1 mCherry and caMKK6 mCherry colon were compared with previously reported human SSAP data⁴⁸. Box plots for relative expression levels of unchanged, upregulated and downregulated genes. SSAP up and down signatures were generated based on the list of classifier genes of human organoids from SSAP (upregulated and downregulated genes, size = 130 and 20, respectively.) A randomly extracted set of 1,000 unchanged genes were used as

controls.

- d. Expression levels of *Lgr5* in bulk RNA-seq analysis of mCherry (m) and GFP (G) cells from control, caMEK1 and caMKK6 colon tissues. Data are presented as the means $\pm$ SDs of biologically independent samples.
- e. Upper: RNA *in situ* analysis of *Lgr5* gene expression in caMEK1 and caMKK6 chimeric mice. Scale bars, 50 μ m. Lower: Quantification of *Lgr5*-positive cells per crypt in mCherry-positive crypts and mCherry-negative crypts in the caMEK1 and caMKK6 colons (n = 3 per group). Data are presented as the means $\pm$ SDs of biologically independent samples. Ordinary one-way ANOVA test, two sided.
- f. Gene expression patterns of caMEK1 mCherry and caMKK6 mCherry in the liver were compared with previously reported aged mouse liver data⁵¹. Box plots for relative expression levels of 1,000 unchanged genes, 965 upregulated genes, and 1,570 downregulated genes.
- g. Gene expression patterns of caMEK1 mCherry and caMKK6 mCherry in the liver were compared with previously reported aged mouse liver data⁵². Box plots for relative expression levels of 1,000 unchanged genes, 813 upregulated genes, and 332 downregulated genes.
- h. Gene expression patterns of the caMEK1 mCherry and caMKK6 mCherry colons were compared with previously reported aged mouse colon data¹⁹. Box plots for relative expression levels of 1,000 randomly extracted unchanged genes, top 100 upregulated genes, and top 100 downregulated genes. The mouse colon aging signature was generated from the reanalyzed data obtained from Zang et al⁴⁹.
- i. Venn diagram showing the overlap of the protein expression signature from aged human plasma⁶¹ (FDR < 0.05 from 2,925 proteins) with the caMEK1 mCherry colon (upper) or caMKK6 mCherry colon (lower).
- j. Left: Immunostaining for ChgA in caMEK1 and caMKK6 chimeric mice. Scale bars, 100 μ m. Right: Quantification of the ChgA-positive cells per crypt in mCherry-positive crypts and mCherry-negative crypts in the caMEK1 and caMKK6 colons (n = 3 per group). Data are presented as the means $\pm$ SDs of biologically independent samples. Ordinary one-way ANOVA test, two sided.
- k.

As box plots in (a)-(c), (f)- (h), the solid lines in each box indicate the median. The bottom

and top of the boxes are the lower and upper quartiles, respectively. Whiskers indicate the ± 1.5 interquartile range (IQR). Kruskal–Wallis test, two sided.

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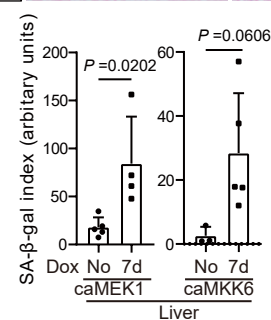
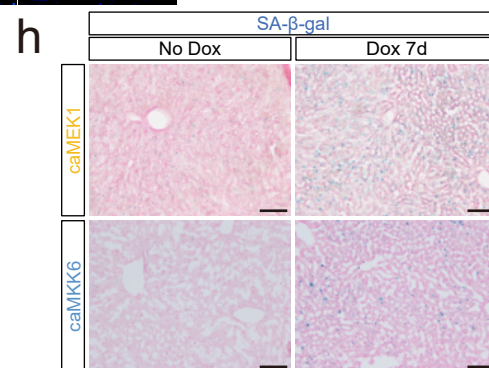
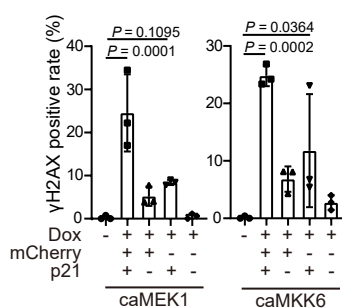
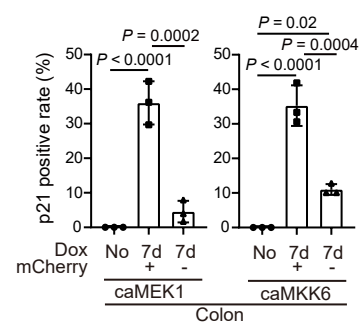
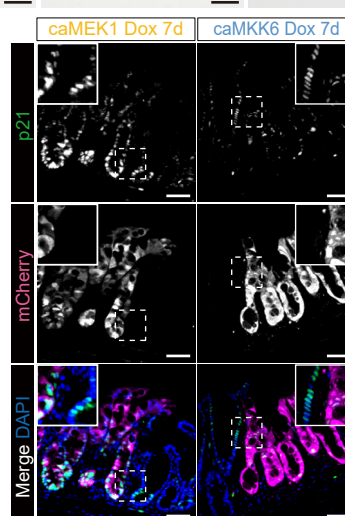
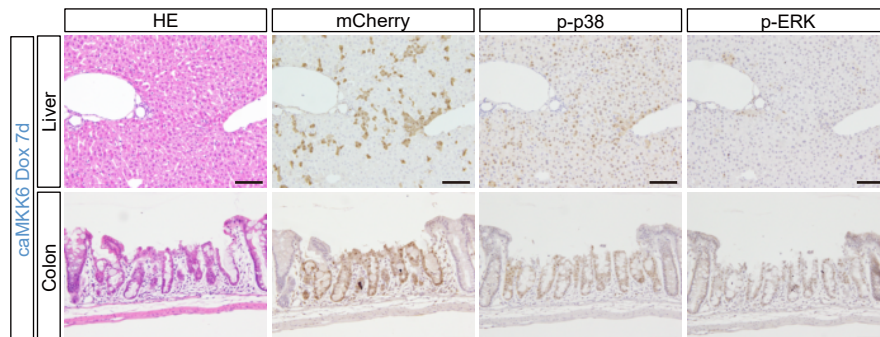
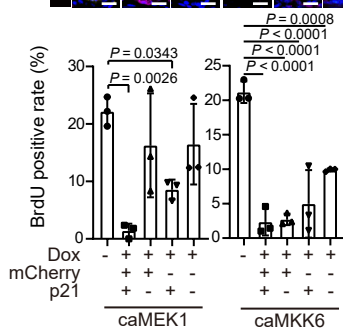
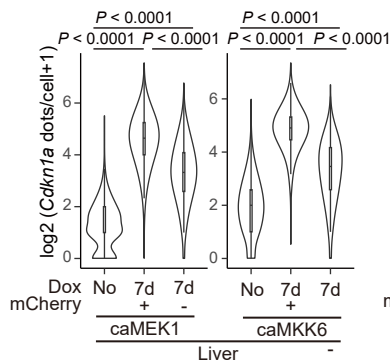
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a



