Single cell transcriptomics of X-ray irradiated *Drosophila* wing discs reveals heterogeneity related to cell-cycle status and cell location

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Abstract
Even seemingly homogeneous populations of cells can express phenotypic diversity in response to environmental changes. Thus, the effects of X-ray irradiation on tissues composed of diverse cell types is likely to be complex. We have used single-cell RNA sequencing to study the effects of X-ray radiation on the wing-imaginal disc of *Drosophila*, a relatively simple tissue that is mostly composed of epithelial cells. Transcriptomic clustering of cells collected from the wing disc generates clusters that are mainly grouped based on cell location in the proximodistal axis. To quantify heterogeneity of gene expression among clusters, we adapted a metric used to study market concentration, the Herfindahl-Hirschman Index. We show that genes involved in DNA damage repair, alleviation of reactive oxygen species, cell-cycle progression, and apoptosis are expressed relatively uniformly. In contrast, genes encoding a subset of ligands, notably cytokines that activate the JAK/STAT pathway, transcription factors implicated in regeneration such as Ets21C, and some signaling proteins are expressed in more restricted territories. Several of these genes are still expressed in a p53-dependent manner indicating that regional and radiation-induced factors combine to regulate their expression. We similarly examined heterogeneity within territories by using a clustering approach based on cell-cycle gene expression. Using this method, we identified a subpopulation characterized by high levels of *tribbles* expression that is mostly found in irradiated discs. Remarkably, this subpopulation accounts for a considerable fraction of radiation-induced gene expression, indicating that cellular responses are non-uniform even within territories. Thus, both inter-regional and intra-regional heterogeneity are important features of tissue responses to X-ray radiation.

Introduction

Ionizing radiation (IR) is a damaging form of energy present in trace amounts in many environments and is commonly used at high doses in the form of X-rays to treat cancers. Worldwide, it is estimated that radiation therapy is an important component of treatment for more than 50% of cancers including those that arise in the brain, breast and prostate. Even in the early years of radiation research, it became apparent that different types of cells varied considerably in their sensitivity to radiation. For example, lymphocytes were rapidly depleted from tissues following radiation, while tissues such as the kidney and liver seemed far more resilient (reviewed by McBride & Schaue, 2020). In general, tissues that turnover rapidly are more sensitive. Furthermore, within tissues, stem cells are most sensitive while differentiated cells are relatively radioresistant. Even within tissues composed of relatively homogenous populations of cells, cells at particular stages of the cell cycle (e.g. G2/M) are more radiosensitive and cells that are more hypoxic tend to be radioresistant (Withers, 1975).

Most of our mechanistic understanding of how cells react to ionizing radiation comes from genetic studies of the DNA damage response (DDR) in yeast and from biochemical studies of cultured mammalian cells (Harper & Elledge, 2007; Pizzul et al., 2022).These studies have identified the sequence of biochemical reactions that are activated by double-stranded breaks in DNA and culminate in DNA Damage repair. They have also highlighted the importance of the reactive oxygen species (ROS) generated by ionizing radiation that contribute to the damage inflicted on cellular macromolecules. Importantly, cellular damage can result in the activation of mechanisms that arrest the cell cycle to enable a restoration of cellular homeostasis or, failing that, to activate pathways that promote apoptotic cell death. A key player in mammalian cells is the p53 protein which is stabilized following DNA damage and activates the transcription of genes that promote both cell cycle arrest and apoptosis (Levine, 2020).

Studies in *Drosophila* have made important contributions to our understanding of the deleterious effects of ionizing radiation. Indeed, the discovery that X-rays generated mutations in a dose-dependent manner was first discovered in Drosophila (Muller, 1927). Subsequent genetic studies identified loci that made flies more susceptible to the effects of DNA-damaging agents – many of these genes encode proteins now known to function in DDR (Sekelsky, 2017). The wing imaginal disc, the larval precursor of the adult wings and part of the thorax (Tripathi & Irvine, 2022), emerged as an attractive model for studying the effects of IR on a relatively simple tissue (Haynie & Bryant, 1977; James & Bryant, 1981). The wing disc derives from a precursor population of approximately 30 cells in the embryo (Worley et al., 2013) and, because of cell proliferation, is composed of more than 30,000 cells by the end of the larval stages (Martín et al., 2009). Most of the wing disc is composed of epithelial cells and a small fraction of myoblasts which are precursors of the adult flight muscles. (Gunage et al., 2014).

Haynie and Bryant (1977) irradiated larval imaginal discs and used clone marking techniques to show that IR at a dose of 1000 rad (10Gy) kills 40-60% of cells and irradiation with 4000 rad kills approximately 85% of cells. Despite this, compensatory proliferation allowed for the development of wings of normal size and shape. In a study using gamma irradiation (James and Bryant, 1981), it was shown that cell death is observed as soon as 4 h after irradiation and continues for up to 44 h. In parallel, there is a dramatic decrease in the number of cells undergoing mitosis within 1 h with a resumption after 8 h. More recent studies using flow cytometry and FUCCI have shown that cells accumulate preferentially in the G2 phase of the cell cycle after irradiation (Ruiz-Losada et al., 2022). Thus, as in mammalian cells, the two obvious cellular responses to IR are cell-cycle arrest and apoptosis. A key difference, however, is that unlike in mammalian cells where p53 functions in both pathways, Drosophila p53 promotes apoptosis but does not seem to function in arresting the cell cycle (Brodsky et al., 2000; Ollmann et al., 2000). In p53 mutants, the expression of genes that function in a wide variety of cellular responses to DNA damage is reduced and apoptosis within 4 h of exposure to IR does not occur. Instead, there is a delayed phase of cell death that involves multiple pathways and aneuploid cells persist in the tissue even into the pupal phase (Akdemir et al., 2007; Brodsky et al., 2004; Brown et al., 2020; Wells et al., 2006; Wells & Johnston, 2012; Wichmann et al., 2006).

Even in a relatively simple tissue such as the wing disc, cells display considerable heterogeneity in their response to radiation. While high levels of cell death are observed in the wing pouch, the dorsal hinge shows reduced levels. The relative radioresistance of this region is dependent upon Wnt and STAT signaling (Verghese & Su, 2016). In more mature discs, more cell death is observed in the intervein regions of the pouch than in regions fated to generate veins (Moon et al., 2005). Thus, even in regions of the disc where there are no obvious morphological differences between cells, the response to radiation can differ considerably. Several studies have documented transcriptional changes at a genome wide level in Drosophila embryos (Akdemir et al., 2007; Brodsky et al., 2004; Kurtz et al., 2019; Lee et al., 2003; Ogura et al., 2009; Zhang et al., 2008) and in imaginal discs (Ledru et al., 2022; Van Bergeijk et al., 2012) following irradiation. However, because these studies have prepared RNA either from whole embryos, entire discs or from cells from specific regions without retaining the single-cell origin of transcripts, they cannot be used to assess the heterogeneity of transcriptional responses throughout the disc.

We and others have used single-cell transcriptomics to characterize differences in the transcriptome of cells from different parts of the wing disc (Bageritz et al., 2019; Deng et al., 2019; Everetts et al., 2021; Zappia et al., 2020). Since the dominant sources of transcriptional variability between cells, or stratifying factors, reflect differences in cell location along the proximodistal (PD) axis, cells from different regions of the disc can easily be identified. Additionally, we and others have characterized transcriptional changes at the single-cell level after ablation of the wing pouch using the tumor necrosis

factor (TNF) ortholog *eiger* (*egr*) (Floc'hlay et al., 2023; Worley et al., 2022) and we have previously identified a pathway downstream of the Ets21C transcription factor that functions during regeneration but not during wing disc development (Worley et al., 2022). These studies provide a foundation for characterizing transcriptional responses of the wing disc to IR and to examine the level of transcriptional heterogeneity between cells.

Here we present a comparison, at the single-cell level, of the transcriptomes of unirradiated and irradiated wing discs from late third-instar larvae. Our studies reveal heterogeneity at two different levels – between territories in the disc and between cells in individual territories. We show that regional heterogeneity is more a feature of some classes of genes over others and use quantitative approaches to investigate heterogeneity more generally.

Results

X-ray irradiation induces widespread DNA damage but apoptosis and cell-cycle arrest occur nonuniformly

The wing disc consists of three major regions that span its PD axis: The notum, which generates most of the adult thorax, the hinge, which develops into the hinge which connects the wing blade to the thorax, and the pouch, which develops into the wing blade (Fig. 1A). We first characterized the primary effects of X-rays on these regions. In order to confirm that X-rays induce DNA damage in each of the major PD regions, we used immunofluorescence to visualize phosphorylated histone H2Av (p-H2Av), a modified histone variant generated after DNA damage that serves as an early mark of double-strand break repair (Madigan et al., 2002).

We found that in unirradiated discs, p-H2Av fluorescence was present in relatively low levels throughout the tissue (Fig. 1B). Thirty minutes after irradiation, a time point conducive to capturing an initial response to DNA damage, we observed a global increase in p-H2Av signal, with little inter-regional variability (Fig. 1C). To test for X-ray induced cell death, performed immunofluorescence (IF) staining using an antibody that targets cleaved Dcp-1, an effector caspase that is active during apoptosis (Song et al., 1997). Unirradiated discs showed low levels of Dcp-1 signal likely associated with normal development (Fig. 1D). Four hours after X-ray exposure, discs showed a marked increase in Dcp-1 signal in each of the major regions of the disc (Fig. 1E). In contrast to p-H2Av fluorescence, anti-DCP1 signal is non-uniform. High levels of apoptosis were observed in the wing pouch but cells along the dorsoventral boundary were spared. Additionally apoptosis is reduced in portions of the wing hinge as has been observed previously (Verghese & Su, 2016).

To examine the proliferative effects of X-ray exposure, we used IF staining against phosphohistone H3 (PHH3), a modified H3 histone state associated with chromatin condensation during mitosis (Hendzel et al., 1997). PHH3 signal was observed across all regions of unirradiated discs (Fig. 1F). In irradiated discs, we observed a global reduction of PHH3 signal as soon as 15 minutes after exposure (Fig. 1G) with greater reduction at 2 and 4 hours after exposure (Fig. 1H-1I). This is consistent with prior observations that cells accumulate in G2 following IR exposure and fail to enter mitosis (Ruiz-Losada et al., 2022). As an additional measure of proliferation, we visualized DNA synthesis using EdU labeling, where positive labeling is associated with an active S-Phase (Salic & Mitchison, 2008). Like PHH3 staining, EdU labeling was observed across all regions of the tissue in unirradiated discs (Fig. 1J). Up to two hours after irradiation, the distribution of EdU labeling resembled that of unirradiated discs (Fig. 1K-1L), while at four hours after exposure, there was a consistent reduction in EdU labeling in the notum and pouch regions of the tissue, while the hinge was largely spared from this reduction (Fig. 1M, Fig. S1_1-S1_2). The persistence of EdU incorporation in the hinge could either indicate that a proportion of cells in this region continue to enter S-phase or, alternatively, that there are higher levels of DNA synthesis related to DNA repair in this region. Taken together, our observations indicate that different regions of the disc show differences in response to radiation both in terms of parameters related to cell-cycle progression and to apoptosis.

Cells of unirradiated and irradiated wing discs show similar patterns of expression of many regionally-expressed genes

Since we have observed differences in cell death and DNA synthesis across the disc, it is likely that there are transcriptomic differences between regions. To explore changes in gene expression on a genome-wide basis across the disc, we used scRNAseq. Previous scRNAseq studies of the disc have shown that genes with patterns of expression restricted to different regions of the PD axis can be used as markers to estimate the approximate anatomical location of cell clusters (Bageritz et al., 2019; Deng et al., 2019; Everetts et al., 2021; Zappia et al., 2020). These include *zfh2* expressed in the hinge (1A, A'') and *nubbin* (*nub)* expressed in the pouch (Fig. 1A', 1A''). In irradiated discs, we found no major changes in the general expression pattern of either gene (Fig. 1B-B''). This suggests that these two marker genes can be used to accurately identify hinge and pouch cells in both conditions.

To examine gene expression on a genome-wide scale, we performed single-cell RNA sequencing (scRNA-seq) on wing discs collected from late third instar larvae 4h after exposure to 4000 rad X-rays, and from larvae of the same stage that were unexposed to irradiation. For both samples two replicates were collected. To generate datasets containing high-quality cells, several filtering steps were applied. In brief, cells that were positively stained with propidium iodide, indicating a compromised cell membrane, were removed via FACS. After sequencing, cells within each dataset that had low numbers of

captured genes were removed from further analysis. For analysis, all four datasets were integrated using Seurat V5's anchor-based canonical correlation analysis (CCA) integration process (see methods for details). Cells were grouped into clusters in the integrated dataset using the Louvain algorithm (default in Seurat V5) with a resolution parameter of 2, resulting in 35 clusters (Fig. 2C).

We found that each dataset was individually stratified across its PD axis, as well as when integrated, as indicated by a separation of PD markers between clusters (Fig. 2D-2H). Clusters were classified as belonging to one of seven broad PD regions based on marker expression: The pouch, hinge-pouch, hinge, hinge-notum, notum, PE, and Myoblasts. For the pouch, hinge-pouch, and hinge: *nub+, zfh2-* positive clusters were annotated as pouch, *nub+, zfh2+* clusters as hinge-pouch, and *nub-, zfh2+* as hinge. For the hinge-notum and notum: *tup+ (or eyg+, Fig.* S2_1A*), zfh2- clusters* were annotated as notum, and *tup+ (or eyg+), zfh2+* as hinge-notum. The one *twi+* cluster was annotated as Myoblasts (Fig. S2_1B). The classification of clusters into broad regions is imperfect, as there are some examples of clusters expressing moderate levels of marker genes belonging to the region that neighbors the one they were classified into (e.g. *zfh2* is expressed at moderate levels in some cells belonging to P2).

To identify unique cluster markers, we performed differential gene expression (DGE) analysis on each individual cluster compared to all other clusters, and compared to all other clusters within the same broad region (Fig. S2_1C, Table S2_T1). Unique markers indicated that many clusters likely correspond to contiguous places in the wing disc with regionally restricted gene expression of one or more genes, e.g. pouch 5, marked by *Optix,* a gene restricted in expression to a small region of the anterior pouch (Seimiya & Gehring, 2000). Other clusters likely correspond to noncontiguous regions in the wing disc with restricted gene expression of one or more genes, e.g. pouch 7, marked by *Doc1*, a gene expressed in two separate locations of the dorsal and ventral pouch (Butler et al., 2003). In two cases, clusters did not correspond to specific regions of restricted gene expression: pouch cluster 9 (P9), which was enriched for cells with S-phase markers, and pouch cluster 3 (P3), which was enriched in irradiated cells expressing the cell cycle gene *trbl* (later discussed at length). In sum, we interpret cluster identity to be primarily associated with genes of restricted, though not necessarily contiguous, regional patterns of expression in the disc with some additional contributions from other parameters such as cell-cycle state.

X-ray induced genes involved in apoptosis, DDR, and reaction to ROS are relatively homogeneous across the disc after irradiation

For an initial broad survey of changes in gene expression after irradiation, we compared the gene expression of all cells of the irradiated condition to all cells of the unirradiated condition. We applied lenient filters, retaining genes that were present in ≥1% of cells in either condition, had an absolute log2FC≥0.1, and an adjusted p value<0.05, resulting in

3,767 genes with increased expression in the irradiated condition and 1,122 genes with decreased expression. Among genes with increased expression were many of those described in previous genome-wide analyses on embryos and wing discs, including genes involved in apoptotic regulation (e.g. *rpr, hid, Corp, egr)* and genes involved in DDR and repair (e.g. *Irbp18, Xrp1, p53, Ku80, Irbp, mre11, rad50)* (Akdemir et al., 2007; Brodsky et al., 2004; Van Bergeijk et al., 2012). Similarly, among genes with decreased expression were many described previously in wing discs, including those involved in DNA replication (e.g. *PCNA, dup, Mcm3, geminin, PolE2*) (Van Bergeijk et al., 2012). For all changes, see Table S3_T1.

There are currently few methods used routinely to assess the heterogeneity of gene expression across cell clusters. There are a variety of ways that heterogeneity is quantified in economics and the social sciences (reviewed in Steele et al., 2022). In order to quantify the heterogeneity of X-ray induced genes across cell clusters, we implemented a variation of the formula used in the Herfindahl-Hirschman Index (HHI), a measure of market concentration used in economics (Herfindahl, 1950; Hirschman, 1945) (here used to measure the “concentration” of gene expression across clusters). If the share of total expression in each cluster is calculated and if those numbers are simply all added, the overall total would be 1. If however, the share of expression in each cluster is squared and then those numbers are added, the total would depend upon the distribution of expression between the clusters. If expression was distributed evenly among N clusters, then each cluster would have 1/N of the total expression. In that case the HHI would be 1/N which is the lowest possible value. In contrast, if all expression was concentrated in one cluster, then the HHI would be 1 which would be the highest possible value and which would be indicative of maximal heterogeneity between clusters. To calculate the HHI score for a given gene, the average gene expression under the irradiated condition for each of the 35 clusters was individually divided by the sum of all average expressions across clusters, squared, and then added together (Fig. 3A, formula on top). Thus, in the case of 35 clusters, the minimum possible score is 0.0286 and the maximum possible score is 1. We consider HHI scoring to be a useful heuristic method for ranking expression concentration in clustered data. However, the interpretation of HHI is limited by the quality and meaning of clusters. Despite its limitations, HHI applied to clusters here, which are primarily defined by genes of spatially restricted expression, allows us to rank genes in terms of their spatial heterogeneity.