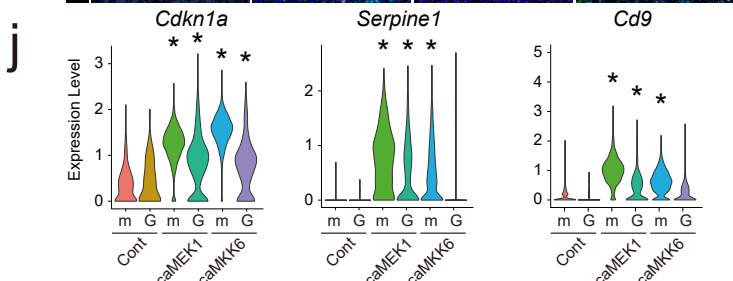
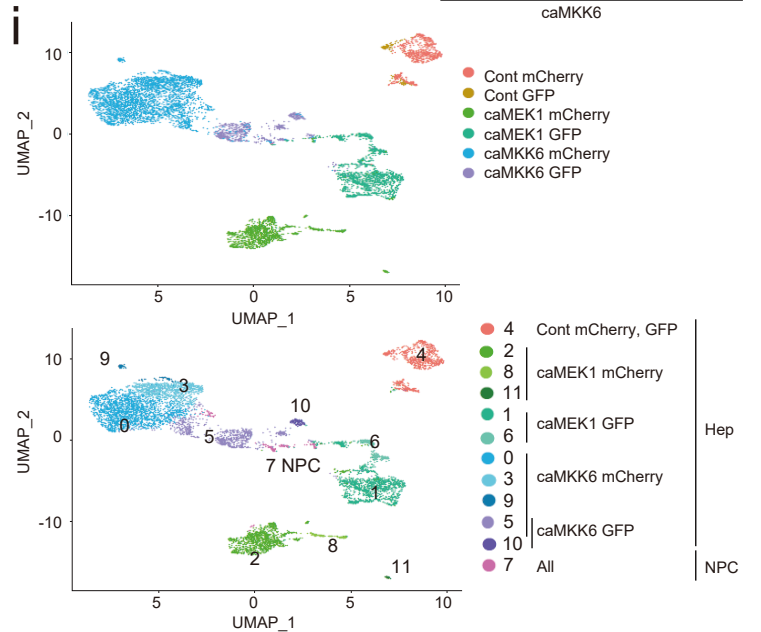
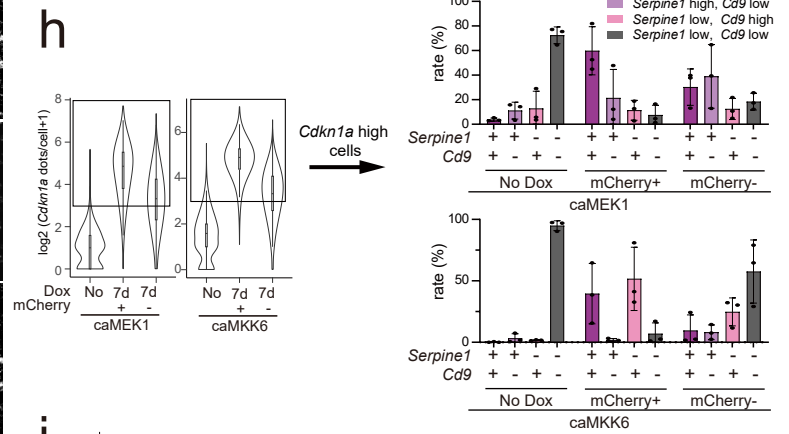
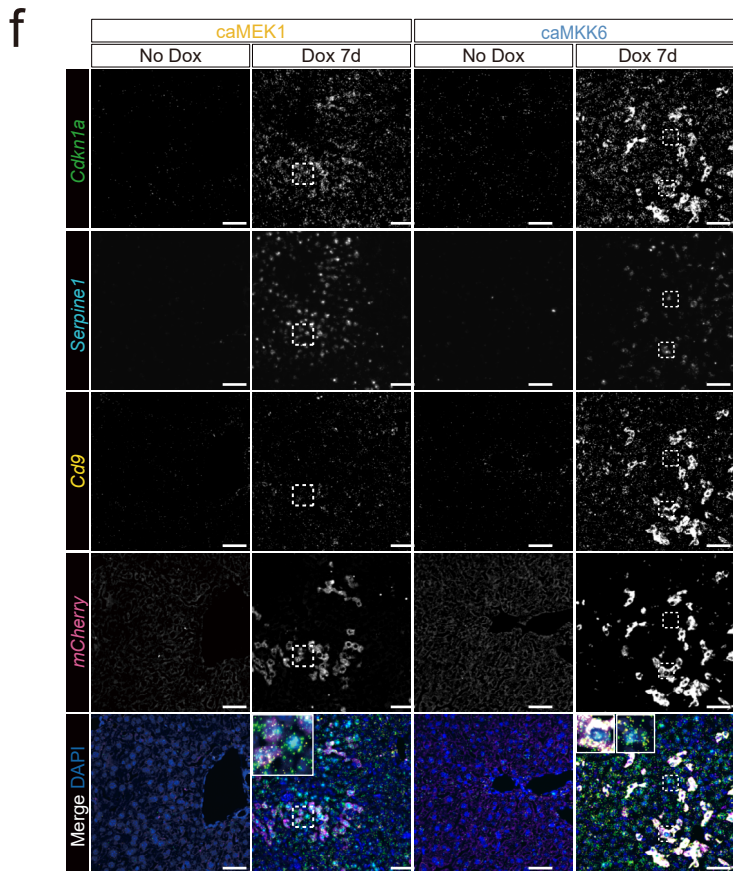
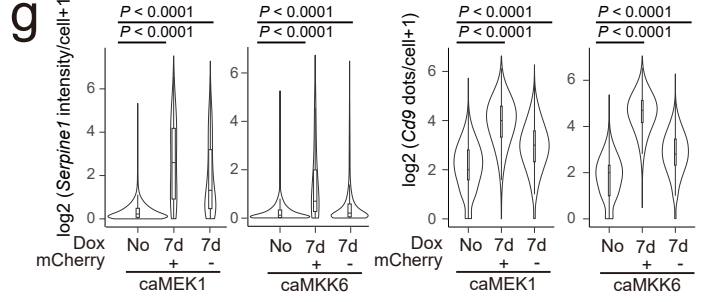
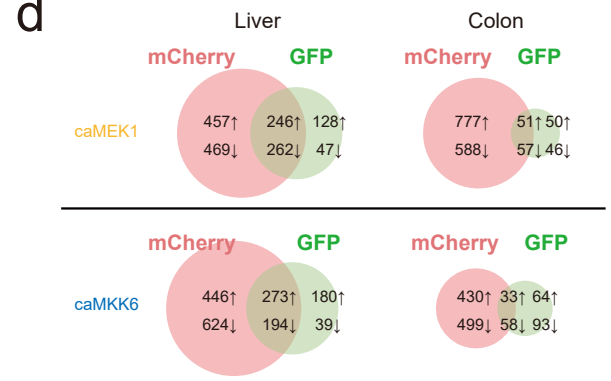
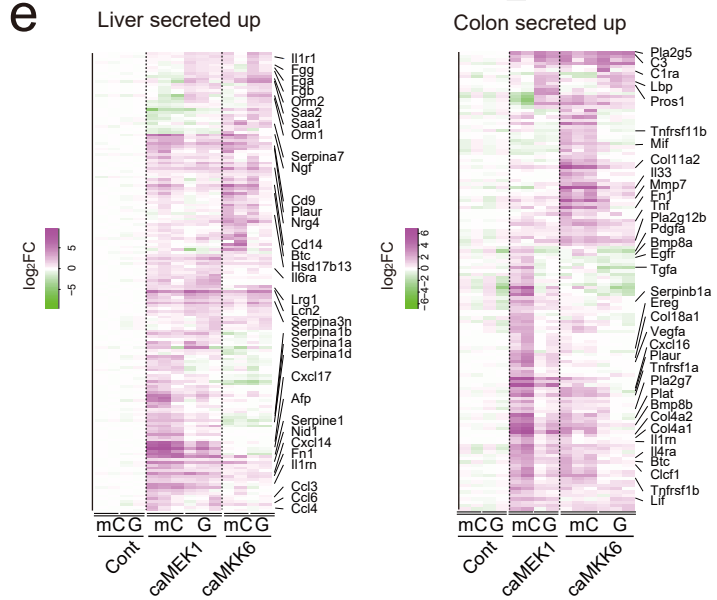
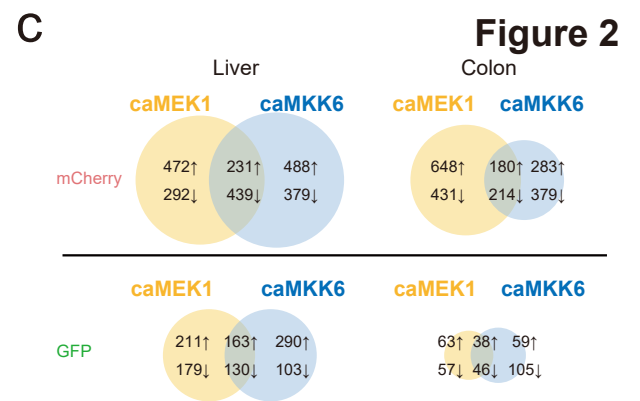
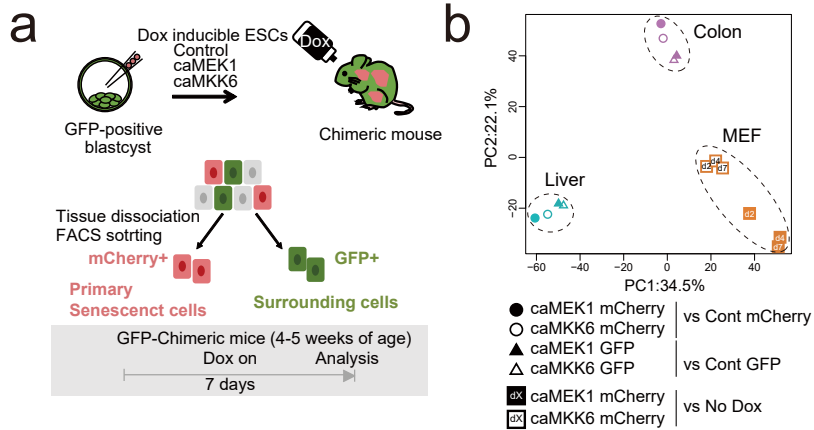
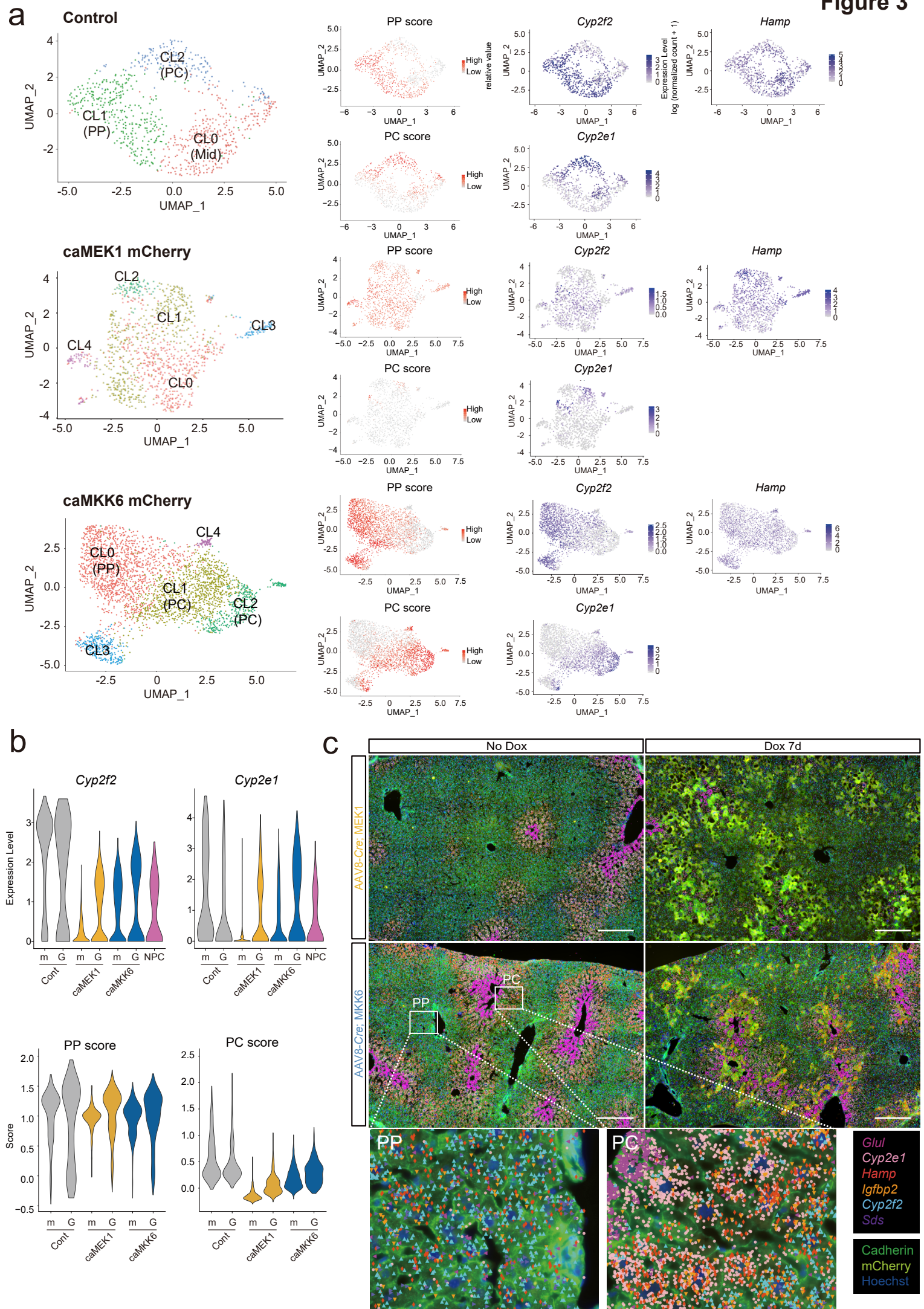


Figure 3

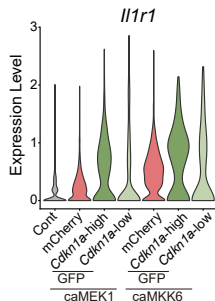


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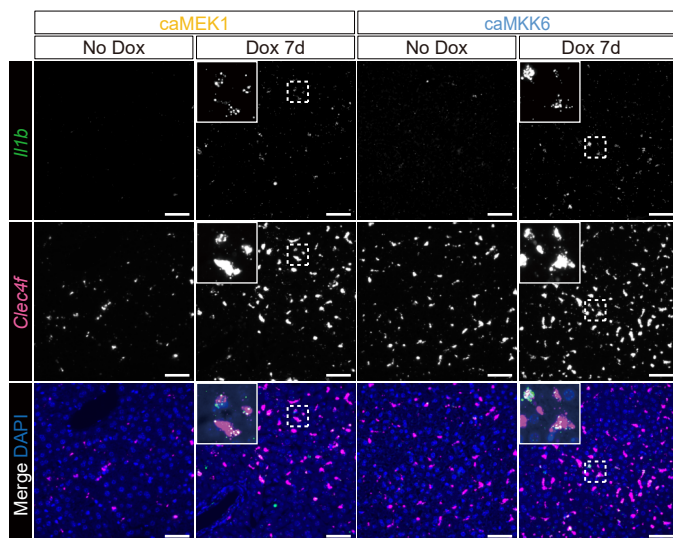
IPA analysis

caMEK1 GFP <i>Cdkn1a</i> -high		caMKK6 GFP <i>Cdkn1a</i> -high	
Upstream Regulator	P value of overlap	Upstream Regulator	P value of overlap
IL6	1.74E-28	IL6	2.62E-16
IL1B	3.47E-19	Immunoglobulin	6.46E-13
STAT3	1.23E-17	IL1B	1.13E-12
CEBPB	1.74E-16	TNF	2.29E-12
OSM	7.41E-16	CEBPB	3.63E-12
NFKBIA	1.8E-15	HFE	1.14E-10
MTDH	8.15E-14	CEBPA	2.47E-09
Stat3-Stat3	8.42E-14	NR1H3	3.25E-09
LIF	1.95E-13	NR1H3	1.06E-08
TNF	2.88E-13	NFE2L2	1.15E-08