To see if there were differences in expression concentration between classes of genes that may be important for X-ray response, we applied HHI using the 35 clusters to 521 genes enriched at 4000 rad that belonged to one of nine categories: Apoptosis, DDR, response to ROS, cell cycle regulation, transcription factors (TFs), phosphatases, kinases, ligands, and receptors (see S3_T2 for complete list of genes used before filtering to 521). The mean HHI for all included genes was 0.037 with a minimum value of 0.029 (*eff)* and a maximum value of 0.25 (*tup)*, the theoretical range being between

0.0286 (least concentrated) and 1 (most concentrated). The classes of genes that have been studied most intensively in the context of cellular responses to radiation (apoptosis, DDR, ROS, cell cycle) are all expressed relatively homogeneously with HHI values less than 0.06. We found that most genes with the highest HHI scores tended to encode either ligands or transcription factors, indicating their concentrated expression in relatively few clusters. A small number of genes that encoded phosphatases, kinases or receptors were also expressed relatively heterogeneously. Another way to visualize the relative heterogeneity of expression is to show the proportion of mRNA of each gene in each of the 35 clusters. Genes with the highest HHI from six different classes are shown demonstrating greater spatial expression concentration of the TFs and Ligands than the other categories. (Fig. 3B). Likewise, expression UMAPS of low HHI genes (Fig. 3D-3F) showed less concentrated patterns of expression when compared to high scoring genes (Fig. 3G).

High scoring ligands (95th+ percentile of included genes, HHI>0.066, n=27) included JAK/STAT pathway activators *upd1* (concentrated in clusters of the hinge, hinge-notum, and PE) and *upd3* (concentrated in clusters of the pouch, hinge-pouch, and hinge). Expression of the VEGF/PDGF orthologs *Pvf1* and *Pvf3,* which bind to the Pvr receptor tyrosine kinase, is most concentrated in clusters of the PE). Expression of *Ilp8*, which encodes a member of the insulin/relaxin family and regulates systemic responses to disc injury is concentrated in the hinge-pouch. All of these genes are known to be upregulated in response to various types of disc injury (Blanco et al., 2010; Floc'hlay et al., 2023; Katsuyama et al., 2015; Worley et al., 2022). Given that radiation induces damage uniformly, the relatively localized expression of these genes was unexpected. The four transcription factors with the highest HHI scores, *stripe* (*sr*), *drumstick* (*drm*), *pannier* (*pnr*) and *tailup* (*tup*) are all known to function in cell fate determination. For HHI scores on all genes included in this analysis, see S3_2-S3_4 and table S3_T3.

When examining gene expression using HHI, we noticed that many X-ray induced genes with concentrated expression at 4000 rad had a lower, but similar pattern of expression at 0 rad (e.g. *daw,* fig. 3G and *upd1*, fig. 4B-4C). To assess whether this property applied to many genes, we implemented a measure of the difference in the pattern of gene expression between conditions on the 3,767 x-ray induced genes. To reduce noise, we further removed genes not found in at least 1% of cells in both conditions, resulting in 3,655 genes. For each gene, we calculated the proportion of mRNA found in each of the seven PD regions (PE, notum, hinge-notum, hinge, hinge-pouch, pouch and myoblasts) under each condition. We chose to use the seven PD regions rather than the 35 clusters to determine large, strong changes in gene expression across the PD axis. Thus each gene could be represented as a point in seven-dimensional space in each of the two conditions. We then calculated the Euclidean distance ("expression distance") between these points. Gene scores for this measure ranged from 0 to 0.7, with higher scores indicating a greater difference in the overall pattern of expression between conditions (Fig. 3C).

Scores formed a right skewed distribution, with the vast majority of genes (>95%) scoring <0.13 (Fig. S3_1C). We found that genes below this threshold, when inspected using expression UMAPs (Fig. S3_5), displayed some level of conserved expression patterns between conditions, with lower scoring genes having more conserved patterns of expression. Examples from this category include the DNA repair protein Rad50 (Fig. 2D), the proapoptotic gene reaper (*rpr*) (Fig. 2E), *GstE6* which responds to oxidative stress (Fig. 2F) and activin-like ligand *dawdle* (*daw*) (Fig. 2G). The genes with the most marked changes in expression pattern, accounting for the top 5% of genes with the highest Euclidean distance between the two conditions, included the TNF ortholog *eiger* (*egr*) and the transcription factor *dysfusion* (*dysf*) (Fig. 3H, 3I). To examine the relationship between expression distance and expression concentration in the seven broad regions, we also calculated the HHI score of genes using the proportion of mRNA in each ("BR-HHI") and plotted them against expression distance (Fig. 3C). Genes with high BR-HHI scores in the 4000 rad condition tended to have relatively low expression distance scores (e.g., *daw, tup, pnr),* indicating that they shared a similar pattern of expression at 0 rad. The reason for this similarity of expression pattern under the two conditions is not obvious. One possible explanation is that genes that are transcriptionally active under unirradiated conditions in some regions of the disc have more accessible chromatin configurations in those cells and are therefore more easily induced in those same cells following irradiation. Another is that induction of these genes occurs by the combined action of region-specific transcription factors and those that are induced by radiation.

The TNF ortholog eiger is expressed in most cells while ligands of the Toll, PDGF/VEGF and JAK/STAT pathway show regionally induced expression

To examine differences in the expression of genes with high HHI scores more closely, we focused on the 14 ligands with the highest maximum FC after X-ray exposure in any cluster. Among the top 14 ligands were genes belonging to the TNF pathway, the Toll pathway, PDGF/VEGF related pathway, and the JAK/STAT pathway.

The most highly induced and uniformly expressed ligand was the tumor necrosis factor (TNF) pathway activator *eiger* (*egr*), increasing more than 16 fold overall after irradiation (Fig. 4A left). In the 4000 rad condition, *egr* had the lowest HHI score among ligands, with expression in all major regions of the PD axis (Fig. 4A right). The two genes encoding *egr* receptors, *grnd* and *wgn,* are expressed in the disc in both conditions. *grnd* had an overall positive fold change after X-ray exposure and is expressed relatively uniformly across clusters while *wgn* has an overall negative fold change and is concentrated in the pouch and hinge-pouch (Fig. S4_2). Eiger activates the JNK pathway which was previously shown to function in promoting p53-independent cell death (McNamee & Brodsky, 2009).

The Toll pathway is well known as a branch of innate immunity in *Drosophila* that is also important in wound healing (Capilla et al., 2017; Carvalho et al., 2014). Two of six Toll

pathway ligands were enriched after X-ray exposure, with *Spz* being among the top 14 induced ligands (the second ligand being *Spz3,* not shown in Fig. 4). The clusters of highest FC belonged to the pouch and hinge-pouch (Fig. 4A left), with expression in the 4000 rad condition being concentrated in these regions (Fig. 4A right). Seven of nine Toll receptors in *Drosophila* were also detected in the wing disc. Toll receptor genes *18w, Tl, Toll-7,* and *Tollo* were detected at high levels, *Toll-9* at low levels, and *Tehao and Toll-6* at barely detectable levels. *18w, Tl, Toll-7* and *Tollo* were expressed in varying patterns across clusters and had overall reduced expression after X-ray exposure (Fig. S4_1).

PDGF/VEGF related signaling in *Drosophila* is known to function in cell migration and wound closure (Tsai et al., 2022). All three *Drosophila* PDGF/VEGF related ligands, *Pvf1, Pvf2,* and *Pvf3,* were among the top 14 enriched after X-ray exposure, with *Pvf1* and *Pvf2* having the highest overall FC (log2FC 2.14 and 1.07, respectively, Fig. 5A left). All three ligands showed a relatively high level of expression at 0 rad in the PE (Fig. S4_1) which was maintained at 4000 rad (4A right). At 4000 rad, *Pvf1* and *Pvf2* were expressed at varying degrees in all major PD regions of the disc, while *Pvf3* had lesser expression in most clusters of the pouch and notum (Fig. S4_1). The sole PDGF/VEGF receptor in *Drosophila*, *Pvr,* is expressed relatively uniformly across all clusters in both conditions, being slightly enriched in the 4000 rad condition, consistent with the possibility that all major regions of the disc are capable of responding to Pvf ligands.

In *Drosophila,* the JAK/STAT pathway is required for normal development but is also known to be important for regeneration of the wing disc (Herrera & Bach, 2019; Katsuyama et al., 2015) . All three *Drosophila* JAK/STAT ligands, *upd1* (Harrison et al., 1998) *, upd2* (Rajan & Perrimon, 2012)*,* and *upd3* (Romão et al., 2021), were among the top 14 most induced ligands in scRNA-seq: *upd1* was primarily induced in the hinge and hinge-notum after irradiation, with concentrated expression in these regions in the 4000 rad condition that matched a similar, but lesser pattern of expression at 0 rad (Fig. 4A and 4B-4C right). *upd2* and *upd3* were expressed at low levels across all clusters at 0 rad (Fig. 4F and 4J). *upd2* was induced in all regions of the disc epithelium as well as the myoblasts, and *upd3* was primarily induced in the pouch and hinge-pouch after X-ray exposure (Fig. 4G-4K right). To validate these findings, we performed hybridization chain reaction (HCR) on upd transcripts in the 0 and 4000 rad conditions. *upd1* displayed a previously characterized pattern of restricted expression in the hinge and hinge-notum that was enhanced after irradiation, which was consistent with its expression UMAP (Fig. 4B, 4C). *upd2* was most highly expressed in the hinge/pouch of the wing disc with low expression in the notum (Fig. 4F-4G). *upd3* was most strongly expressed in the pouch and hinge pouch with low/undetectable expression in the hinge consistent with its expression UMAP (Fig. 4K). The sole JAK/STAT receptor in *Drosophila*, *dome,* is expressed in all clusters at 0 rad with a slight increase in expression at 4000 rad (Fig. S4_2).

The expression of *upd2* and *upd3* in *RasV12* tumors has been shown to be dependent on p53 and confer tumor radiation resistance in *Drosophila* (Dong et al., 2021). We therefore sought to determine if radiation induced expression of the upd genes required *p53* in normally developing irradiated wing discs. In wing discs carrying a near-complete deletion of *p53* (Xie & Golic, 2004), *upd2* and *upd3* showed little to no induction after X-ray exposure (Fig. 4H and 4I, 4L and 4M) unlike their wildtype counterparts. However, *upd1* in *p53* mutants had a normal pattern of expression in regions of the hinge before irradiation (Fig. 4D) with an increase of expression after irradiation (Fig. 4E), similar to wild type wing discs. Together, these results indicate that the normal pattern of *upd1* expression, and its induction after irradiation, is independent of *p53*. In contrast, X-ray induced *upd2* and *upd3* expression requires *p53*.

Transcription factors Dif and Ets21C are expressed heterogeneously in the wing disc, while expression of p53, Irbp18, and Xrp1 is relatively homogenous

Similarly to ligands, we focused our attention on the TFs with the maximum FC in any cluster after x-ray exposure. Among these 14 TFs were those involved in DDR previously shown to be upregulated after IR including *p53, Irbp18, and Xrp1* (Akdemir et al., 2007; Brodsky et al., 2004; Khan & Baker, 2022), and *dysf*, a TF important for the development of several structures including the trachea (Jiang & Crews, 2003), but yet to be characterized in the context of X-ray response. Also included were genes involved in regeneration (*Ets21C)*, immunity (*Dif*), and several predicted TFs with unknown functions (*CG14441, CG15435, CG2678, CG1729*).

Xrp1 and *Irbp18* are two transcription factors important for DDR that form a heterodimeric unit and are upregulated after irradiation. Both *Irbp18* and *Xrp1* are transcriptional targets of *p53,* a master transcriptional regulator of DDR and apoptosis*,* which itself is upregulated after exposure to X-rays. *Irbp18, Xrp1,* and *p53* were enriched in every scRNA-seq cluster after irradiation, with relatively homogenous expression across them at 4000 rad (Fig. 5A, right). We confirmed the homogenous expression patterns of *p*53 and *Xrp1* using HCR in the wing disc at 0 and 4000 rad (Fig. 5B-5C, 5Q-5R, S5_1B for increased gain at 0 rad), which were indeed evenly expressed across the disc.

Two TFs with heterogeneous enrichment after irradiation were the pro-regenerative TF *Ets21C* (Worley et al., 2022) and the Toll pathway inducer *Dif* (Ip, 1993)*.* In scRNA-seq, *Dif* was expressed most highly in the pouch of the disc, though was also present in other regions in the 4000 rad condition (Fig. 5A right, 5T left). An HCR of *Dif* confirmed this pattern of expression (Fig. 5T Right). *Dl*, the other TF inducer of the Toll pathway was present in all scRNA-seq clusters at 4000 rad (Fig. S5_1A). In the 4000 rad condition, *Ets21C* scRNA-seq expression was concentrated in hinge, with lesser but present expression in other regions of the disc (Fig. 5A right, 5H left). An HCR of

Ets21C in irradiated wing discs confirmed this, with regions of high expression present in both the dorsal and ventral hinge (Fig. 5H right). We next sought to determine if *Ets21C* required *p53* for its induction after x-ray exposure and performed HCR on *Ets21C* in *p53* mutant wing discs. *Ets21C* showed little to no expression before or after X-ray exposure in *p53* mutant wing discs (Fig. 5J, 5K), indicating its dependence on *p53* for X-ray induction. This is consistent with previous work showing that p53 can activate the JNK pathway (Shlevkov & Morata, 2012)*,* and that *Ets21C* expression is JNK dependent, at least in tissues with overgrowth perturbations (Toggweiler et al., 2016), although it is also possible that p53 directly activates Ets21C transcription but only in some regions of the disc.

Another TF that drew our interest was *dysf*, which is required for the development of the larval tracheal system and adult leg joints (Córdoba & Estella, 2018; Jiang & Crews, 2003), but has yet to be described in the context of exogenous stressors. In scRNA-seq, *dysf* was less concentrated in expression across clusters relative to *Ets21C* and *Dif,* but more so than *p53, Xrp1, or Irbp18* at 4000 rad (Fig. 5A, right)*.* HCR of *dysf* revealed expression in the dorsal and anterior-ventral hinge of the disc at 0 rad (Fig. 5L, left), which was reflected in the scRNA-seq expression pattern (Fig 5L, right). At 4000 rad, *dysf* was additionally induced in all major PD regions (Fig. 5M, right, see left for matched scRNA-seq expression). However, this induction was only observed in a subset of discs, as some irradiated discs showed no additional *dysf* expression after irradiation (Fig. 5N). Importantly, irradiated discs with no induced *dysf* expression did show increased *Ets21C* and *p53* expression similar to those with X-ray induced *dysf* (Fig. 5D, 5I), supporting the fact that they weren't excluded from X-ray exposure. These discs also displayed the developmentally-regulated expression pattern of *dysf* present in unirradiated discs, indicating that the HCR probes used in this experiment were capable of detecting *dysf* RNA. The reason for the dramatic difference in *dysf* induction between discs is not known, but one possible explanation is that discs may vary slightly in maturity and that *dysf* might simply not be induced in discs that are slightly more mature as has been observed for several damage-responsive genes (Harris et al., 2020).

Interestingly, in those discs with additional *dysf* expression at 4000 rad, X-ray induced *dysf* was specifically localized to nuclei (Fig. 5M, Fig. S5_1B for higher magnification). In contrast, the regions of *dysf* expression present at 0 rad had no discernible biased subcellular localization in either condition. The specific nuclear localization of X-ray induced *dysf* was unique among the genes we visualized with HCR.

When unirradiated *p53* mutant discs were probed for *dysf,* the wildtype expression pattern was present (Fig. 5O). After X-ray exposure, no *p53* mutant discs were found to have additional *dysf* expression, though the expression pattern found in unirradiated discs was present (Fig. 5P). Together, these results indicate that the pattern of *dysf* expression found in unperturbed wing discs is *p53* independent and the RNA is not restricted to the nucleus. In contrast, X-ray induced *dysf* expression depends upon *p53,*

the RNA is mostly localized to the nucleus, and appears to occur in most but not all wild-type discs.

Taken together these results indicate that, for IR-responsive transcription factors, some are induced homogeneously while others are expressed in specific regions. Moreover, at least two of the IR-induced transcription factors, *Ets21C* and *dysf,* require p53 for their induction as has been shown for *Xrp1* by others (Brodsky et al., 2004; Khan & Baker, 2022). It is therefore likely that p53 functions in combination with other region-specific factors to enable IR-induced expression of *Ets21C* and *dysf*.

Cell cycle centric clustering of cells reveals an emergent, high *trbl* transcriptional state with enriched expression of several secreted signaling genes

So far, we have characterized heterogeneity at the territorial level. However, for prior studies, we know that there must be heterogeneity in cellular responses to radiation even within territories. This is because, at the dose of radiation used, a majority of cells are expected to die while the remaining cells survive and typically resume proliferation. It is likely that these two classes of cells are interspersed among each other. For clustering thus far, we have used Seurat V5's standard pipeline, which calls upon the 2000 most variable genes in the dataset for clustering and dimensionality reduction, including UMAP. This results in clusters that are stratified based on the greatest sources of transcriptional variation at the global level. In the wing disc, the major sources of variation appear to be in genes that differ in expression along the PD axis and thus the UMAP bears some similarities to the layout of the wing disc itself where individual clusters are drawn from particular regions of the disc. In such a UMAP, cells that differ in response to radiation are likely to be found in each cluster.

We therefore considered other methods of clustering that might emphasize differences in transcriptional response that are not dependent upon the location of the cell within the disc. One approach would be to base the clustering on genes that are known to vary between cells in a location-independent manner. Our previous work has shown that in populations of relatively homogeneous cells, such as the myoblasts of the wing disc, that cell cycle genes can drive cell clustering and IR is known to affect the distribution of cells within the cell cycle.

To explore the relationship between cell cycle state and X-ray response in our data, we applied a cell cycle centric clustering approach. We performed dimensionality reduction and clustering on a manually curated list of 175 cell cycle genes (Table S6_T1). We note that separating cells into distinct phases of the cell cycle based on transcriptional profile alone is a notoriously difficult task for which several approaches have been developed (comprehensively reviewed in Guo & Chen, 2024). One type of approach, like the one used here, relies upon manually selected cell cycle marker genes to classify cells into different cell cycle phases. Importantly, the accuracy of these approaches,

including ours, is dependent on the quality of selected marker genes. The 175 cell cycle genes used here are drawn from several sources (see methods), and represent our best guess at genes with core functions in different phases of the cell cycle.