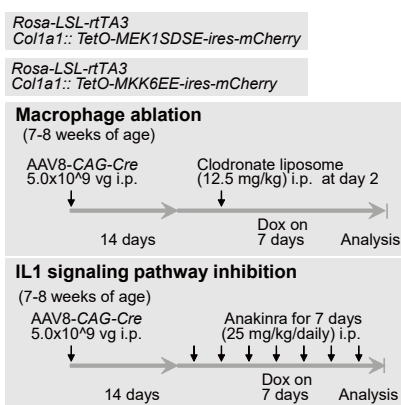
b



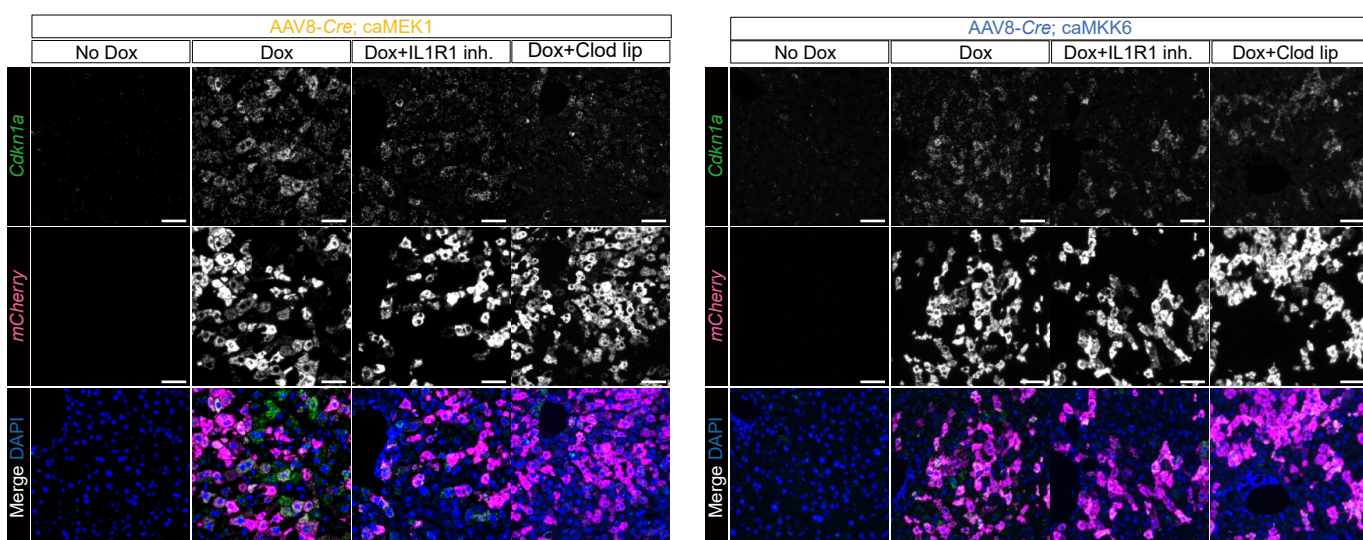
d



e



f



c

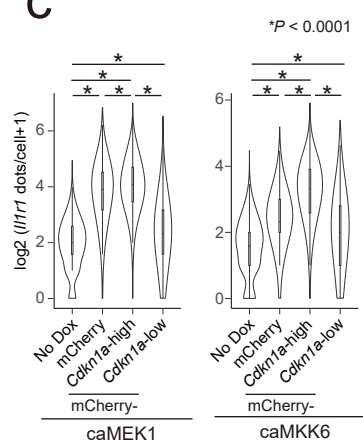
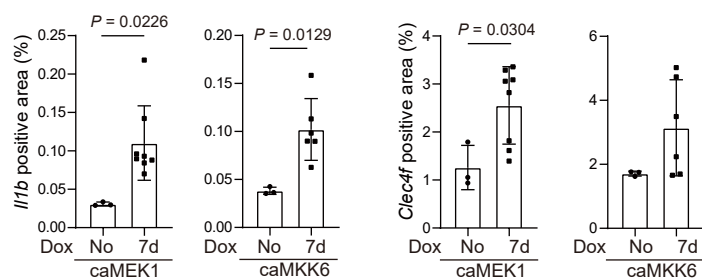
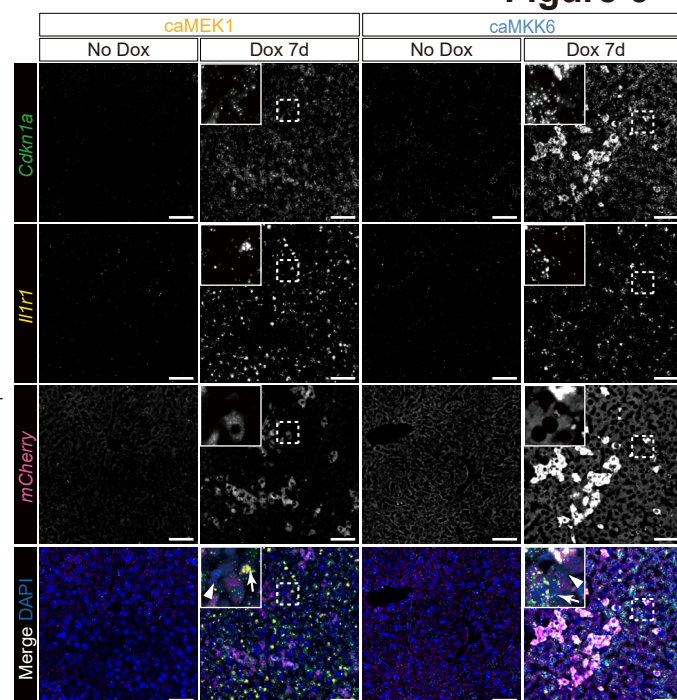


Figure 6



g

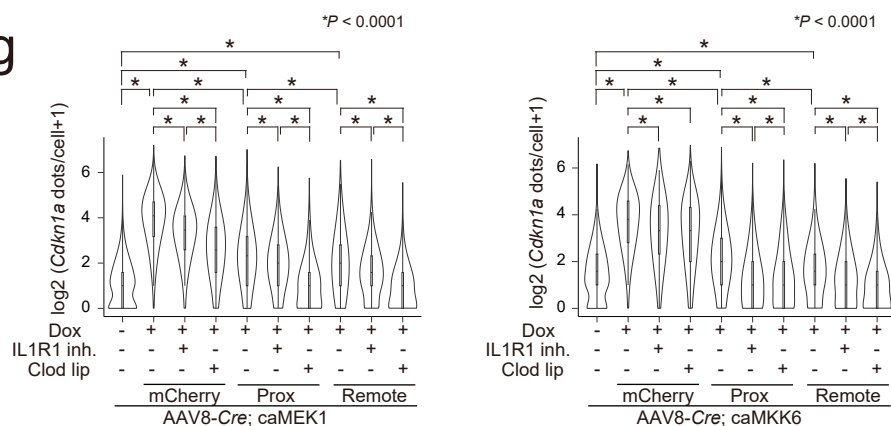


Figure 4

a

caMEK1 mCherry vs Control DEG	gene number	
	up	down
CL0 vs Control	161	637
CL1 vs Control	188	613
CL2 vs Control	203	625
CL3 vs Control	152	658
CL4 vs Control	175	895
Common	103	489

b

Upregulated subcluster specific genes
caMEK1 mCherry

GO:0071560~cellular response to transforming growth factor beta stimulus

GO:2000377~regulation of reactive oxygen species metabolic process

GO:0060333~interferon-gamma-mediated signaling pathway

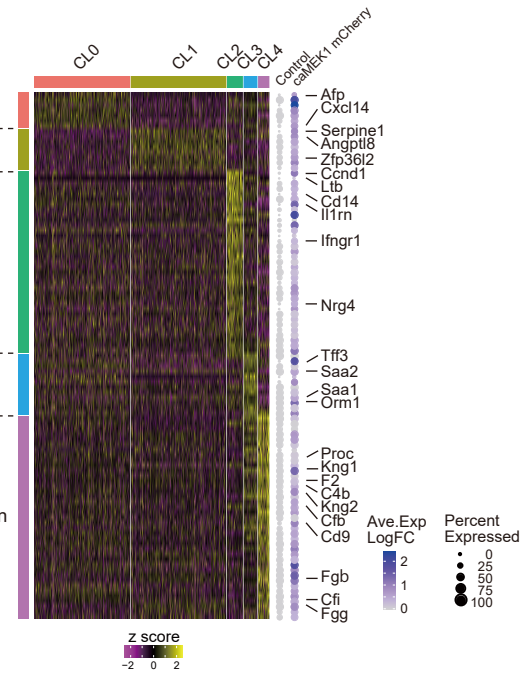
GO:0032760~positive regulation of tumor necrosis factor production

GO:0006953~acute-phase response

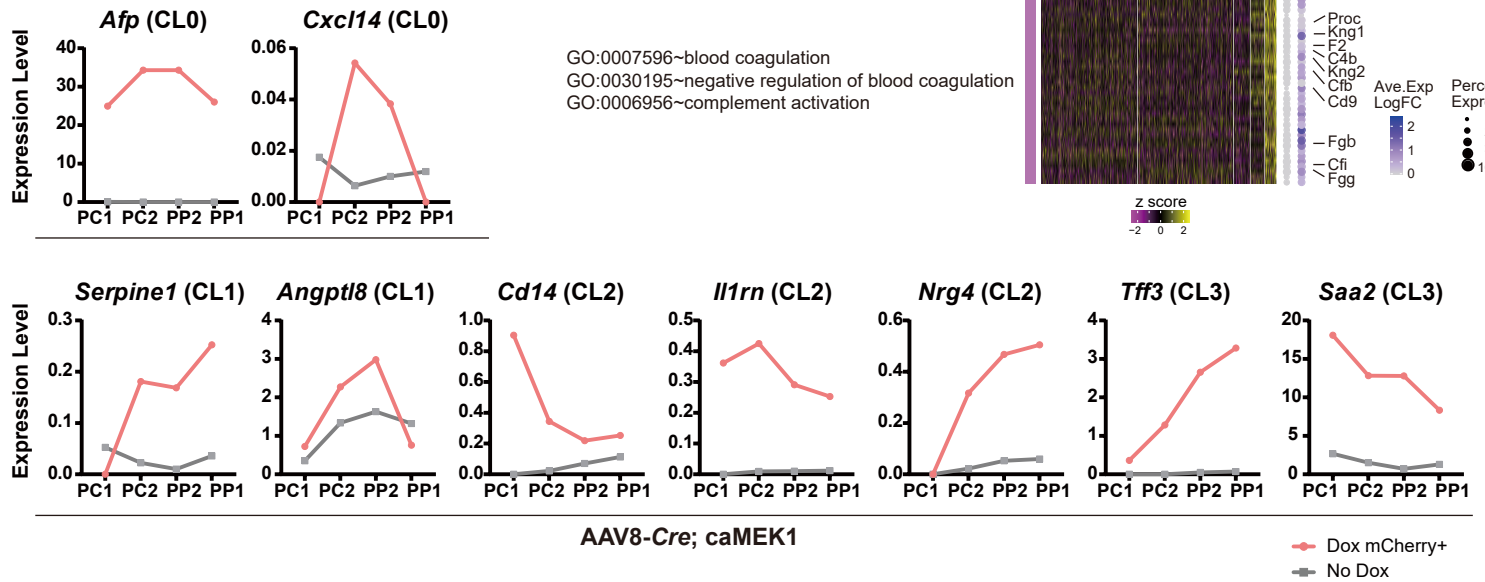
GO:0007596~blood coagulation

GO:0030195~negative regulation of blood coagulation

GO:0006956~complement activation



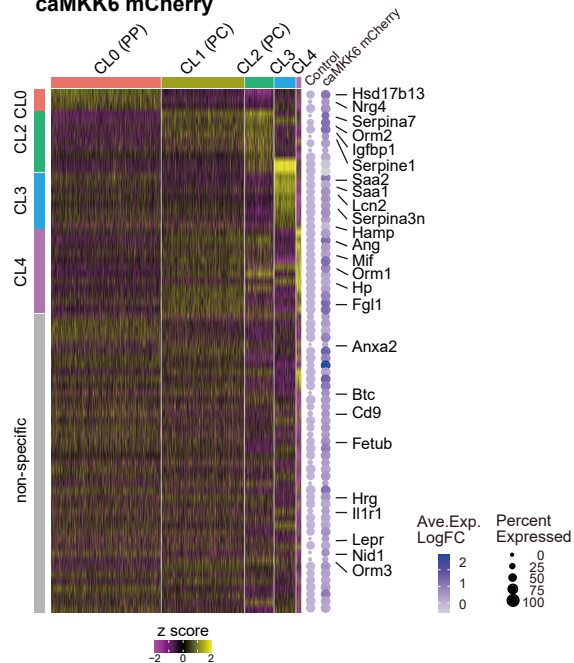
c



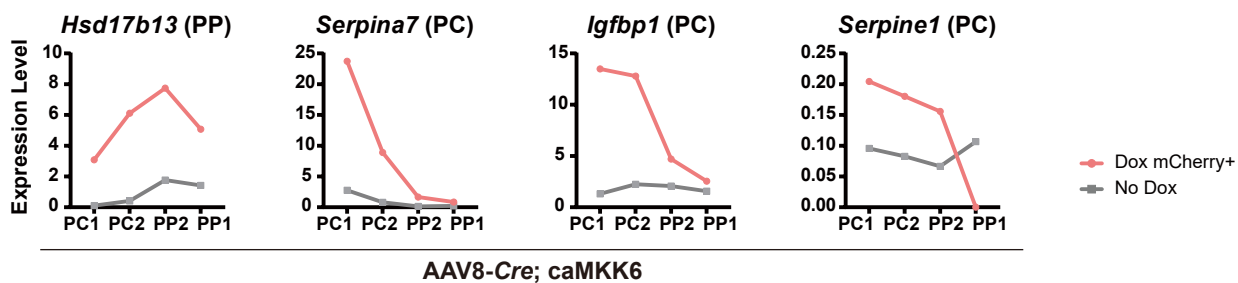
d

caMKK6 mCherry vs Control DEG	gene number	
	up	down
CL0 (PP) vs Cont CL1 (PP)	236	392
CL1 (PC) vs Cont CL2 (PC)	239	443
CL2 (PC) vs Cont CL2 (PC)	230	420
CL3 vs Cont CL1 (PP)	143	284
CL4 vs Cont CL0 (Mid)	277	403
Common	74	205

e

Upregulated secreted genes
caMKK6 mCherry

f



a

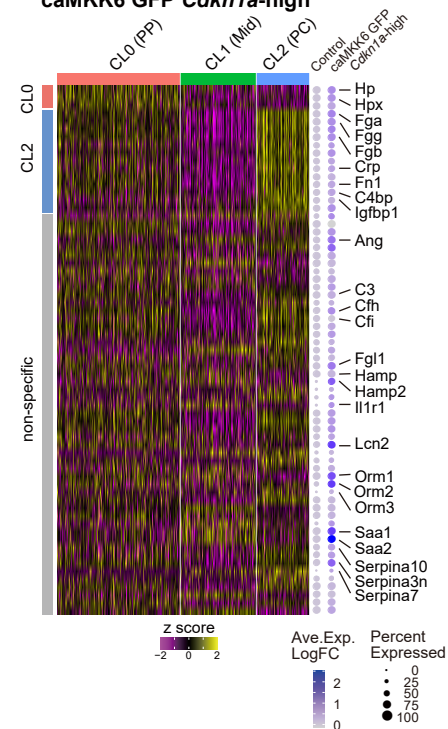
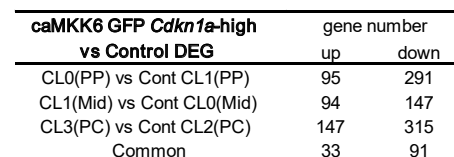
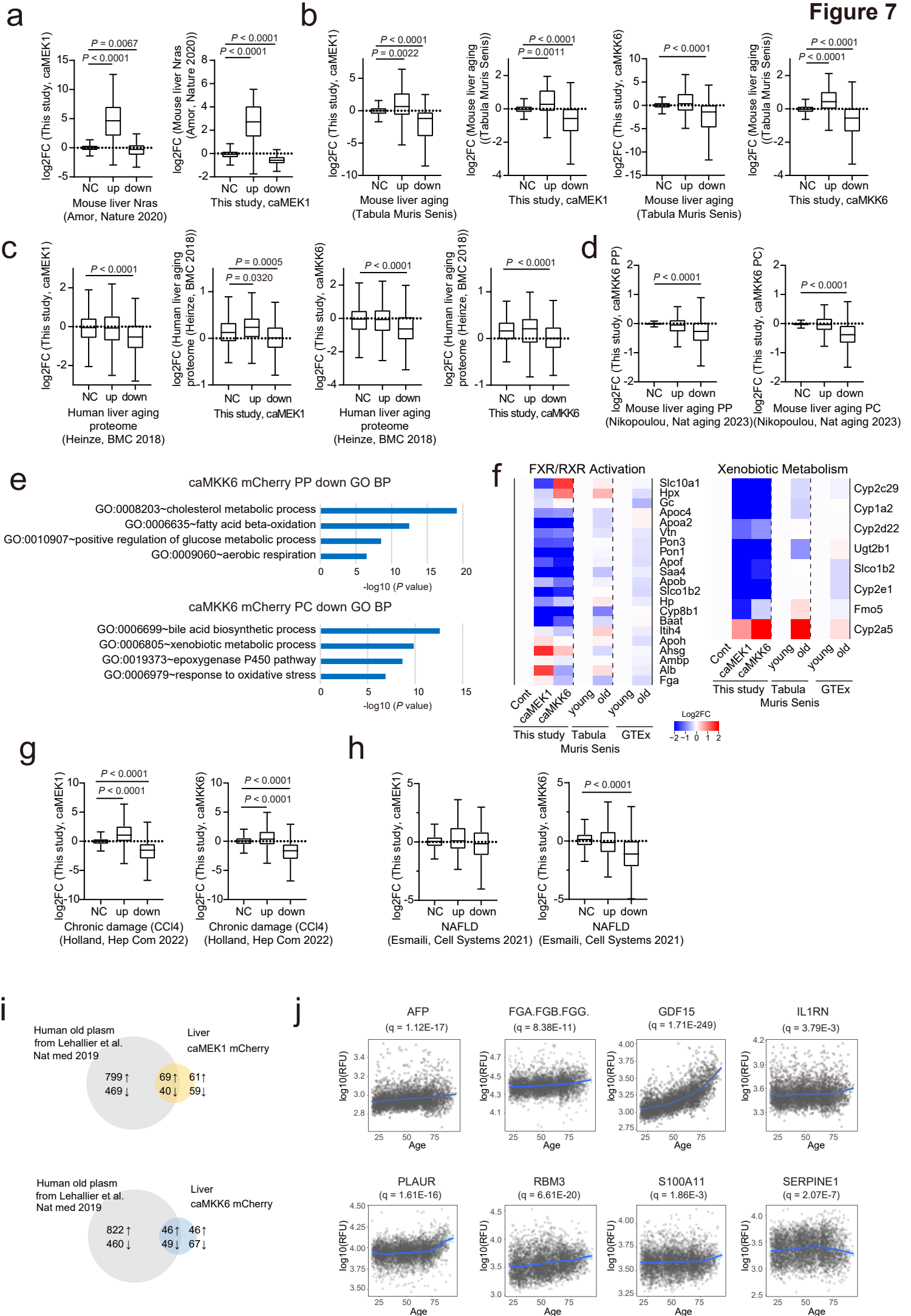
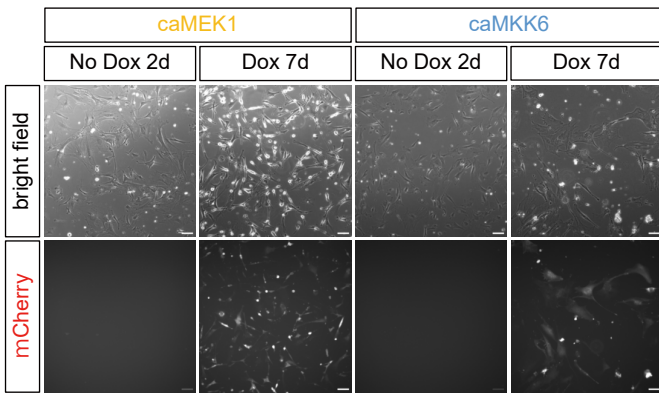