For clustering, we applied the Louvain algorithm with a resolution parameter of 0.5, using the first 30 PCs (cell embeddings transformed with CCA based integration) of the 175 cell cycle genes as variables, resulting in 6 total clusters. For visualization of clusters, we ran UMAP on these same 30 PCs (Fig. 6A-6C'). Of the 175 cell cycle genes used for clustering, cluster 3 was high in S-phase genes including *PCNA, Ts, Claspin,* and *Mcm5*. Cluster 5 was also high in many of the same S-phase genes albeit to a lesser extent, including *PCNA*, but was also marked by three M-phase genes, *aurA, aurB,* and *bora* (Fig 6D, Table T6_1). The high S-phase markers of cluster 3 (hereafter "High-*PCNA* Cluster-a") are consistent with this cluster containing cells in the G1/S phase of the cell cycle. The high but slightly lower levels of these S phase markers including *PCNA* in cluster 5, along with the expression of *aurA*, *aurB*, and *bora,* which are required for entry into mitosis, is consistent with this cluster containing cells in late S/G2 (hereafter "High-*PCNA* Cluster-b"). There was a notable reduction in the proportion of cells belonging to the High-*PCNA* Cluster-a from 0 rad (~21% of cells) to 4000 rad (~8% of cells) (Fig. 6E), indicating a reduction in the proportion of cells in this putative G1/S phase transcriptional state after X-ray exposure.

Cluster 4 was marked by the expression of *trbl*, which encodes a kinase-like protein that generates a G2/M cell cycle arrest by inducing the degradation of the mitosis promoting *Cdc25* proteins String (Stg) and Twine (Twe) (Mata et al., 2000). Cluster 4 (hereafter "High-*trbl* Cluster") had the highest expression of *p53*, high levels of genes involved in both DNA synthesis and repair, such as *RPA1, RPA2, RPA3 and Spn-a,* and relatively low expression of *Stg* (See Table T7_1 for complete list of cell cycle markers). High *trbl* and low *Stg* expression are consistent with this cluster containing cells arrested, or entering arrest, at the G2/M phase of the cell cycle. The High-*trbl* Cluster contained exceptionally few cells at 0 rad (~2%) with markedly increased representation at 4000 rad (~18%) (Fig. 6E). The large increase in the number of cells in this transcriptional state after irradiation is compatible with the large increase in G2/M cells after irradiation observed in vivo by other groups using the cell cycle reporter FUCCI and DNA content quantification using FACS (Ruiz-Losada et al., 2022). For cluster stability at different clustering resolutions, see Fig. S6_1. For cluster relationships, see Fig. S6_2.

To see how cells belonging to the High-*PCNA* and High-*trbl* Clusters were distributed amongst clusters that were previously generated at a global level from the 2000 most variable genes in the dataset, we transferred cell cycle-based cluster annotations onto our previously clustered data. We found that cells belonging to the High-*PCNA* and the High-*trbl* clusters were distributed among each of the seven broad PD regions (Fig. 6D-6D'). As expected, *PCNA* and *trbl* expression matched the distribution of High-*PCNA* and High-*trbl* Cluster identities (Fig. 6G-6I').

Next we focused on differences in highly induced genes ("HIGs", defined as X-ray induced genes >log2FC 1 between conditions, n=359) between cell cycle clusters. At 4000 rad, both High-*PCNA* Clusters had relatively low levels of many X-ray induced genes including those involved in apoptosis (*rpr, hid, Corp, egr*). This was a general trend, with the average scaled expression across HIGs being slightly lower in the High-*PCNA* Clusters than in other clusters (Fig. 7A). Both of these clusters had few positive X-ray induced markers (Table S7_T1). In comparison, the High-*trbl* Cluster showed a strong enrichment for many HIGs, having the highest average scaled expression across all of these genes (303/359) compared to other clusters in the 4000 rad condition (Fig 7A).

The High-*trbl* cluster was enriched in HIGs involved in apoptosis (*rpr, hid, Corp, egr*), DNA damage repair (e.g. *rad50, Irbp18, Ku80, mre11*) and ROS related genes (*GstE6, GstE7, GstD1*). Additionally, this cluster was enriched in HIGs encoding a subset of TFs (*Ets21C, dysf,* and *Dif),* and secreted proteins *(Pvf2, upd2, upd3, and spz)* (For complete list of markers, see Table T7_1). Most of these gene categories were represented among the top 24 HIGs with highest expression in the High-*trbl* Cluster (Fig. 7B). Though the majority of HIGs were highest in the High-*trbl* cluster, 56/309 were generally more homogenous across clusters (Fig. 7C), with none having relative expression in other clusters as high as those enriched in the High *trbl* cluster (Fig. 7A, 7C). These results suggest that a High-*trbl* transcriptional cell cycle state, which accounts for approximately 18% of cells, is associated with the bulk of X-ray induced gene expression.

The high *trbl* cluster was also enriched in two noteworthy secreted HIGs: *Swim* and *Arc1. Swim* is a secreted protein that binds to *wg* and has been proposed to aid in its spreading, though this point is contended (McGough et al., 2020; Mulligan et al., 2012; Simões et al., 2022). *Arc1* is a retroviral gag protein primarily described as a regulator of neural plasticity in *Drosophila*, but was recently shown in a *RasV12* tumor model to be expressed in tumor associated hemocytes, with a loss of function being associated with larger tumors, decreased pJNK, and decreased Dcp-1 activity in tumors (Khalili et al., 2023).

In conclusion, our results are consistent with transcriptional states that likely correspond to early and late S-phase being reduced in proportion after X-ray exposure, and cells accumulating in what might be a normally uncommon transcriptional state that could correspond to a G2/M arrest. Cells in the putative G1/S and S/G2 states have relatively low expression of many HIGs. In contrast, cells in the putative M/G2 stalled state show relatively high expression of many HIGs and seem to be responsible for the bulk of X-ray induced gene expression.

Simultaneously visualizing two levels of heterogeneity

To compare the level of heterogeneity that was observed using the two clustering approaches for individual genes, we plotted their two HHI scores on a two dimensional plot where the X-axis shows the score obtained using cell-cycle-based clustering and the Y-axis shows the score obtained from the clusters obtained with the standard Seurat pipeline (Fig. 8). For these plots we used transformed HHI scores where the original HHI scores which ranged from 1/N to 1 (where N represents the number of clusters) were mapped to a range from 0 to 1 using a linear transformation. Importantly, the two parameters cannot be considered to be truly orthogonal since the cell-cycle state likely makes some contribution to the clustering using the standard pipeline and because a cell's location in the disc might affect its behavior with respect to the cell cycle. Additionally, the distribution HHI scores with respect to any parameter changes with the number of clusters being analyzed - thus the values on the two axes are not directly comparable. With these caveats in mind, these plots provide us with a sense of the extent of heterogeneity of expression that each gene displays when assessed in each of these two ways.

As can be seen from the plots, genes encoding components of the DNA-damage response and regulators of ROS levels, apoptosis and cell-cycle progression are expressed broadly in the disc since their HHI scores are low when the standard clustering pathway is used. However, genes in each of these categories have a diversity of HHI values when the cell-cycle-based clustering is used, indicating that some of these genes show variations between cells that correlate with their cell-cycle state (Fig. 8A - D). Genes encoding transcription factors (Fig. 8E) or ligands (Fig. 8H) display a variety of HHI scores along both axes indicating that they have both inter-regional and intra-regional heterogeneity. Signaling proteins such as phosphatases, kinases and receptors (Fig. 8F, G. I) show an intermediate level of variation with respect to both parameters.

Discussion

An important shift in our understanding of biological systems in recent decades has been an increasing awareness of heterogeneity. This applies at multiple levels – from molecules to cells. At the level of cells, even individual cells in microbial communities have considerable phenotypic diversity, especially in response to changes in environmental conditions (Ackermann, 2015). Thus, it is likely that cells in mammalian tissues, which are often composed of many different types of cells, will display diverse responses to external stressors such as ionizing radiation. Only recently have single-cell approaches been used to study the effects of ionizing radiation on complex tissues, for example (Lu et al., 2023; Mills et al., 2025; Morral et al., 2024; Mukherjee et al., 2021; Sheng et al., 2020; Yuan et al., 2023). These studies have mostly focused on changes

in cellular composition following radiation, or in characterizing particular subpopulations, rather than attempting to address the overall types of heterogeneity in response within and between cell types.

To make the study of heterogeneity of cellular responses to radiation more tractable, we have used a relatively simple and homogenous tissue, the Drosophila wing disc which is mostly composed of two different cell types (reviewed by Tripathi & Irvine, 2022). Each wing disc is composed of a little over 30,000 epithelial cells (Martín et al., 2009) and approximately 2,500 myoblasts (Gunage et al., 2014). This has allowed us to look for differences in the way cells in different parts of the wing disc respond to radiation without the complication of a diversity of cell types. Our studies have uncovered two kinds of heterogeneity (Fig. 9). The first kind, inter-regional heterogeneity, is where some genes are upregulated far more in some territories of the wing disc than in others (Fig 9A). The other kind of heterogeneity, intra-regional heterogeneity, is manifest in all regions of the wing disc. Here cells with different types of radiation-responsive transcriptomes are interspersed among each other (Fig 9B).

Using the Herfindahl-Hirschman Index to assess heterogeneity of single-cell datasets

Studies that use single-cell RNAseq or multiomics frequently characterize differences in gene expression between clusters, but they typically do not use quantitative approaches to examine the extent of heterogeneity between clusters on a genome-wide level. To develop a means of ranking genes with respect to heterogeneity of expression between clusters, we have used a simple mathematical tool, the Herfindahl-Hirschman Index (HHI), which is most typically used by economists to study market concentration (Herfindahl, 1950; Hirschman, 1945). This and other mathematical ways of calculating heterogeneity are summarized by (Steele et al., 2022). In the context of our application, a low HHI index value indicates a lower level of concentration or more homogenous expression, while a higher value indicates that most of the expression occurs in one or a few clusters. While the value of the index for any given gene is not particularly useful in isolation, ranking genes has allowed us to identify those genes that are expressed more homogeneously among clusters as opposed to those that are expressed primarily in a few clusters. When we have used the conventional Seurat pipeline, a higher HHI typically identifies genes that are expressed in a subset of territories in the wing disc i.e. inter-regional heterogeneity. When applied to clusters generated using a curated set of cell-cycle genes, a high HHI indicates that that gene is preferentially expressed in a subset of cells that have a particular transcriptome state of cell cycle genes. This type of intraregional heterogeneity is observed in most, if not all, parts of the disc. Thus, these

two types of heterogeneity can be shown simultaneously using a two-dimensional plot (Fig. 8). Given that the same tissue can be analyzed using a variety of cellular parameters (e.g. chromatin accessibility, proteomic approaches), cells can be clustered using each of these approaches, and an HHI score for each gene could be calculated with respect to that parameter. In principle, each gene could then be assigned a multi-dimensional HHI score that reflects its heterogeneity with respect to each parameter.

Characterization of inter-regional heterogeneity

Since DNA damage, as assessed by p-H2Av immunofluorescence, is uniformly distributed throughout the disc, it is unsurprising that most genes that are upregulated in response to DNA damage are also expressed relatively uniformly. These include genes encoding proteins that repair DNA damage, inactivate ROS as well as those that regulate cell-cycle responses and apoptosis in response to DNA damage. This also applies to the key transcription factor considered to regulate many of the transcriptional responses to DNA damage, p53. We find that heterogeneous expression with respect to geographical territories in the wing disc is most evident among some genes encoding transcription factors and secreted ligands and found occasionally among receptors and signal transducers such as kinases and phosphatases.

Why are these genes expressed heterogeneously? By manually inspecting gene expression we find that in many cases, the expression pattern following irradiation is similar to that in unirradiated discs. Like our approach with the HHI, we have used a Euclidean distance measure in seven-dimensional space to rank the difference in expression pattern with respect to the seven major regions of the disc under the two conditions (unirradiated versus irradiated) (Fig. 3C). Genes with an especially low Euclidean distance have more similar patterns of expression between the two conditions. Clear examples are *dawdle* (*daw*) which encodes a ligand for an activin receptor, and *upd1* which encodes a ligand upstream of the JAK/STAT pathway, which have similar patterns of expression under the two conditions but are expressed at higher levels in the same regions after irradiation. Why might this happen? One possibility is that the expression of this gene requires both a region-specific transcription factor and a transcription factor induced by irradiation. An alternative possibility is that the chromatin state of such genes might reflect their pattern of expression in the unirradiated disc; a radiation-induced transcription factor might bind more readily to genes that already have more accessible chromatin. Both explanations are consistent with our observation that the non-uniform upregulation of several radiation induced

genes including *upd2*, *upd3* and *Ets21* require the function of *p53* which is induced by X-ray irradiation yet expressed in all territories of the disc.

For some genes such as the JAK/STAT ligands *upd2* and *upd3*, expression is clearly non-uniform after radiation. Yet in the unirradiated condition, expression is not detected. This pattern of induction is more difficult to explain. It is possible that *upd2* and *upd3* might be expressed at extremely low levels in unirradiated discs in patterns similar to their induction. Also possible is that the chromatin state of these genes, even if they are not expressed in the unirradiated disc, shows inter-regional heterogeneity. This phenomenon was also observed with the transcription factor *Ets21C* which is known to be necessary for regeneration of the wing pouch after its ablation (Worley et al., 2022). Identification of many such examples of interregional differences in the induction of genes that likely play an important role in response to radiation indicate that this phenomenon is relatively widespread and that its mechanistic basis merits further investigation.

Intraregional heterogeneity: cells with a particular cell-cycle transcriptome show the highest levels of upregulation of damage-responsive genes

Since the standard Seurat pipeline, when applied to the wing disc, generated clusters that most reflect a cell's location in the wing disc, we tried alternative approaches that could separate cells into clusters that were agnostic to their location in the disc. Using a curated set of cell-cycle genes, we were able to separate cells into six clusters. One cluster accounted for only 2% of cells in the unirradiated state but 18% of cells following irradiation. This cluster was marked by high expression of the *trbl* gene which encodes a pseudokinase that promotes the degradation of the Cdc25 ortholog String (Mata et al., 2000). Based on their transcriptome, cells in this cluster are likely to be in G2 and prevented from entering mitosis.

Unexpectedly, *p53*, as well as genes involved in DNA repair, apoptosis and inactivation of ROS were all most highly expressed in this cluster at 4000 rad as were the majority of radiation-induced genes. Thus, rather than being expressed uniformly in all cells, expression of many radiation-induced genes shows strong intra-regional heterogeneity with the highest expression found in a subpopulation that occupies a particular cell-cycle transcriptomic state. This same high-*trbl* cluster also expresses higher levels of the transcription factor *Ets21C* as well as genes encoding a variety of secreted ligands such as *Pvf2, upd2, upd3* and *spz*. Studies of regeneration in recent years have identified situations where subsets of cells function to organize regenerative proliferation by secreting factors that promote the survival and proliferation of other cells (Aztekin et

al., 2019;) . Indeed, following ablation of the wing pouch, a localized subpopulation of cells likely arrested in G2 (Cosolo et al., 2019) promotes the proliferation of surrounding cells (Worley et al., 2022). In the case of diffuse damage, such as that elicited by X-ray exposure, the analogous population of organizing cells could be dispersed throughout the disc and interspersed with other cells. Cells belonging to the high-*trbl* cluster could potentially represent such a population.

Functional consequences of heterogeneity

Many of the genes that display intraregional heterogeneity have been implicated previously in regulating cell survival and proliferation as well as in activating mechanisms that function during regeneration. These include the *upd2* and *upd3* genes, and the transcription factor *Ets21C*. Previous work has shown that a region of the wing disc, the dorsal hinge, has less apoptosis following irradiation that is dependent upon increased JAK/STAT signaling in that region (Verghese & Su, 2016). It is therefore likely that many of the examples that we have observed of interregional heterogeneity have functional consequences. A systematic disruption of all genes that display heterogeneous patterns of expression would give us a more complete view of this phenomenon.

The observation that many of the highly inducible genes are expressed in a subset of cells that have a particular cell-cycle state was an unexpected finding of our analysis. This could imply that cells must be in this state to most effectively turn on these genes. Alternatively, the suite of genes that are expressed most after irradiation could push the cells to a particular cell-cycle state. Such a state might be more conducive to the repair of cellular damage and to promote local and systemic responses to radiation that facilitate recovery. Further study of this phenomenon could enable ways of using cell cycle manipulations to alter radiosensitivity.

Concluding remarks

Our studies have focused on one time point at one developmental stage in a relatively simple tissue. The levels of heterogeneity that we have uncovered here show that heterogeneity in response to radiation must be far more widespread and complex in tissues such as mammalian organs that are composed of many different types of cells. The approach and the methods of analysis that we have used here should be applicable, with appropriate modifications, to these more complex situations.

Materials and Methods

Fly strains used

Oregon R was used for wildtype experiments and p53[5A-1-4] (BDSC #6815) was used for p53 mutant experiments.

Antibodies used

Primaries: Rabbit anti-H2AvD-pS13 (#600-401-914, Rockland), 1:250; Rabbit anti-Dcp-1 (#9578S, Cell Signaling), 1:250 ; Rabbit anti-PHH3 (#06-570, Millipore), 1:500 ; Rat anti-Zfh2 (), 1:200; *Secondaries:* Goat anti-rat Alexa Fluor 555 (A21434, Invitrogen), 1:500; Goat anti-rabbit Alexa Fluor 555 (A21428, Invitrogen), 1:500;

Single-Cell Sequencing Experiments

Preparation of Petri Dish Food Plates for Rearing and Irradiation

For scRNA-seq experiments: Fly food prepared using the Bloomington *Drosophila* Stock Center (BDSC) formula was melted in the microwave and poured to a height of 10 +-2 mm into clear 15 mm x 60 mm petri dishes. After cooling, condensation was wiped from the lid, and ~1/8th tsp of dry active yeast was added on top of the food. Plates were stored at room temperature for 1-2 days before use.