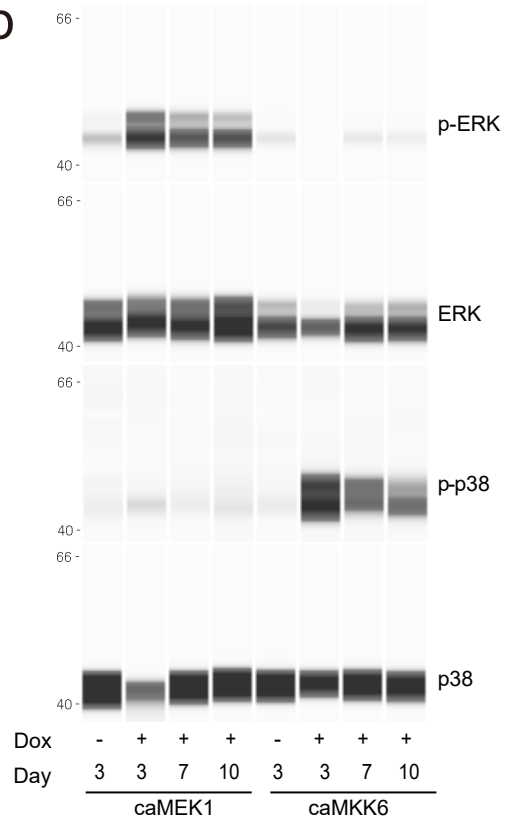
Figure 7



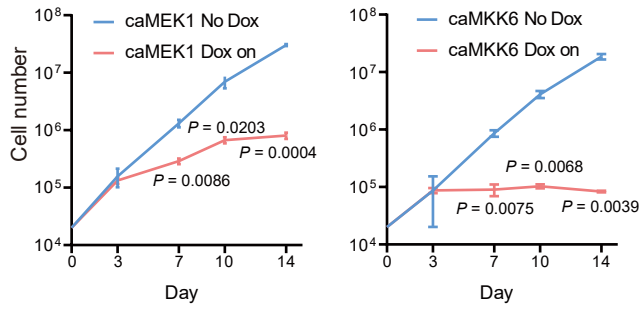
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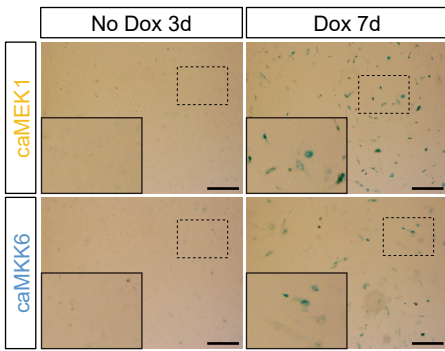
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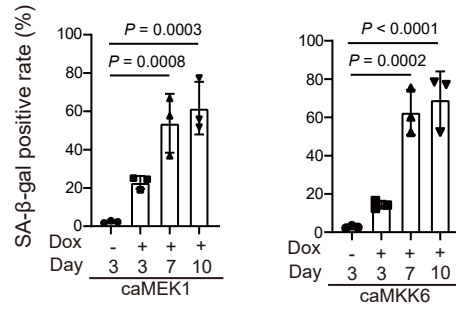
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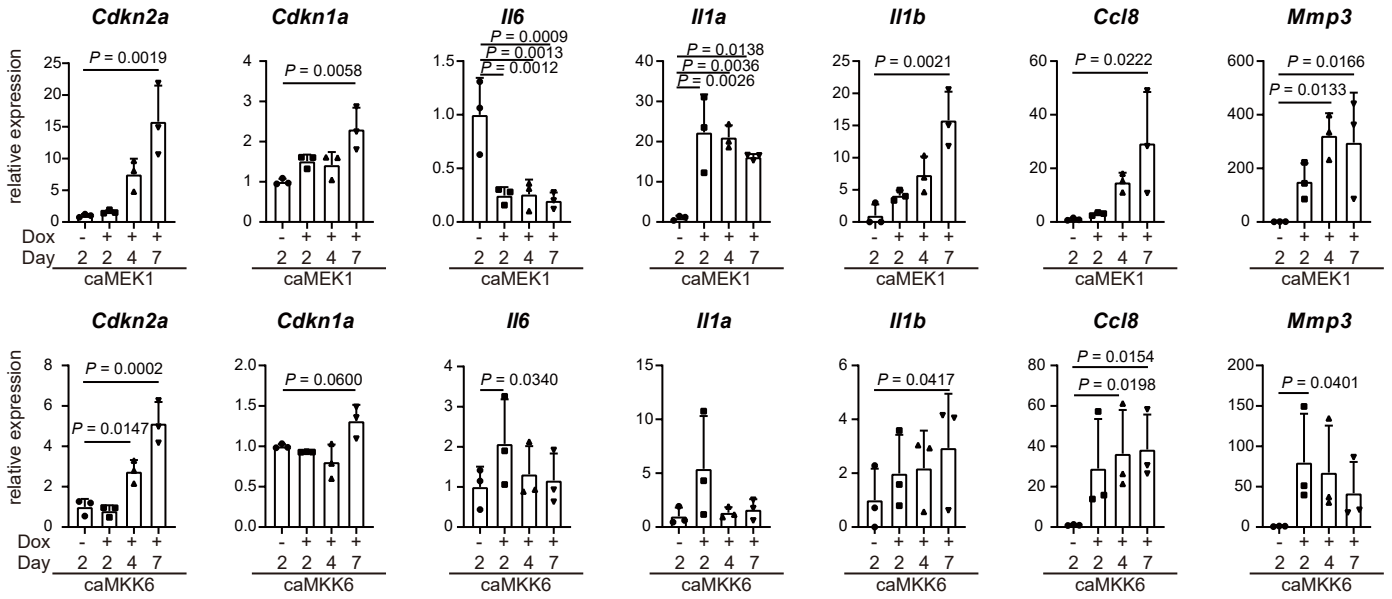
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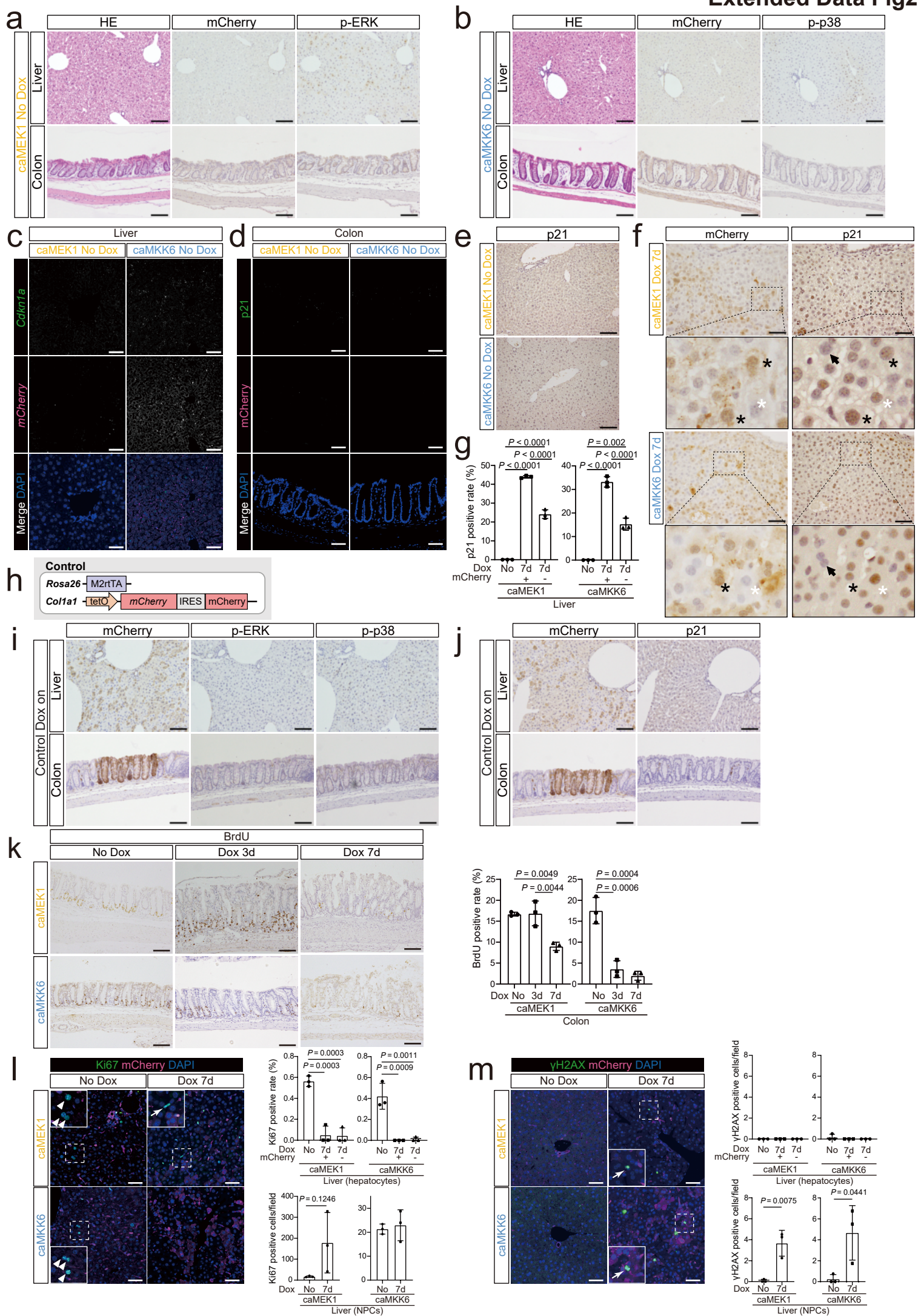


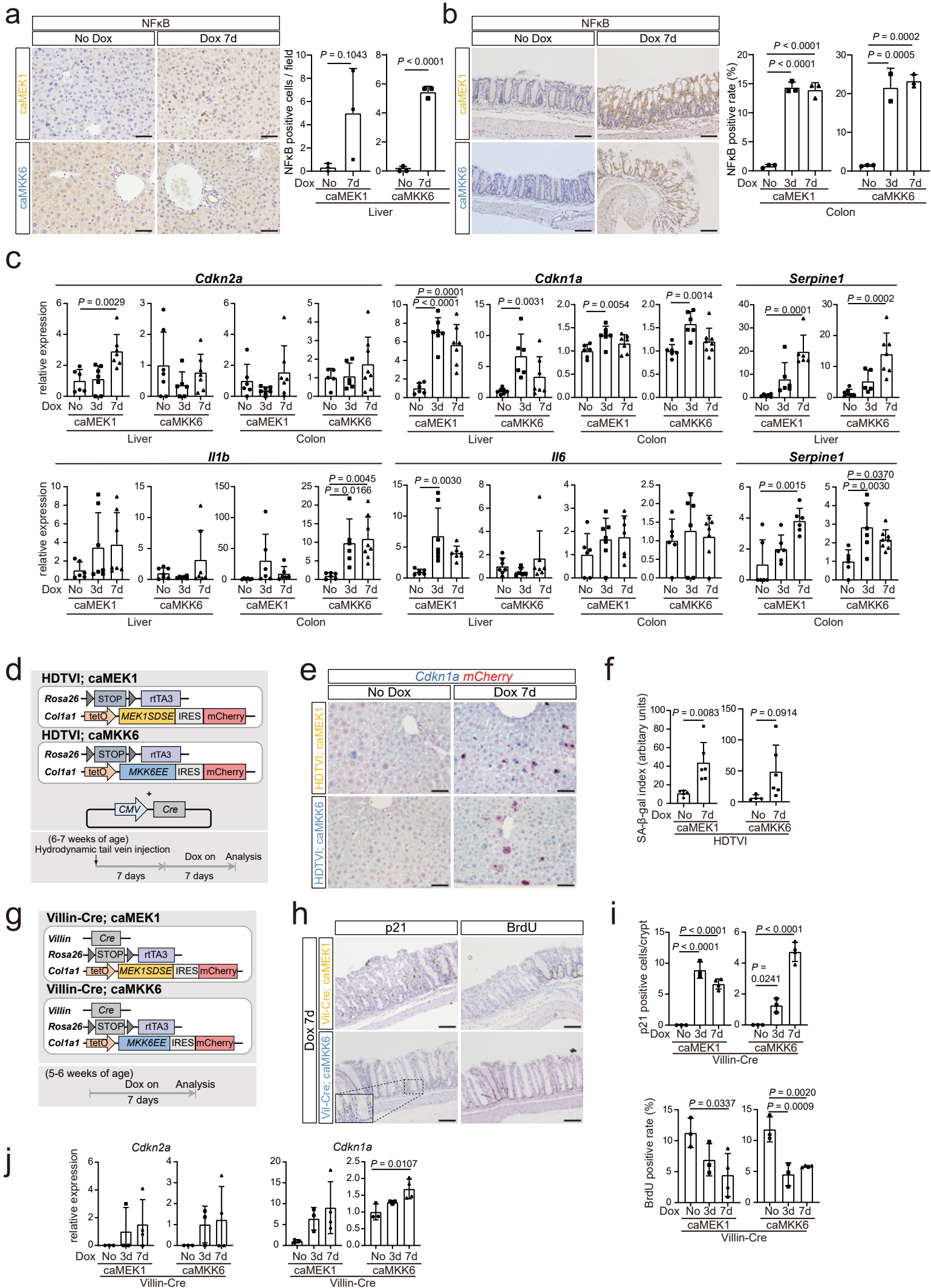
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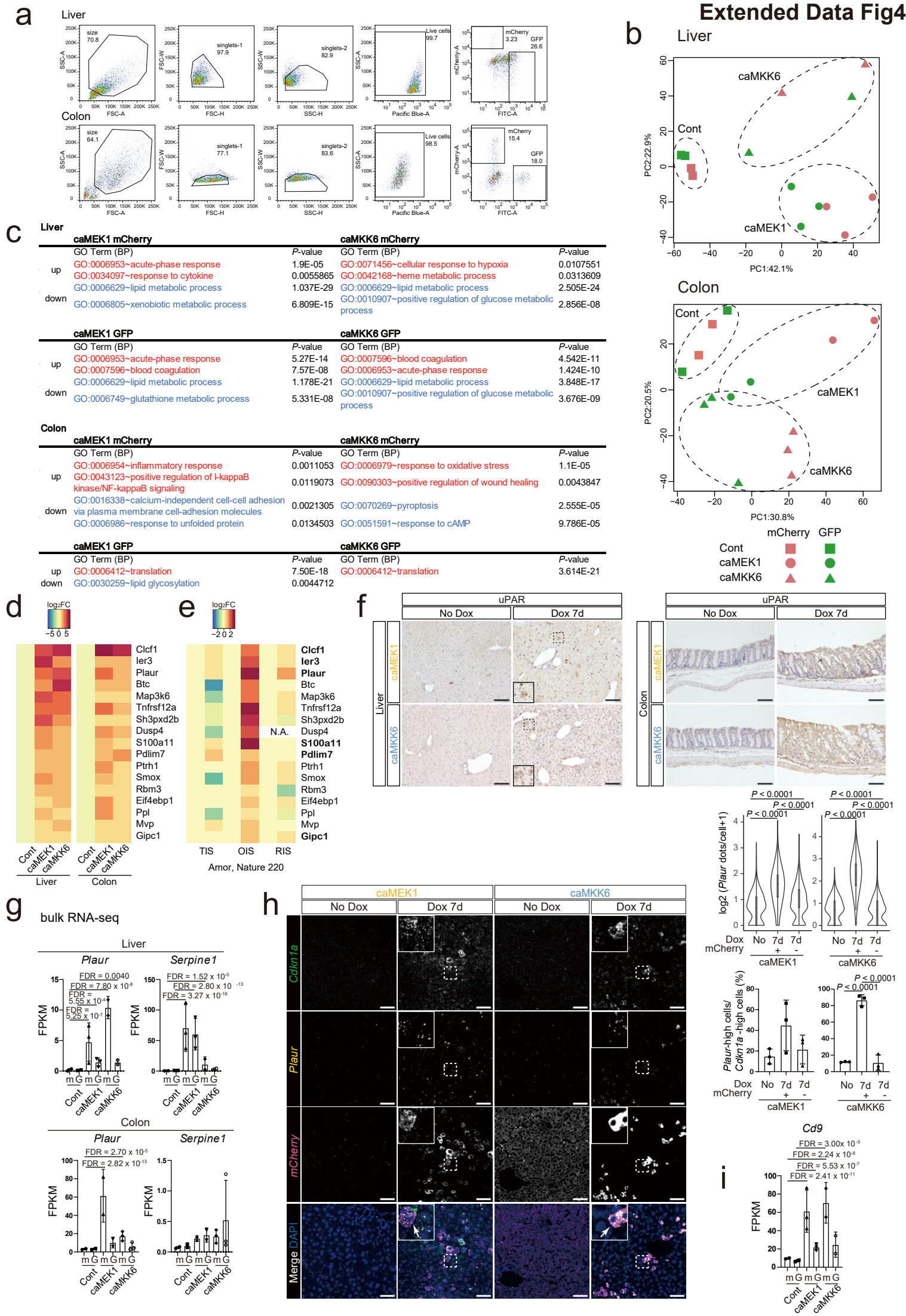
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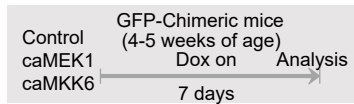