Egg Collections and Rearing

All single-cell experiments were performed using the “Oregon R” wildtype fly strain. Egg lays and rearing were performed at 25C. Groups of 15 males and 35-40 females were sorted into food vials and allowed to recover from CO2 incapacitation. Flies were then tapped into egg lay bottles containing a grape agar plate topped with a dollop of yeast paste and pre-incubated for 1-2 days to increase egg yield. On the day of egg collection, flies were tapped into egg-lay bottles containing fresh grape agar plates and yeast paste, and incubated for 4 hours. After the egg lay period, grape plates were collected and yeast paste was removed. Eggs were incubated for 24 hours, after which 1st instar larvae were picked using a poker tool dipped in yeast paste for adhesion. 50 larvae were deposited into each petri dish food plate, and incubated until their time of irradiation/dissection.

Dissections, Tissue Dissociations, and FACS

All microcentrifugations between washes were performed at 5,000 RPM (~2,000 g) at room temperature. All samples and media were stored on ice unless otherwise noted. Larvae were collected into 1X PBS and dissected in supplemented schneider’s media (SSM) at room temperature over one hour. Wing discs were collected into SSM on ice, pooled into 1.5 ml Eppendorf tubes, and washed with Rinaldini solution. Wing discs were dissociated at 37C in 0.25% Trypsin EDTA (Sigma T4049) for 10 minutes. Dissociated cells were washed once in PBS-10% FBS and twice in PBS-1% FBS. Cells were pooled together in a final suspension of 500 ul PBS-1% FBS and passed through a 35 uM filter into a FACS tube. Cell suspensions were sorted on a BD FACSAria Fusion. Forward scatter and side scatter were used to sort out debris and doublets.

Propidium iodide (PI) was added to the sample and a Texas Red filter was used to remove low quality/dead cells: PI fluorescence intensity revealed a bimodal distribution of cells, and a gate was drawn between the two distributions. The upper distribution was sorted out. Cell concentrations were determined using a hemocytometer, and brought to an appropriate final concentration in accordance with 10X Genomics v3.1 guidelines.

Library Preparation, Sequencing, and Alignment
cDNA libraries were generated using 10X Genomics v3.1 chemistry and hardware. All libraries were sequenced at a depth of around 1 billion paired end reads. Sequences were aligned to *Drosophila* transcriptome v6.55 using Kallisto-Bustools v0.28.2 (Kallisto v0.50.1, Bustools v0.43.2) with default settings. FlyBase gene IDs were translated to gene symbols using org.Dm.eg.db v3.18.0 R package. A table containing all gene symbols used in this dataset, their Flybase IDs, and their annotation symbols are included as a supplement.

Data Processing and Analysis
After alignment, all datasets were individually filtered in the same manner before integrated analysis.
1) Genes which were captured in 3 or less cells, and cells which contained less than 200 unique genes were removed.
2) Unique genes captured per cell were visualized with a density plot (typically used for continuous data, but used here heuristically), revealing roughly bimodal distributions in each sample. One maxima was near zero (representing “low quality” cells) and the other maxima was between 2000 and 5000 unique genes. A cutoff was drawn at the lowest density value between the two maxima, and the lower half of cells were removed.
3) Cells which were in the 98.5 percentile of unique features and above (potential doublets) were removed

Immunofluorescence
All larvae were reared at 25C. For each experiment, wandering L3 larvae were picked from non-synchronized stocks and placed into separate vials. They were then irradiated with 4000 rad, or left outside of the irradiator for control. At the end of irradiation, larvae were allowed 4 hours of recovery at 25C. After recovery, 9-15 larvae were picked, dissected in 1x PBS, and fixed. After fixing for 20 minutes in 4% PFA in PBS, carcasses then washed 3x in 0.1% PBST for 10 minutes. Then carcasses were blocked in 10% NGS in 0.1% PBST. After blocking, samples were incubated with primary antibodies in blocking solution overnight at 4C. The next day, samples were washed 3x in PBST for 15 minutes and then incubated with secondary antibodies in blocking solution at 1:500 for 2.5 hours at 25C or overnight at 4C. Samples were washed once in PBST for 5 minutes and then incubated with 1:1000 DAPI in PBST for 20 minutes. Afterwards, samples were washed 2x in PBS for 10 minutes and wing discs were mounted. Wing

discs were imaged on an epifluorescent microscope with apotome attachment.

EdU Assays

Larvae were dissected and incubated in Schnieder's Drosophila Medium (Thermo Fisher 21720024) at room temperature. Discs were incubated in EdU for 30 minutes. After incubation, discs were fixed in 4% PFA for 15 minutes. EdU staining protocol followed the Click-iT EdU Cell Proliferation Kit, Alexa Fluor 647 (Thermo Fisher C10340).

Hybridization Chain Reaction

All larvae were reared at 25C. For each experiment, about 50 third instar larvae for each condition were placed into vials containing bloomington food for irradiation. Half of the vials were then irradiated at a dose of 4000 rad while the other half were left outside of the irradiator. Larvae were given a four hour recovery time at 25C after irradiation before the beginning of dissection. Dissection took approximately one hour to complete and carcasses were kept on ice until fixation. Our HCR protocol was adapted from Molecular Instruments for use on larval carcasses and used Molecular Instruments reagents. In brief, after fixing in formaldehyde, samples were permeabilized in detergent solution and pre-hybridized in "Probe Hybridization Buffer" for 30 minutes at 37°C. Probe sets for any given gene were made against mRNA sequences conserved between all predicted gene isoforms. After pre-hybridization, samples were incubated in a probe solution consisting of "Probe Hybridization Buffer" and probesets overnight (14-17 hours) at 37°C. Following hybridization, samples were washed using "Probe Wash Buffer" and SSCTween. Samples were then pre-incubated in "Amplification Buffer" for 30 minutes. After pre-incubation in "Amplification Buffer", samples were left in hairpin solution consisting of "Amplification Buffer" and complementary hairpins in the dark at room temperature overnight (12-15 hours). Samples were then washed, incubated with DAPI, and mounted using Invitrogen's Diamond Antifade Mountant. Slides were imaged using a confocal microscope and images were processed using Fiji. For image processing, a max-projection was generated of all images and the signal from each channel of the irradiated samples were auto-adjusted. These adjustments were propagated to all other wing-disc images of the same genotype.

Supplementary Tables

Supplementary Table S2_T1. Top Markers for each cluster.

Contains the following columns (**Column;** description): **Subregion;** cluster name **Marker_Integrated_Global;** Top marker gene when comparing cluster to all other cells in the integrated data. **Pct_Diff_Integrated_Global;** Percent of cells expressing top marker in cluster of interest minus percent of cells expressing in all other cells in the integrated data. **Marker_R4K_Global;** Top marker gene when comparing cluster to all

other clusters in the 4000 rad condition. **Pct_Diff_R4K_Global;** Percent of cells expressing top marker in cluster of interest minus percent of cells expressing in all other cells in the 4000 rad condition. **Marker_R0K_Global;** Top marker gene when comparing cluster to all other clusters in the 0 rad condition. **Pct_Diff_R0K_Global;** Percent of cells expressing top marker in cluster of interest minus percent of cells expressing in all other cells in the 0 rad condition. **Marker_Integrated_Region;** Top marker gene when comparing cluster to all other clusters within its broad PD region in the integrated data. **Pct_Diff_Integrated_Region;** Percent of cells expressing top marker in cluster of interest minus percent of cells expressing in all other cells within its broad PD region in the integrated condition. **Marker_R4K_Region;** Top marker gene when comparing cluster to all other clusters within its broad PD region in the 4000 rad condition. **Pct_Diff_R4K_Region;** Percent of cells expressing top marker in cluster of interest minus percent of cells expressing in all other cells within its broad PD region in the 4000 rad condition. **Marker_R0K_Region;** Top marker gene when comparing cluster to all other clusters within its broad PD region in the 0 rad condition. **Pct_Diff_R0K_Region;** Percent of cells expressing top marker in cluster of interest minus percent of cells expressing in all other cells within its broad PD region in the 0 rad condition. Markers were only considered if (1) they were expressed in at least 10% of cells in the cluster being considered or its comparison group, (2) there was minimum difference of 10% between the cluster being considered or its comparison group, and (3) there was an enrichment of at least log2FC=1 in the cluster being considered. Ties were broken by taking the marker with the higher log2FC.

Supplementary Table S3_T1. DEG 4000 rad vs 0 rad.
Contains the following columns (**Column;** description): **gene_name**; Gene name. **p_val;** unadjusted p-value of Wilcoxon rank-sum test. **avg_log2FC;** average log2FC of all cells in 4000 rad condition versus all cells in 0 rad condition. **pct.1;** Percent of cells expressing given gene in 4000 rad condition. **pct.2**; Percent of cells expressing given gene in 0 rad condition. **p_val_adj;** Bonferroni corrected p-value from p_val column (corrected on total genes captured = 13,384). Genes were only included in this table if they were present in at least 1% of cells in either condition, had an adjusted p-value <0.05, and had an average log2FC of ≥ 0.1. This table was produced using the Findmarkers() function in Seurat v5.

Supplementary Table S3_T2. Apoptosis, DNA damage response (DDR), response to reactive oxygen species (ROS), cell cycle regulation, transcription factors (TFs), phosphatases, kinases, ligands, and receptors genes considered for HHI comparison (pre-filter).
Contains the following columns (**Column;** description): **genes_this_data;** All genes captured in this data belonging to the considered categories, 1716 total. **Category;** Category each gene is classified into.

Supplementary Table S3_T3. Apoptosis, DNA damage response (DDR), response to reactive oxygen species (ROS), cell cycle regulation, transcription factors

(TFs), phosphatases, kinases, ligands, and receptors genes considered for HHI comparison (post-filter).
Contains the following columns (**Column;** description): **gene_name;** Gene name. Includes all 521 genes used in 3A. **HHI_4KR;** HHI score in the 4000 rad condition. **max_logFC;** Maximum log2FC when looking at the change in expression within a cluster between conditions. **max_pct;** Maximum percentage of cells expressing in any cluster. **Group;** Gene category. **Broad_Group;** Gene category where DDR, ROS, Apoptosis, and Cell Cycle are condensed into a category called "D/R/A/C."

Supplementary Table S6_T1. Cell Cycle Gene Markers for Cell Cycle Based Clusters.
Contains the following columns (**Column;** description): **gene;** Gene name. **avg_scaled_…;** Average scaled expression in each cell cycle cluster (integrated). 6 columns. **Percent_Expressed_…;** Percentage of cells expressing given gene in each cell cycle cluster (integrated). 6 columns. This table includes all 175 genes used in cell cycle based clustering.

Supplementary Table S7_T1. Highly Induced Gene Markers for Cell Cycle Based Clusters.
This table includes the 359 genes that are highly induced after irradiation. These genes are expressed in at least 1% of cells in either condition, have an average $\log_2 FC \geq 1$, and an adjusted p-value < 0.05. Contains the following columns (**Column;** description): **gene;** Gene name. **avg_scaled_…;** Average scaled expression in each cell cycle cluster in the 4000 rad condition. 6 columns. **Percent_Expressed_…;** Percentage of cells expressing given gene in each cell cycle cluster in the 4000 rad condition. 6 columns.

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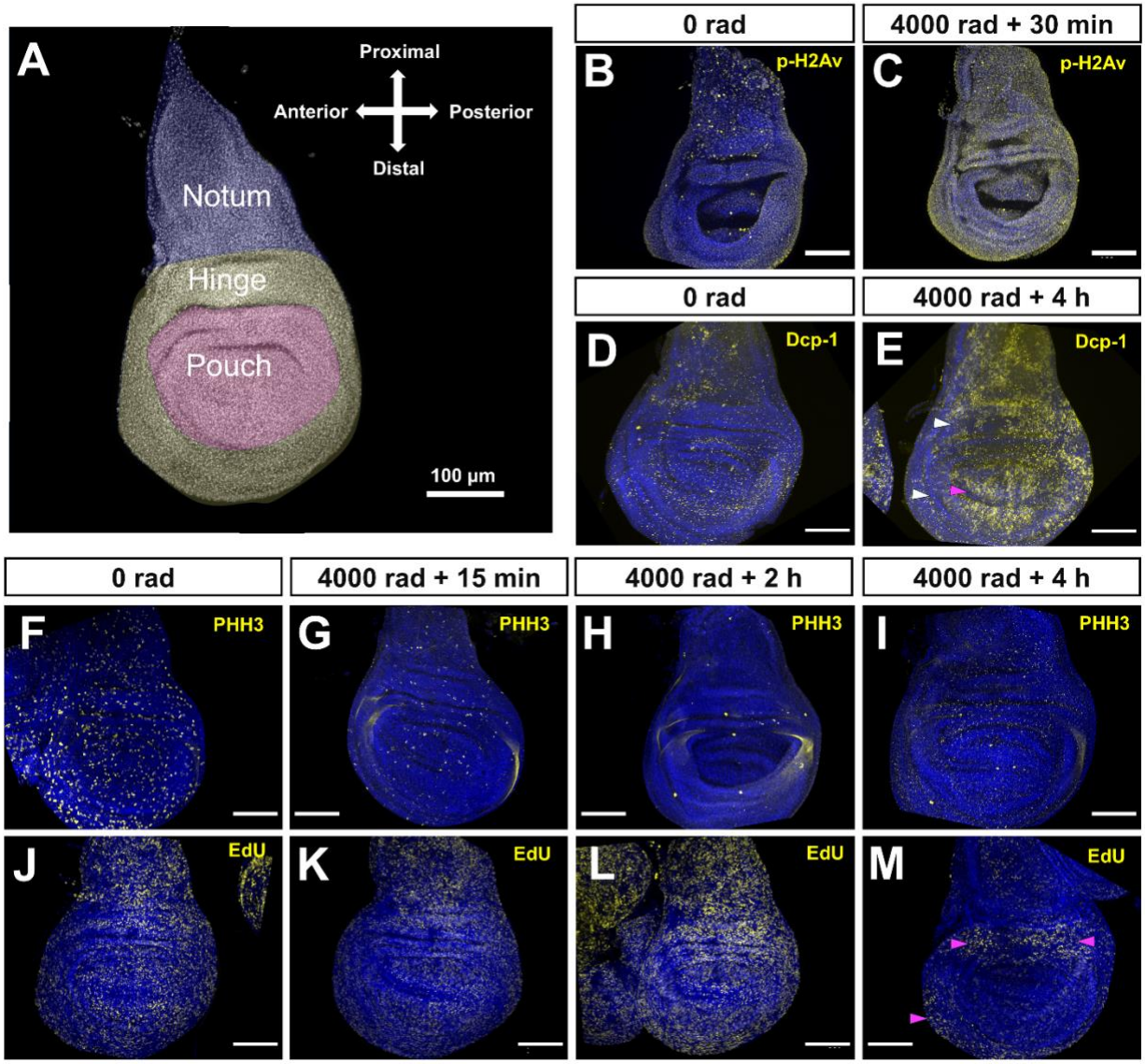


Figure 1. Effects of X-ray irradiation of DNA damage, apoptosis and cell-cycle progression

(A) Cartoon overlay of wing disc showing the PD regions. **(B, C)** IF of p-H2AX at 0 rad **(B)** and 4000 rad of irradiation, 30 min after exposure **(C)**. **(D, E)** IF of cleaved Dcp-1 at 0 rad **(D)** and 4000 rad 4 h after exposure **(E)**. Magenta arrowhead points to the DV boundary and white arrowheads point to regions of the hinge, regions with less Dcp-1 signal. **(F - I)** IF of PHH3 at 0 rad **(F)** and 4000 rad 15 min **(G)**, 2 h **(H)**, and 4 h (**I**) after exposure. **(J - M)** EdU incorporation at 0 rad **(J)** and 4000 rad 15 min **(K)**, 2 h **(L)** and **(M)** 4 h after exposure. Magenta arrowheads point to regions of high EdU staining at 4 h. All scale bars are 100 µm.