Extended Data Fig4

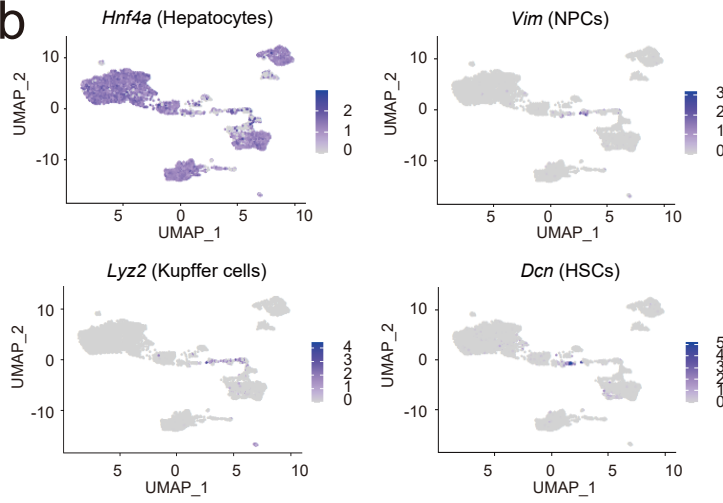


Extended Data Fig5

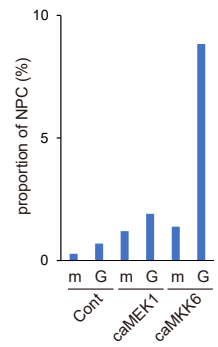
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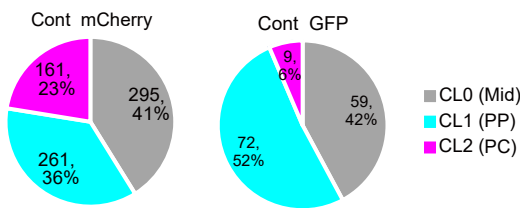
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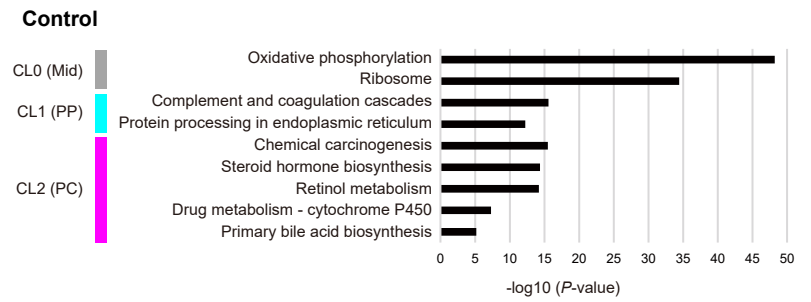
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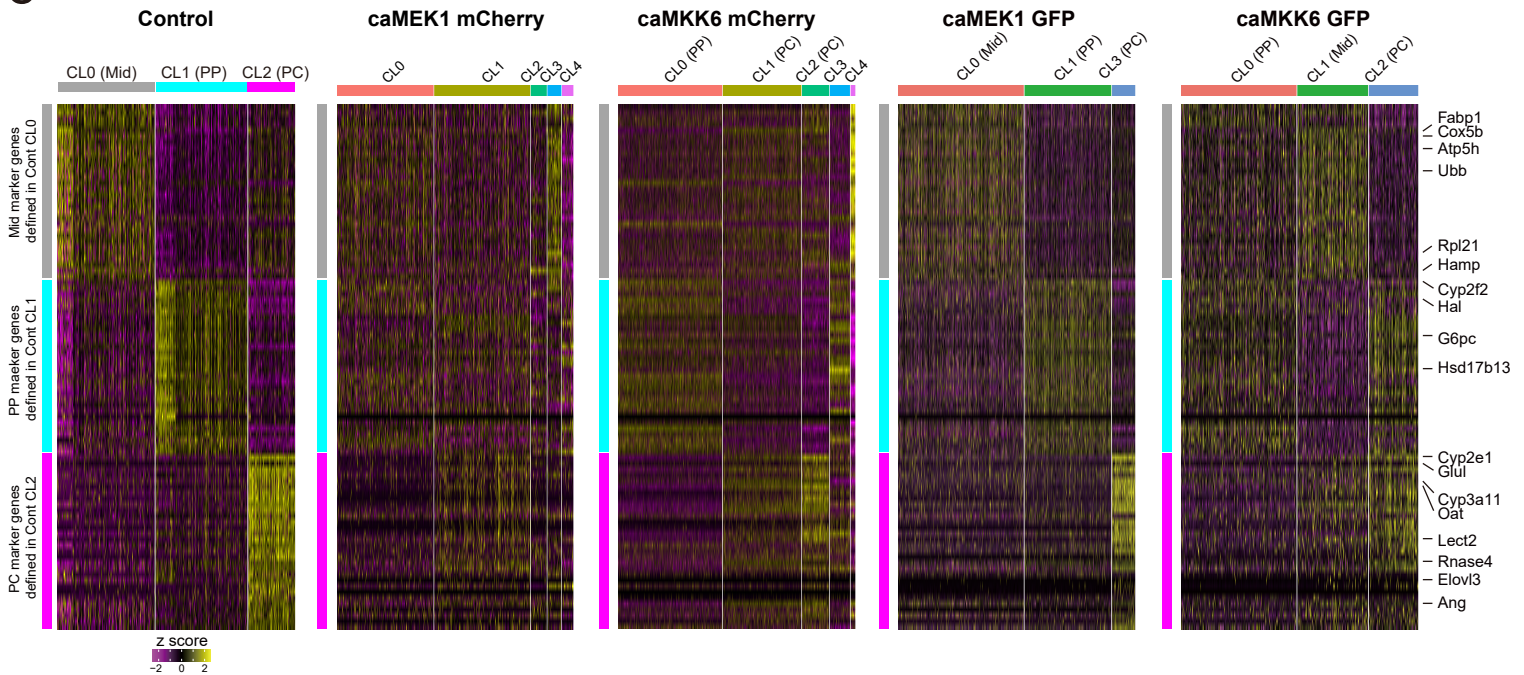
d



f



e



g