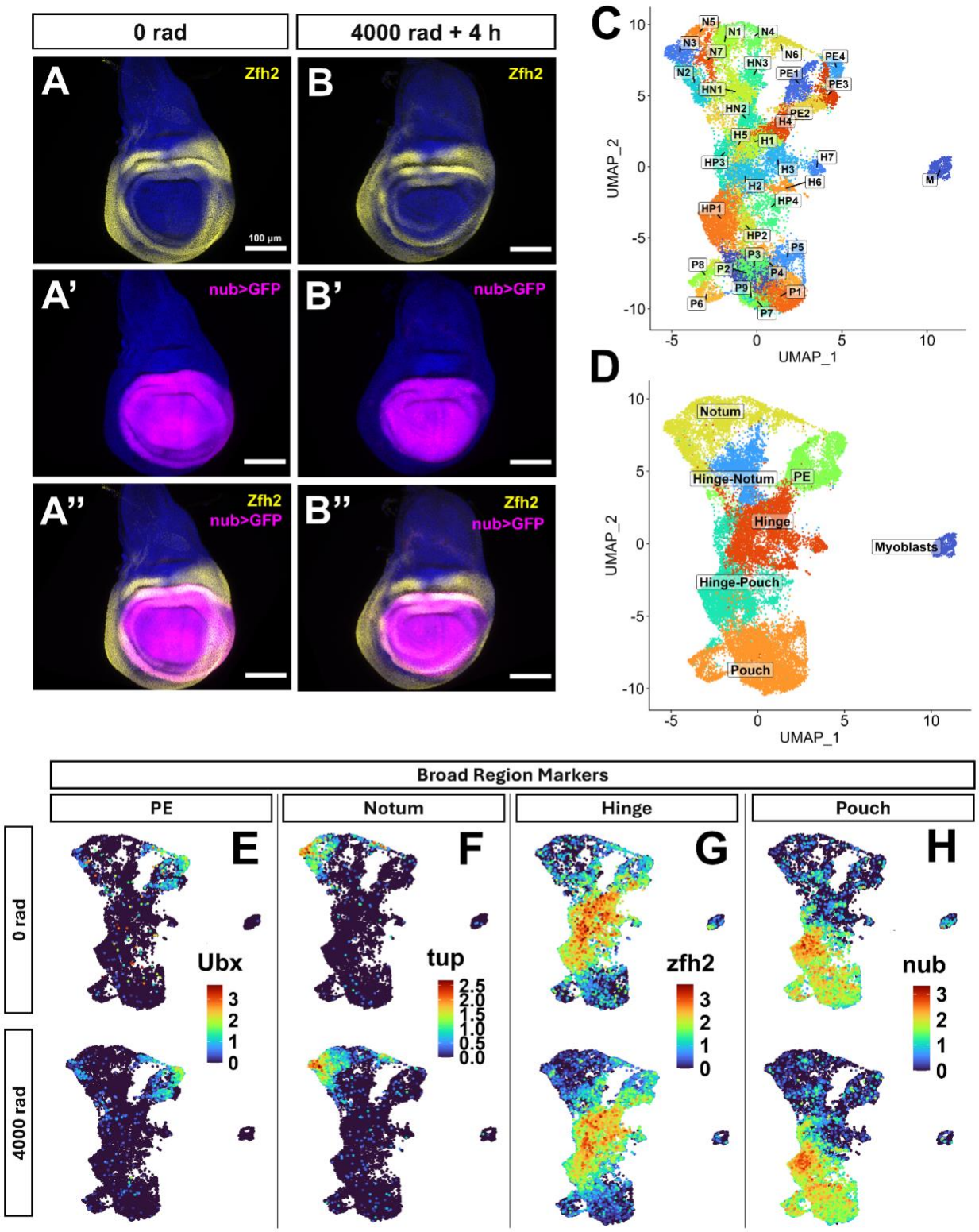

0 rad
4000 rad + 4 h
A
Zfh2
100 µm
B
Zfh2
A'
nub>GFP
B'
nub>GFP
A''
Zfh2
nub>GFP
B''
Zfh2
nub>GFP
C
N5
N1
N4
N3
N7
N6
PE4
N2
HN3
PE1
PE3
HN1
HN2
PE2
H4
H5
H1
HP3
H7
H3
H6
M
H2
HP4
HP1
HP2
P5
P3
P8
P2
P4
P9
P1
P6
P7
UMAP_2
UMAP_1
D
Notum
Hinge-Notum
PE
Hinge
Myoblasts
Hinge-Pouch
Pouch
UMAP_2
UMAP_1
Broad Region Markers
PE
Notum
Hinge
Pouch
0 rad
4000 rad
E
Ubx
3
2
1
0
F
tup
2.5
2.0
1.5
1.0
0.5
0.0
G
zfh2
3
2
1
0
H
nub
3
2
1
0

Figure 2. Territories of the wing disc shown by immunofluorescence and UMAP plots.
IF of Zfh2 **(A, B)**, *nub>GFP* **(A', B')**, and merged images of both **(A'', B'')** at 0 rad **(A - A'')** or 4 h after exposure to 4000 rad **(B–B'')**. All scale bars are 100 µm. (**C**) UMAP showing 35 cluster annotations with cluster designations based on PD region. (**D**) UMAP showing broad epithelial region annotations. **(E-H)** UMAP plots showing the expression of markers used to annotate specific epithelial regions at 0 rad (top row) and 4000 rad (bottom row); *Ubx* for PE **(E)**, *tup* for notum **(F)**, *zfh2* for hinge **(G)**, *nub* for pouch **(B)**. Combinations were used to determine hinge-notum and hinge-pouch regions. Plots for eyg (also used for the notum) and *twi* (used for the myoblasts) are shown in Figure S2_1.

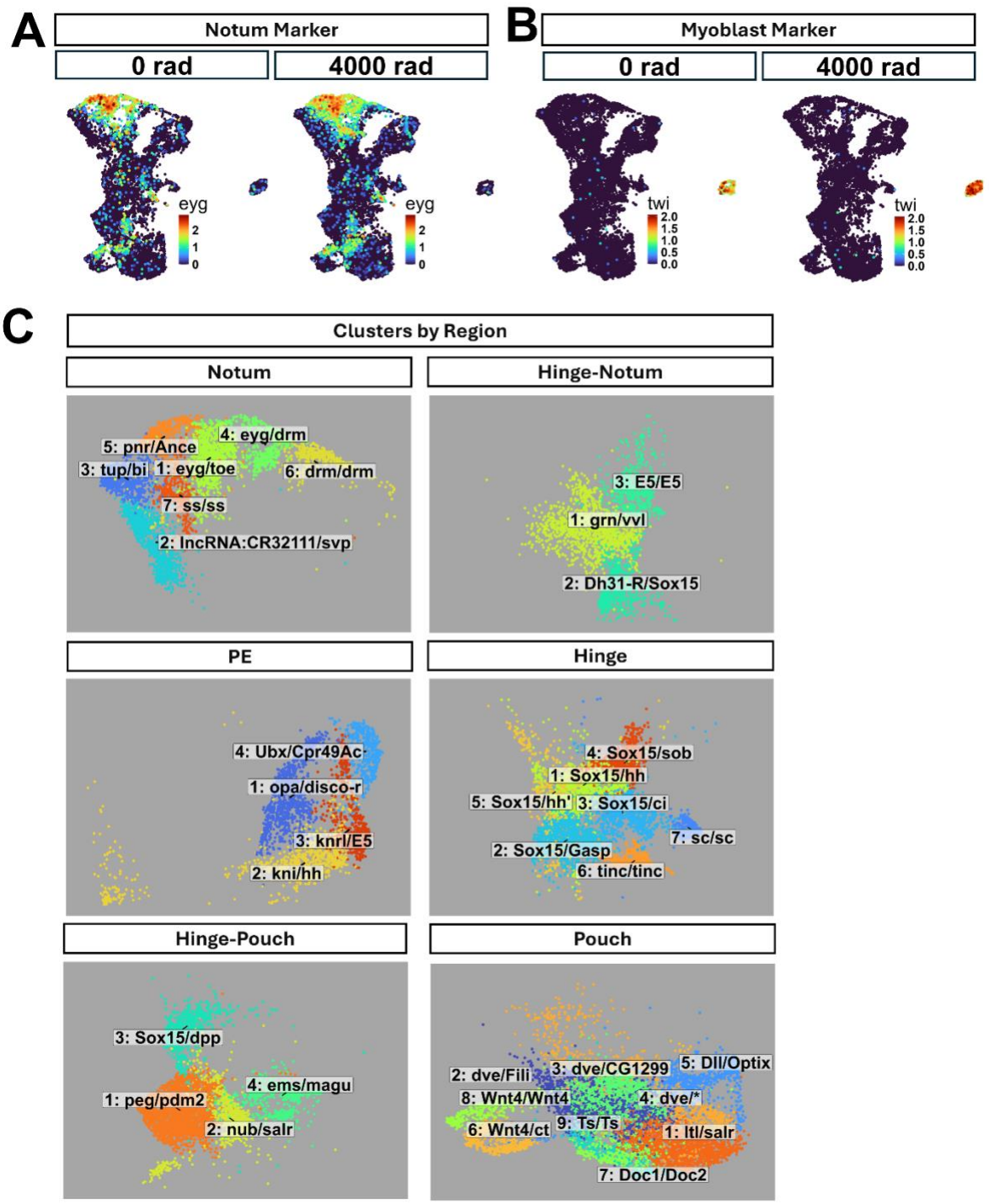

A
Notum Marker
0 rad
4000 rad
eyg
2
1
0
B
Myoblast Marker
0 rad
4000 rad
twi
2.0
1.5
1.0
0.5
0.0
C
Clusters by Region
Notum
5: pnr/Ance
4: eyg/drm
3: tup/bi
1: eyg/toe
6: drm/drm
7: ss/ss
2: lncRNA:CR32111/svp
Hinge-Notum
3: E5/E5
1: grn/vvl
2: Dh31-R/Sox15
PE
4: Ubx/Cpr49Ac
1: opa/disco-r
3: knrl/E5
2: kni/hh
Hinge
4: Sox15/sob
1: Sox15/hh
5: Sox15/hh'
3: Sox15/ci
7: sc/sc
2: Sox15/Gasp
6: tinc/tinc
Hinge-Pouch
3: Sox15/dpp
4: ems/magu
1: peg/pdm2
2: nub/salr
Pouch
2: dve/Fili
3: dve/CG1299
5: Dll/Optix
8: Wnt4/Wnt4
4: dve/*
6: Wnt4/ct
9: Ts/Ts
1: ltl/salr
7: Doc1/Doc2

Figure S2_1. UMAP plots of Top Cluster Markers by Region.
(A) Expression UMAPs of *eyg* at 0 rad (left) and 4000 rad (right). **(B)** Expression UMAPs of *twi* at 0 rad **(left)** and 4000 rad **(right)**. **(C)** Cropped and zoomed in UMAP plots of clusters belonging to each of the major PD regions. Each cluster is labeled by its cluster number and the genes that are most highly expressed in it when compared to all clusters (left of the slash) and when compared to other clusters within the same PD region (right of the slash).

$$HHI = \left(\frac{\text{Cluster 1 Average Expression}}{\text{Sum Average Cluster Expression}}\right)^2 + \left(\frac{\text{Cluster 2 Average Expression}}{\text{Sum Average Cluster Expression}}\right)^2 \ldots + \left(\frac{\text{Cluster n Average Expression}}{\text{Sum Average Cluster Expression}}\right)^2$$

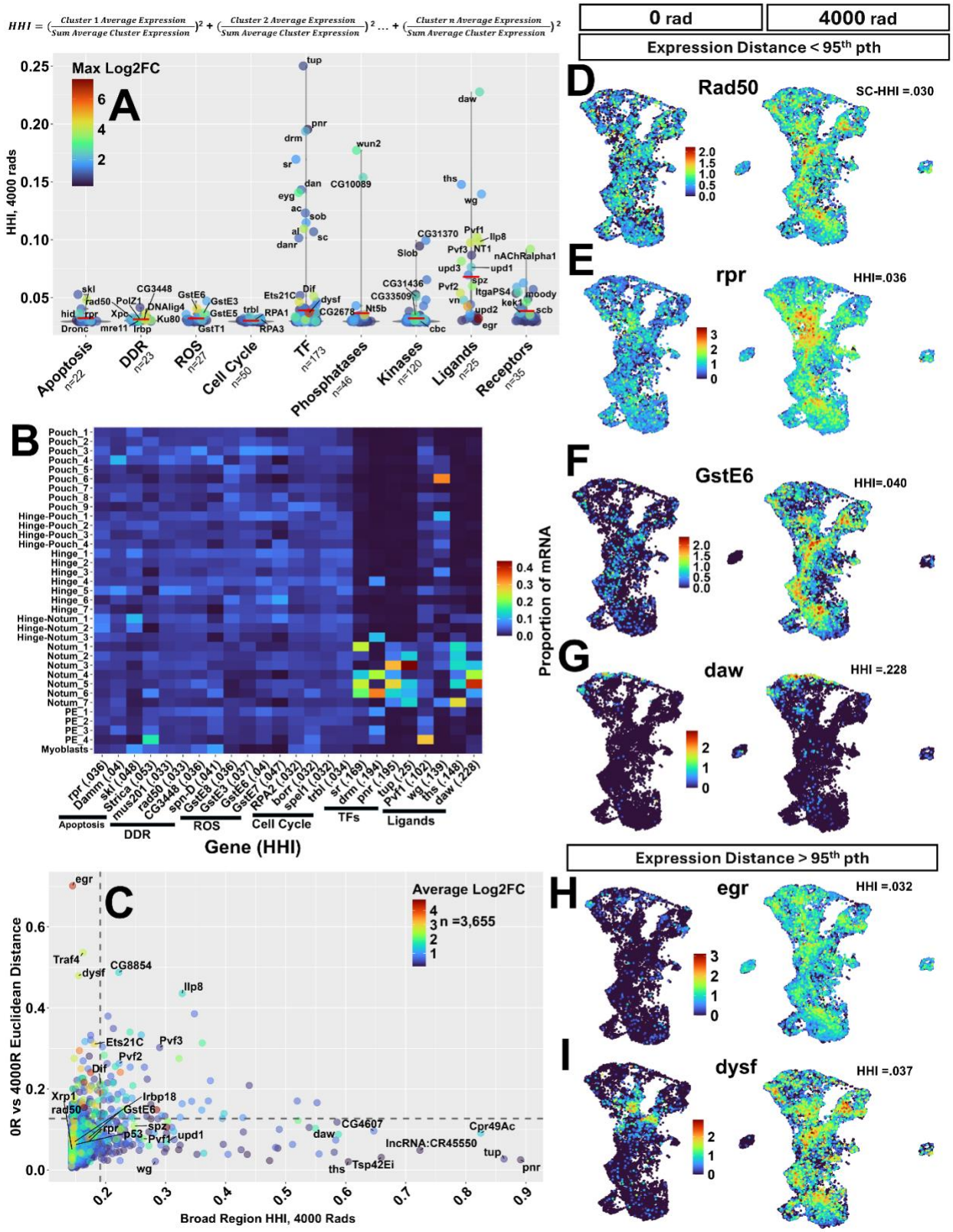
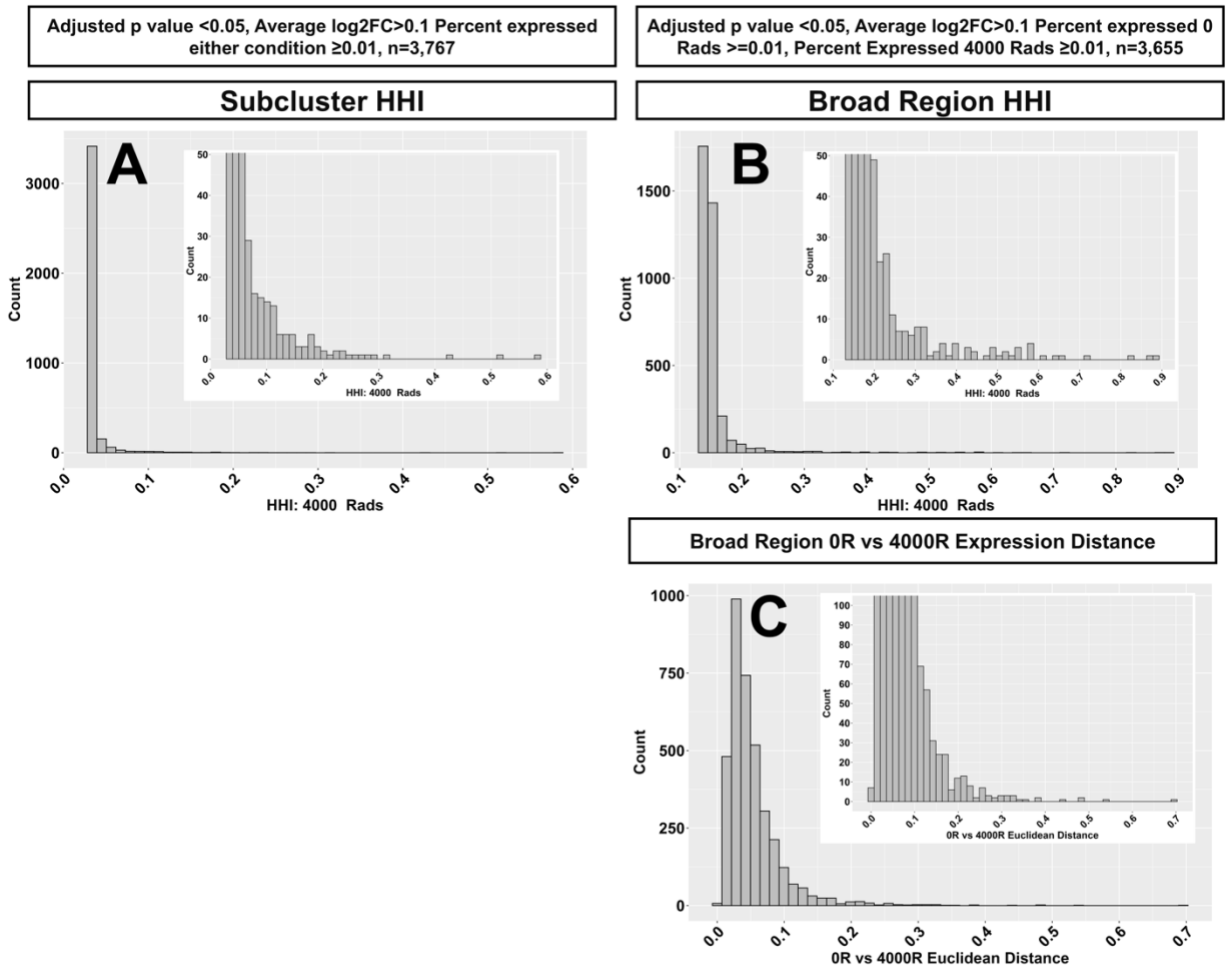
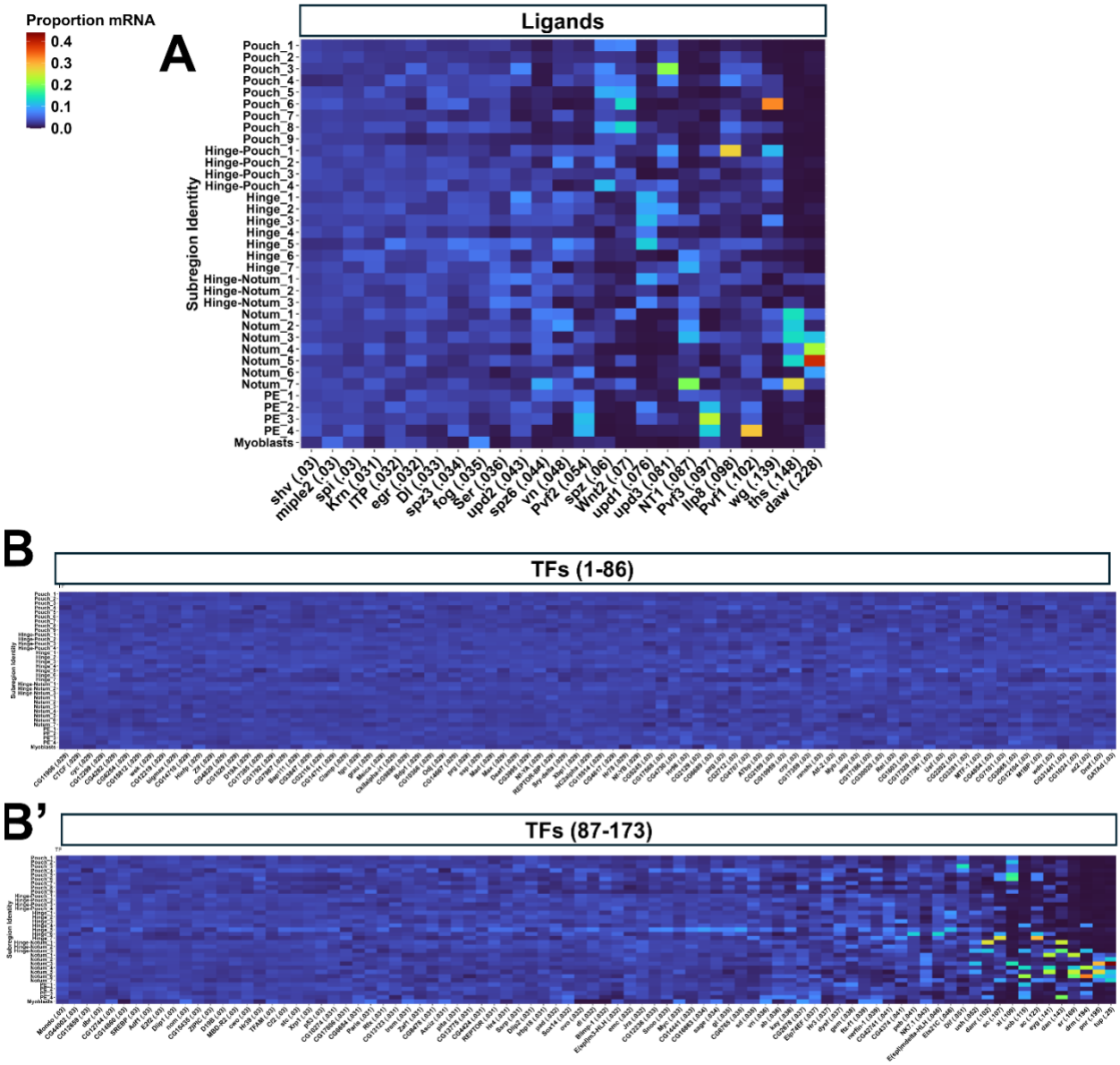


Figure 3. Heterogeneity of gene expression across the wing disc following radiation exposure

(A) 521 genes across 9 functional categories plotted as points by their HHI score at 4000 rad. Point color represents the $\log_2$FC between conditions in the cluster that has the highest $\log_2$FC. The horizontal red bar represents the mean HHI score for the genes of that category. Genes with max $\log_2$FC > 2 or HHI > 0.075 were labeled where space permitted. The equation used to calculate the Herfindahl-Hirschman Index (HHI) is shown above the panel. **(B)** Heat map of 4 highest HHI scoring genes in apoptosis, DDR, ROS, cell cycle (low HHI scoring categories), and TF and ligands (categories with high HHI scoring genes). Box colors represent the proportion of mRNA found in each subregion relative to the sum of all mRNA found in all clusters (the values used to calculate HHI). **(C)** X-ray induced genes plotted by their HHI score (calculated on 7 broad regions, not the 35 clusters) and the Euclidean distance in gene expression in seven-dimensional space at 0 rad vs 4000 rad (calculated using the 7 broad regions as described in the text). Color represents the $\log_2$FC of each gene when comparing all cells from the 4000 rad condition to all cells of the 0 rad condition. The dotted lines are drawn at the 95th percentile of each of the two parameters. **(D-G)** Gene expression UMAPs of example genes with <95th percentile Euclidean distance score belonging to the DDR **(D)**, apoptosis **(E)**, ROS **(F)**, and ligand **(G)** groups. **(H-I)** Gene expression UMAPs of two genes with large differences in expression pattern between 0 rad and 4000 rad identified by a Euclidean distance in the top 5%. Genes in **(D-I)** are arranged from high (top) to low (bottom) HHI score.

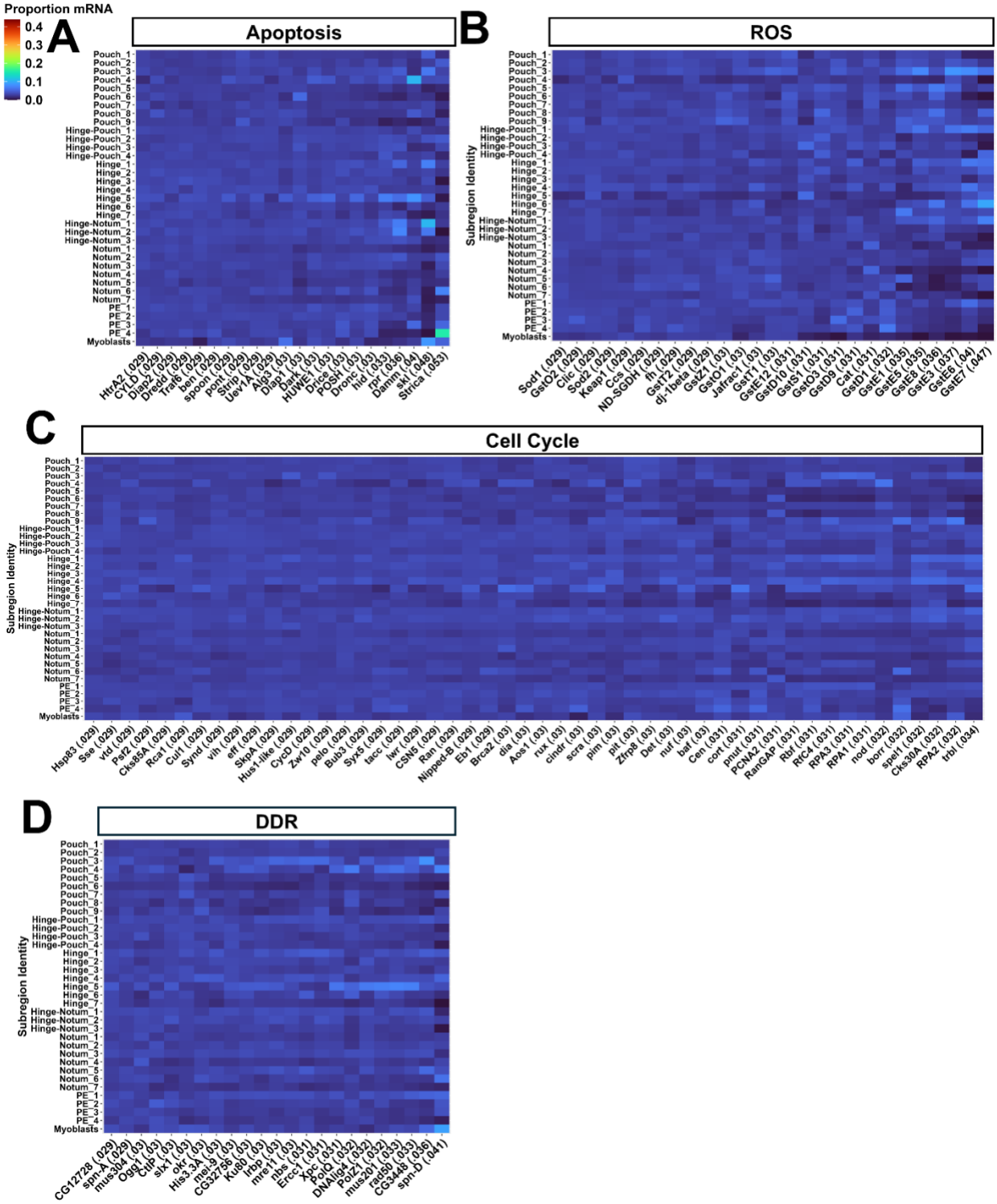


Supplement S3_1. Histogram of cluster HHI scores, Broad Region HHI scores, and Euclidean Expression Distance scores.
(A) Histogram of cluster HHI scores of 3,767 X-ray induced genes that pass the following filters: Adjusted p value < 0.05, Average $\log_2$FC > 0.1, Percent expressed *either* condition ≥0.01. Inset is the same histogram cropped on the y-axis to better view the distribution. **(B)** Histogram of Broad Region HHI scores of 3,655 X-ray induced genes that pass the following filters: Adjusted p value <0.05, Average log2FC>0.1, Percent of cells expressing in *both* conditions ≥ 1%. Inset is the same histogram cropped on the y-axis to better view the distribution. **(C)** Histogram of Expression Euclidean Distances for the same 3,655 genes as in **(B)**. Inset is the same histogram cropped on the y-axis to better view the distribution.



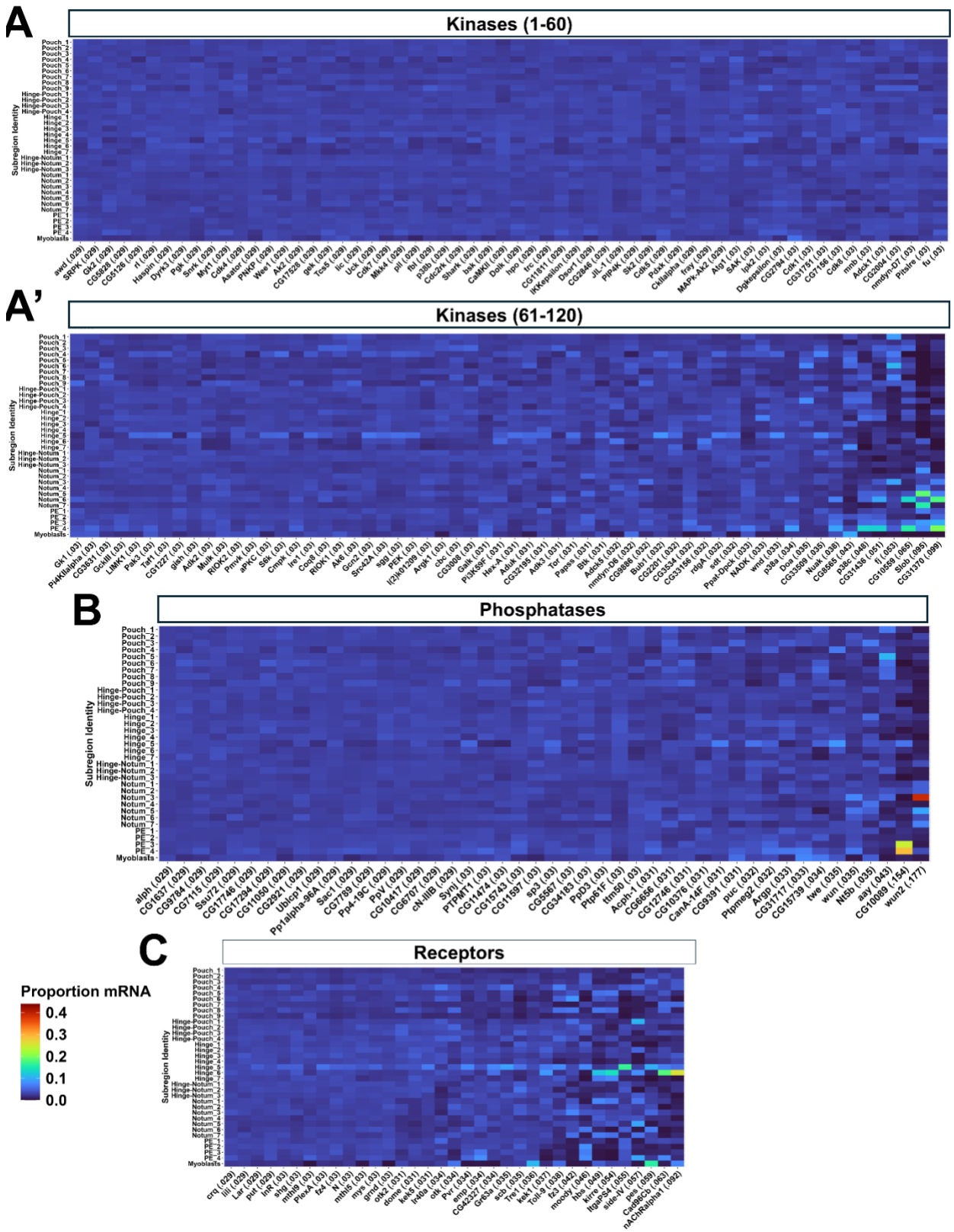
Supplement S3_2. Proportion mRNA heatmaps of Ligands and Transcription Factors

Heatmaps showing the proportion of mRNA found in each of the 35 clusters in the 4000 rad condition for all genes in the Ligand **(A)** and TF **(B-B')** gene categories.


Proportion mRNA
0.4
0.3
0.2
0.1
0.0
A
Apoptosis
B
ROS
C
Cell Cycle
D
DDR
Subregion Identity
Pouch_1
Pouch_2
Pouch_3
Pouch_4
Pouch_5
Pouch_6
Pouch_7
Pouch_8
Pouch_9
Hinge-Pouch_1
Hinge-Pouch_2
Hinge-Pouch_3
Hinge-Pouch_4
Hinge_1
Hinge_2
Hinge_3
Hinge_4
Hinge_5
Hinge_6
Hinge_7
Hinge-Notum_1
Hinge-Notum_2
Hinge-Notum_3
Notum_1
Notum_2
Notum_3
Notum_4
Notum_5
Notum_6
Notum_7
PE_1
PE_2
PE_3
PE_4
Myoblasts
HtrA2 (.029)
CYLD (.029)
Diap2 (.029)
Dredd (.029)
Traf6 (.029)
ben (.029)
spoon (.029)
pont (.029)
Strip (.029)
Uev1A (.029)
Alg3 (.03)
Diap1 (.03)
Dark (.03)
HUWE1 (.03)
Drce (.03)
POSH (.03)
Dronc (.03)
hid (.03)
rpr (.036)
Damm (.04)
ski (.046)
Strica (.055)
Sod1 (.029)
GstO2 (.029)
Clic (.029)
Sod2 (.029)
Keap1 (.029)
Ccs (.029)
ND-SGDH (.029)
fh (.029)
GstT2 (.029)
dj-1beta (.029)
GstZ1 (.03)
GstO1 (.03)
Jafrac1 (.03)
GstT1 (.03)
GstE12 (.031)
GstD10 (.031)
GstS1 (.031)
GstO3 (.031)
GstD9 (.031)
Cat (.031)
GstD1 (.032)
GstE1 (.035)
GstE5 (.035)
GstE8 (.036)
GstE3 (.037)
GstE6 (.04)
GstE7 (.047)
Hsp83 (.029)
Sse (.029)
vtd (.029)
Psf2 (.029)
Cks85A (.029)
Rca1 (.029)
Cul1 (.029)
Synd (.029)
vih (.029)
eff (.029)
SkpA (.029)
Hus1-like (.029)
CycD (.029)
Zw10 (.029)
pelo (.029)
Bub3 (.029)
Syx5 (.029)
tacc (.029)
lwr (.029)
CSN5 (.029)
Ran (.029)
Nipped-B (.029)
Eb1 (.029)
Brca2 (.03)
dia (.03)
Aos1 (.03)
rux (.03)
cindr (.03)
scra (.03)
pim (.03)
pit (.03)
Zfrp8 (.03)
Det (.03)
nuf (.03)
baf (.03)
Cen (.031)
cort (.031)
pnut (.031)
PCNA2 (.031)
RanGAP (.031)
Rbf (.031)
RfC4 (.031)
RPA3 (.031)
RPA1 (.031)
nod (.032)
borr (.032)
spel1 (.032)
Cks30A (.032)
RPA2 (.032)
trbl (.034)
CG12728 (.029)
spn-A (.029)
mus304 (.03)
Ogg1 (.03)
CtIP (.03)
six1 (.03)
oKr (.03)
His3.3A (.03)
mei-9 (.03)
CG32756 (.03)
Ku80 (.03)
Irbp (.03)
mre11 (.03)
nbs (.031)
Ercc1 (.031)
Xpc (.031)
PolQ (.032)
DNAlig4 (.032)
PolZ1 (.032)
mus201 (.033)
rad50 (.033)
CG3448 (.036)
spn-D (.041)

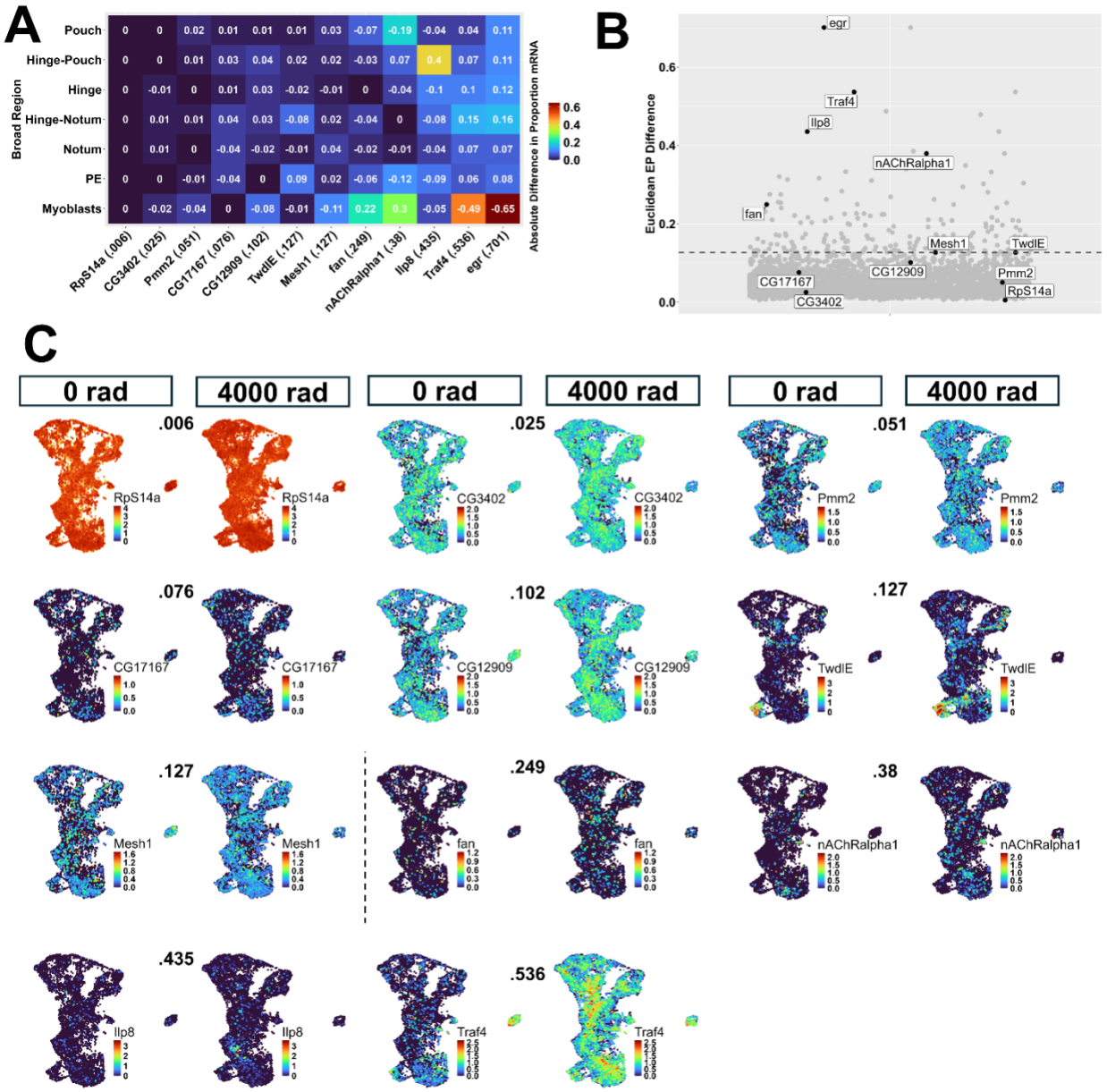
Supplement S3_3. Proportion mRNA heatmaps of Apoptosis, ROS, Cell Cycle, and DDR Genes

Heatmaps showing the proportion of mRNA found in each cluster in the 4000 rad condition for all genes in the Apoptosis **(A)** ROS **(B)**, Cell Cycle **(C)** , and DDR **(D)** gene categories.


A
Kinases (1-60)
A'
Kinases (61-120)
B
Phosphatases
C
Receptors
Subregion Identity
Proportion mRNA
0.4
0.3
0.2
0.1
0.0

Supplement S3_4. ***Proportion mRNA heatmaps of Kinase, Phosphatase, and Receptor Genes.***

Heatmaps showing the proportion of mRNA found in each cluster in the 4000 rad condition for all genes in the Kinase **(A-A')**, Phosphatase **(B)**, and Receptor **(C)** gene categories.



Supplement S3_5. Examples of Genes Spanning Range of Expression Distance Score

(A) Heat map showing the difference in the proportion of each PD region between 4000 and 0 rad. Numbers in the tiles are the numeric values of this difference, with positive values indicating a higher proportion at 4000 rad for a given region, and negative values indicating a lower proportion at 4000 rad. Euclidean distance was calculated on the absolute values of these differences. Genes were selected to span the range of Expression Distances. **(B)** Plot showing the distribution of Expression Distances for the 3,655 genes plotted in Fig. 3C. The dotted line is drawn at the 95th percentile of scores. Genes from panel A are highlighted. **(C)** Expression UMAPs of genes spanning the

range of Expression Distance scores, ordered in ascending order of Expression Distance (number above UMAPs). The dotted line separates genes below (left of the line) and above (right of the line) the 95th percentile.

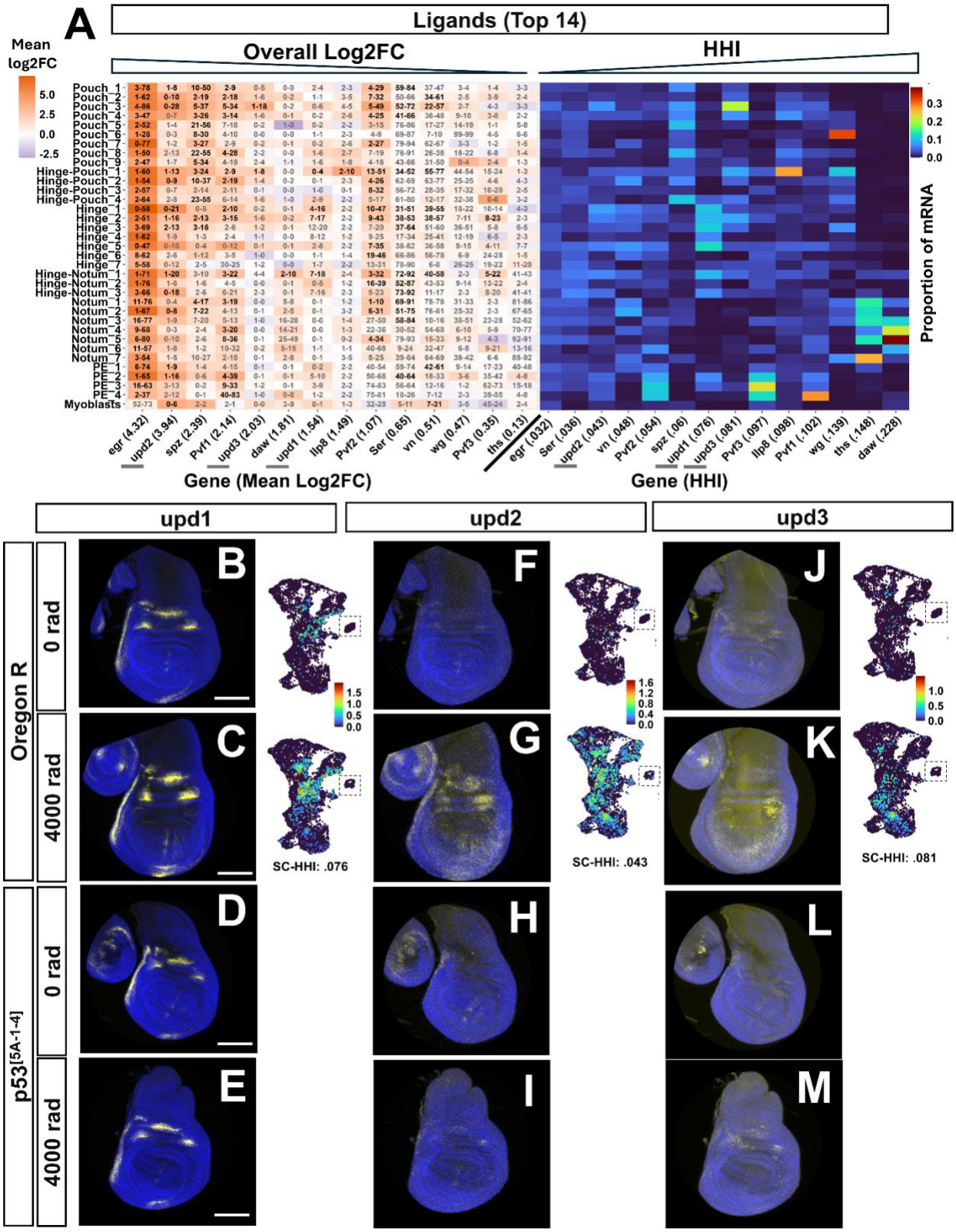
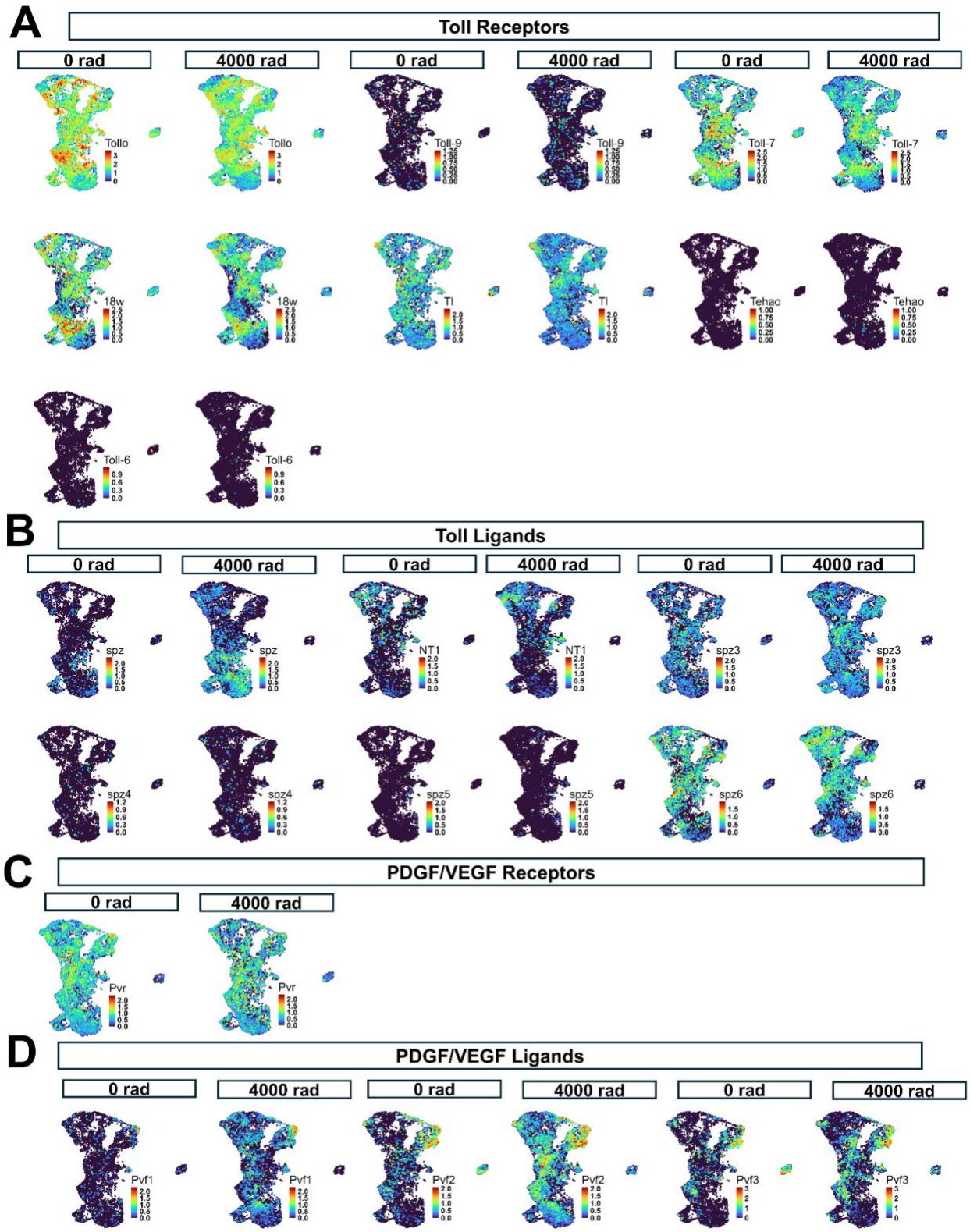
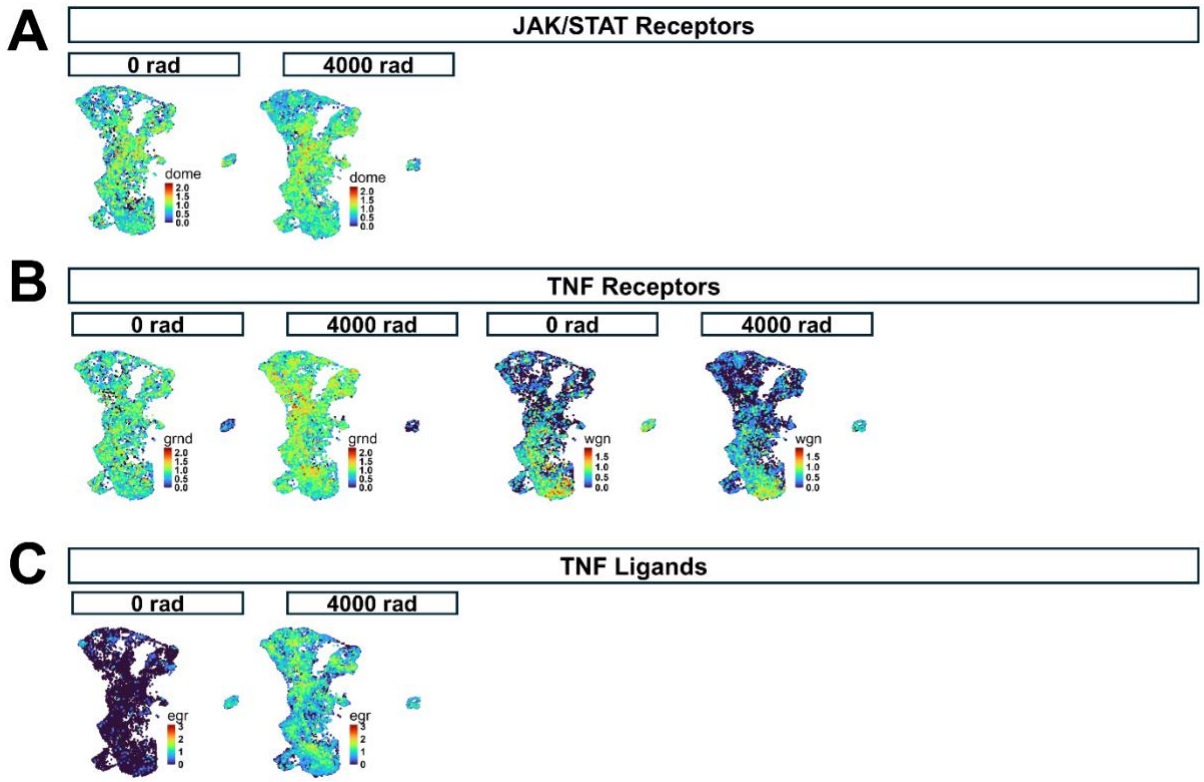

A
Ligands (Top 14)
Overall Log2FC
HHI
Mean log2FC
5.0
2.5
0.0
-2.5
Pouch_1
Pouch_2
Pouch_3
Pouch_4
Pouch_5
Pouch_6
Pouch_7
Pouch_8
Pouch_9
Hinge-Pouch_1
Hinge-Pouch_2
Hinge-Pouch_3
Hinge-Pouch_4
Hinge_1
Hinge_2
Hinge_3
Hinge_4
Hinge_5
Hinge_6
Hinge_7
Hinge-Notum_1
Hinge-Notum_2
Hinge-Notum_3
Notum_1
Notum_2
Notum_3
Notum_4
Notum_5
Notum_6
Notum_7
PE_1
PE_2
PE_3
PE_4
Myoblasts
egr (4.32)
upd2 (3.94)
spz (2.39)
Pvf1 (2.14)
upd3 (2.03)
daw (1.81)
upd1 (1.54)
Ilp8 (1.49)
Pvf2 (1.07)
Ser (0.65)
vn (0.51)
wg (0.47)
Pvf3 (0.35)
ths (0.13)
egr (.032)
Ser (.036)
upd2 (.043)
vn (.048)
Pvf2 (.054)
spz (.06)
upd1 (.076)
upd3 (.081)
Pvf3 (.097)
Ilp8 (.098)
Pvf1 (.102)
wg (.139)
ths (.148)
daw (.228)
Gene (Mean Log2FC)
Gene (HHI)
0.3
0.2
0.1
0.0
Proportion of mRNA
upd1
upd2
upd3
Oregon R
0 rad
4000 rad
p53[5A-1-4]
0 rad
4000 rad
B
C
D
E
F
G
H
I
J
K
L
M
1.5
1.0
0.5
0.0
SC-HHI: .076
1.6
1.2
0.8
0.4
0.0
SC-HHI: .043
1.0
0.5
0.0
SC-HHI: .081

Figure 4. X-ray-induced expression of genes encoding ligands
(A) Heatmaps showing log_2FC between conditions for each cluster (left) and proportion of total mRNA in each cluster at 4000 rad (right) of top 14 ligands ranked by max log_2FC in any cluster. **A Left**: Boxes are colored based on the average log_2FC from 0 rad to 4000 rad for each cluster. The left number in each box is the percentage of cells in the cluster expressing at least one transcript at 0 rad; the right number is the same at 4000 rad. Dark black numbers indicate that the change is statistically significant in that cluster (adjusted p value < 0.05, Wilcoxon Rank Sum Test, Bonferroni correction), while light gray numbers indicate that it is not (adjusted p value ≥0.05). Genes are sorted from left to right in descending order of the mean log_2FC when comparing all cells of 0 rad to all cells of 4000 rad ("overall mean log_2FC"); The overall mean log_2FC value is in parentheses next to gene names (all significant). **A Right**: Boxes are colored based on the proportion of mRNA expressed in that cluster vs the total amount of mRNA expressed in all clusters. Genes are sorted from left to right in ascending order of HHI score at 4000 rad. HHI score is noted in parentheses next to gene name. Genes shown in panels **(B-G)** are underlined with gray bars in **(A)**. **(B-M)** In each panel: HCR of *upd1, upd2, upd3* are in yellow and DAPI in blue at 0 rad **(B, D, F, H, J, L)** or 4000 rad **(C, E, G, I, K, M)**. Wild type discs **(B, C, F, G, J, K)** are compared to p53 mutant discs **(D, E, H, I, L, M)**. To the right of each wild-type HCR image is an expression UMAP of each gene in each condition. The myoblasts were cropped into the UMAP images for space, indicated by the dotted box around them.


A
Toll Receptors
0 rad
4000 rad
0 rad
4000 rad
0 rad
4000 rad
Tollo
Tollo
Toll-9
Toll-9
Toll-7
Toll-7
18w
18w
Tl
Tl
Tehao
Tehao
Toll-6
Toll-6
B
Toll Ligands
0 rad
4000 rad
0 rad
4000 rad
0 rad
4000 rad
spz
spz
NT1
NT1
spz3
spz3
spz4
spz4
spz5
spz5
spz6
spz6
C
PDGF/VEGF Receptors
0 rad
4000 rad
Pvr
Pvr
D
PDGF/VEGF Ligands
0 rad
4000 rad
0 rad
4000 rad
0 rad
4000 rad
Pvf1
Pvf1
Pvf2
Pvf2
Pvf3
Pvf3

Supplement S4_1. Toll and PDGF/VEGF ligands and receptors
Expression UMAPs of Toll receptors **(A)**, Toll ligands **(B)**, PDGF/VEGF receptors **(C)**, and PDGF/VEGF ligands **(D)**.



Supplement S4_2. JAK/STAT receptors and TNF ligands and receptors
Expression UMAPs of JAK/STAT receptors **(A)**, TNF receptors **(B)**, and TNF ligands **(C)**.

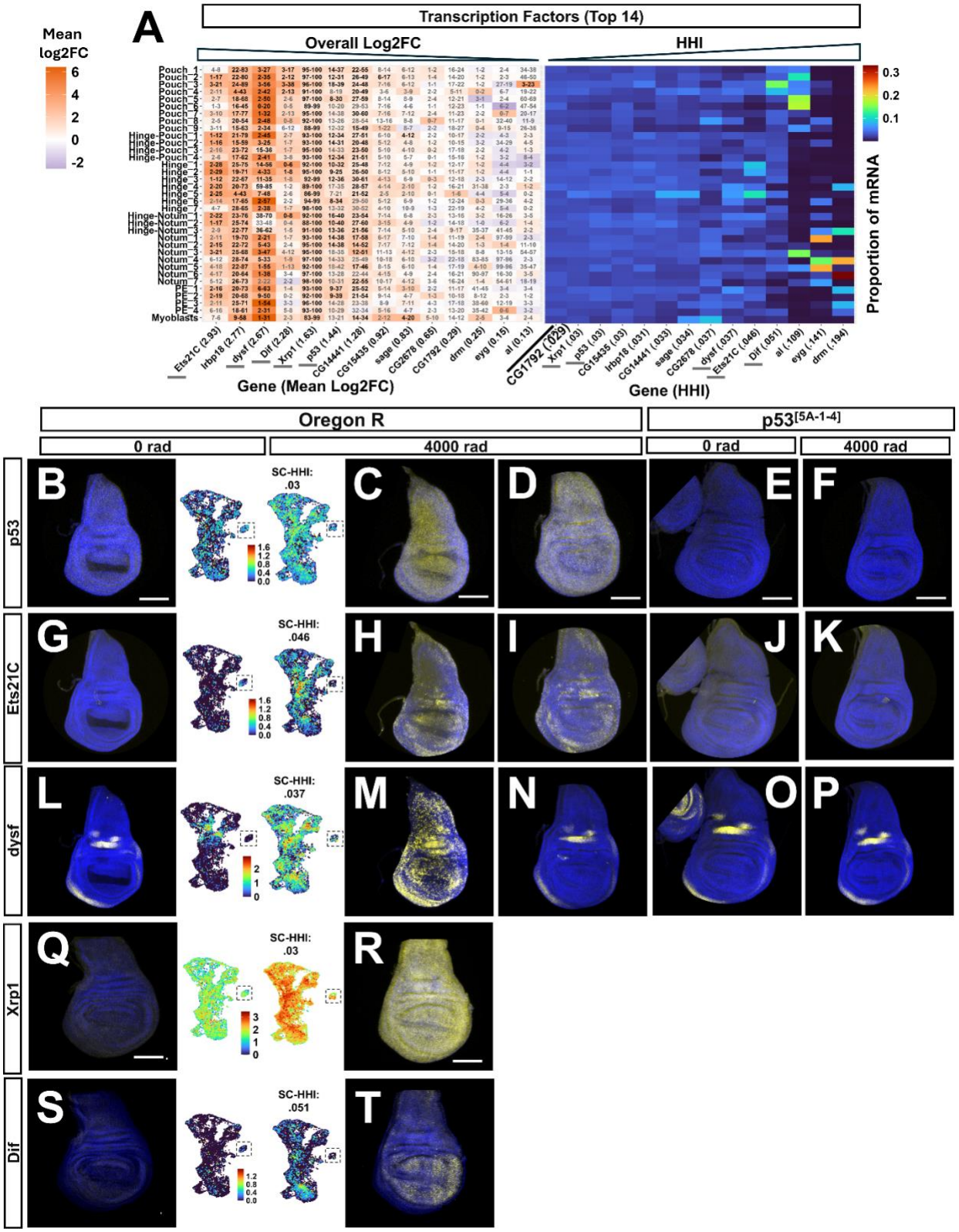
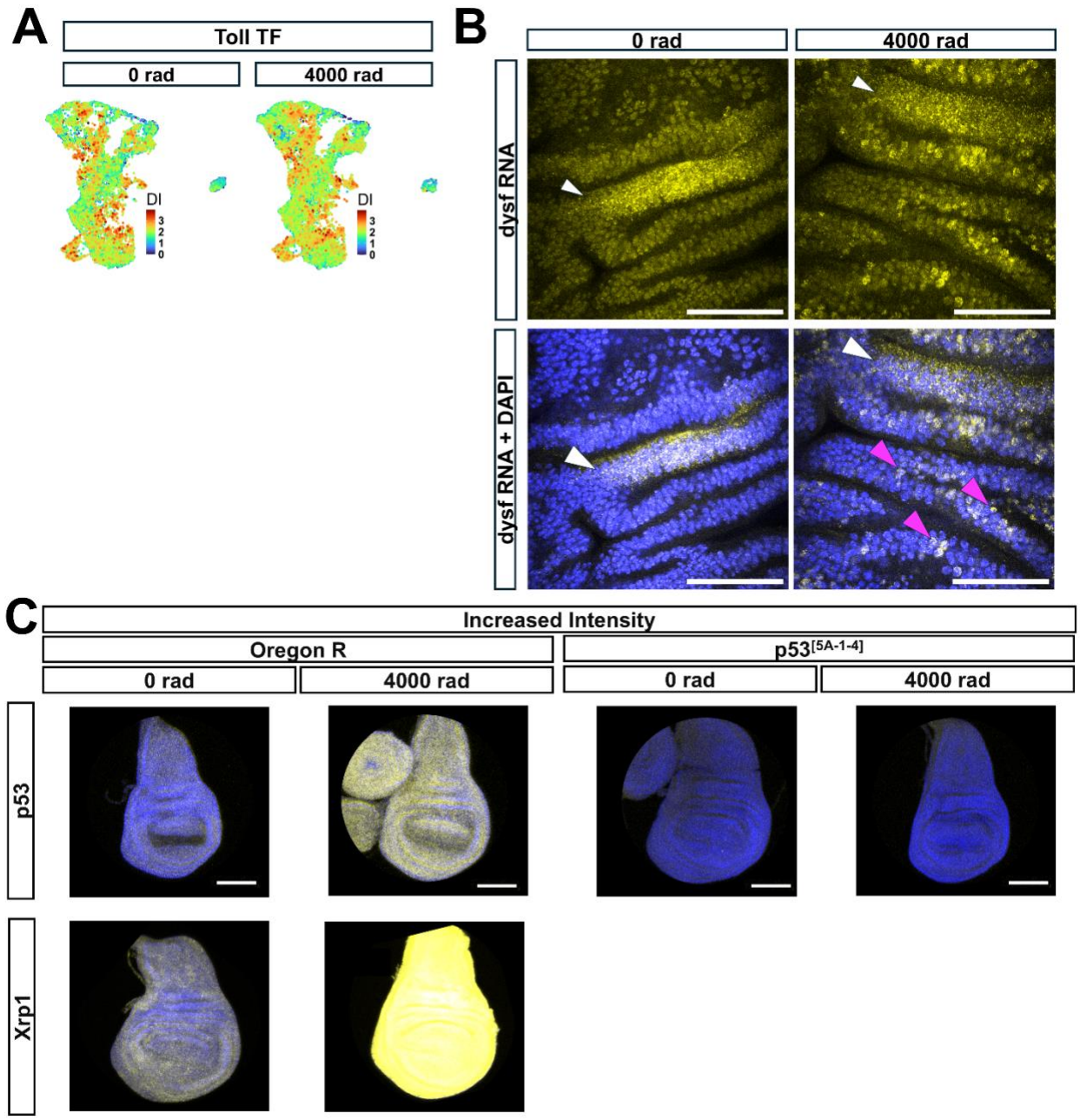

A
Transcription Factors (Top 14)
Overall Log2FC
HHI
Mean log2FC
Proportion of mRNA
Gene (Mean Log2FC)
Gene (HHI)
Oregon R
p53[5A-1-4]
0 rad
4000 rad
p53
Ets21C
dysf
Xrp1
Dif
SC-HHI: .03
SC-HHI: .046
SC-HHI: .037
SC-HHI: .03
SC-HHI: .051

Figure 5. X-ray Induced Expression of Transcription Factors.
(A) Heatmaps showing log2FC between conditions for each cluster **(left)** and proportion of total mRNA in each cluster at 4000 rad **(right)** of top 14 TFs by max log2FC in any cluster. Generated in the same way as figure 5A. Genes shown in figures B-T are underlined with gray bars in A. **(B,G,L,Q,S)** HCR of *p53, Ets21C, dysf, Xrp1,* and *Dif* at 0 rad in Oregon R wing discs. (**C,H,M,R,T)** HCR of the same genes at 4000 rad in Oregon R wing discs. HCR in yellow and DAPI in blue. To the right of 0 rad and left of 4000 rad HCR image is an expression UMAP of each gene in each condition. (D,I,N) alternative irradiated wing disc showing non-induction of *dysf* after X-rays. The myoblasts were cropped into the UMAP images for space, indicated by the dotted box around them. **(E,J,O)** HCR of *p53*, *Ets21C* and *dysf* in *p53[5A-1-4]* mutant wing discs at 0 rad. **(F,K,P)** HCR of the same genes at 4000 rad in *p53[5A-1-4]* mutant wing discs. HCR in yellow and DAPI in blue.



Supplement S5_1.
(A) Heatmap of the Toll TF *Dl.* **(B)** 63X magnification images of *dysf* HCR. White arrowheads point to the location of normal developmental expression of *dysf* in the posterior hinge (pouch is below this location). Magenta arrowheads point to X-ray induced *dysf* RNA which is localized to the nucleus. Scale bars are 50 µm. **(C)** HCR of *p53* and *Xrp1* with increased gain. Scale bars are 100 µm.

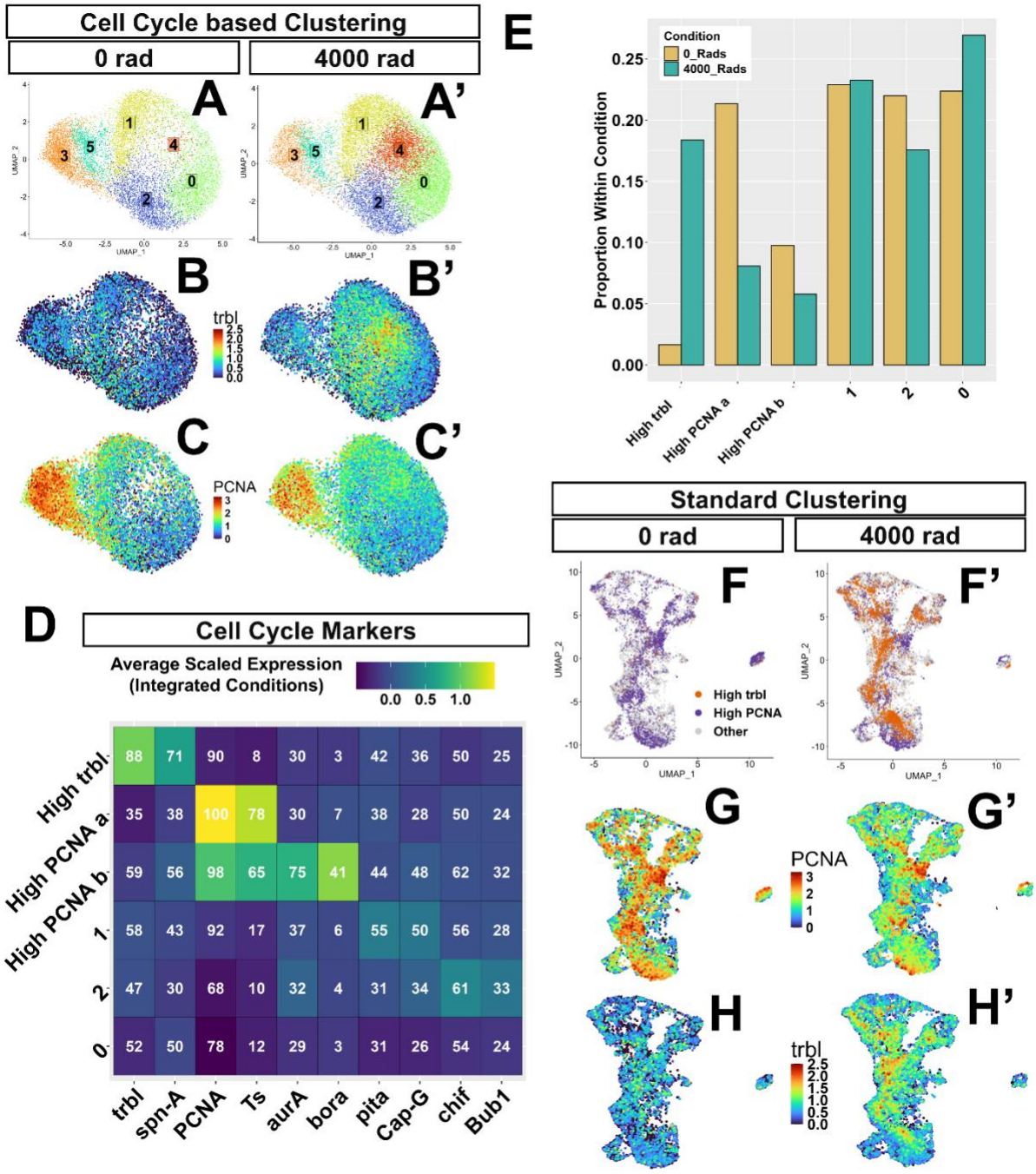
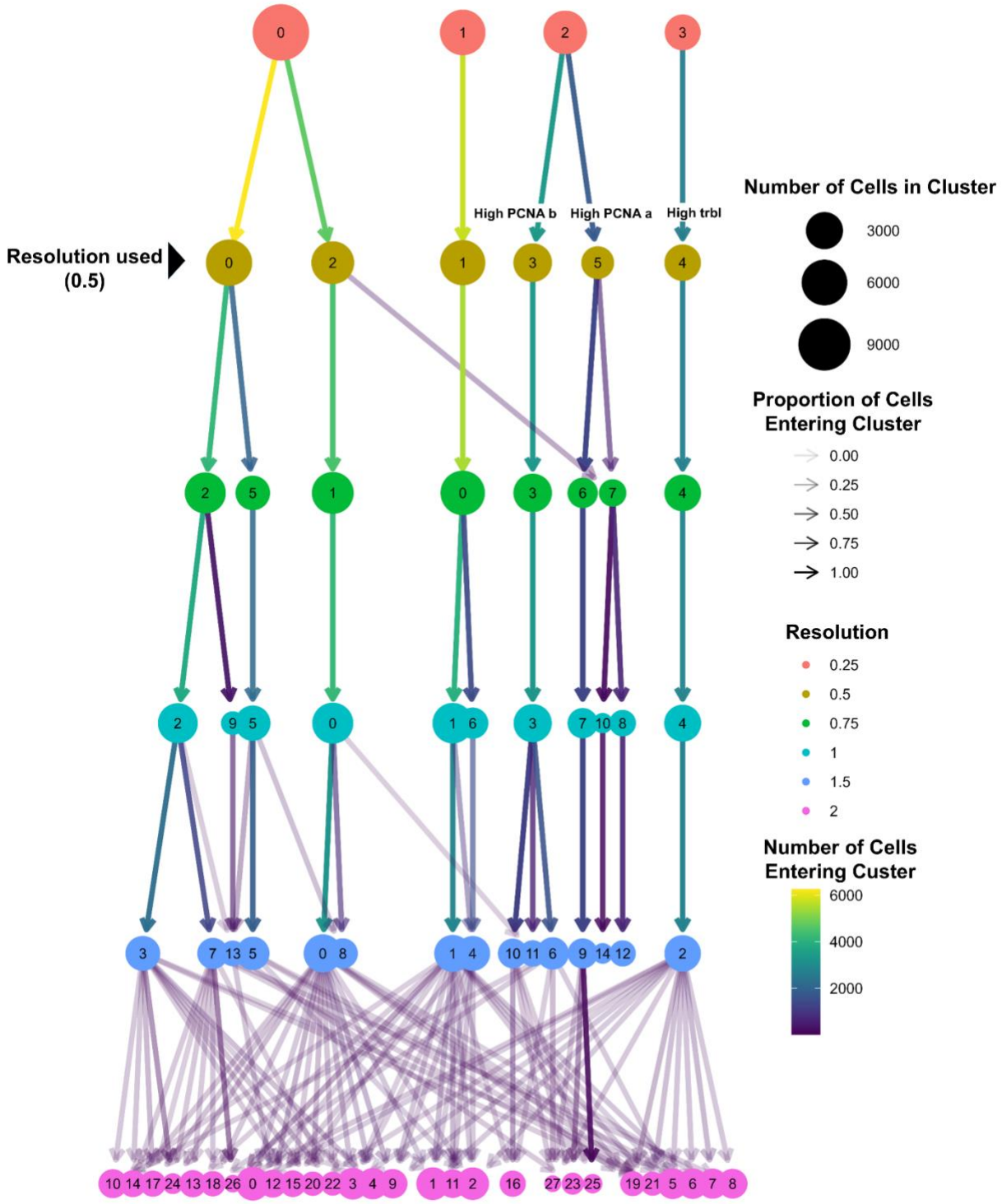


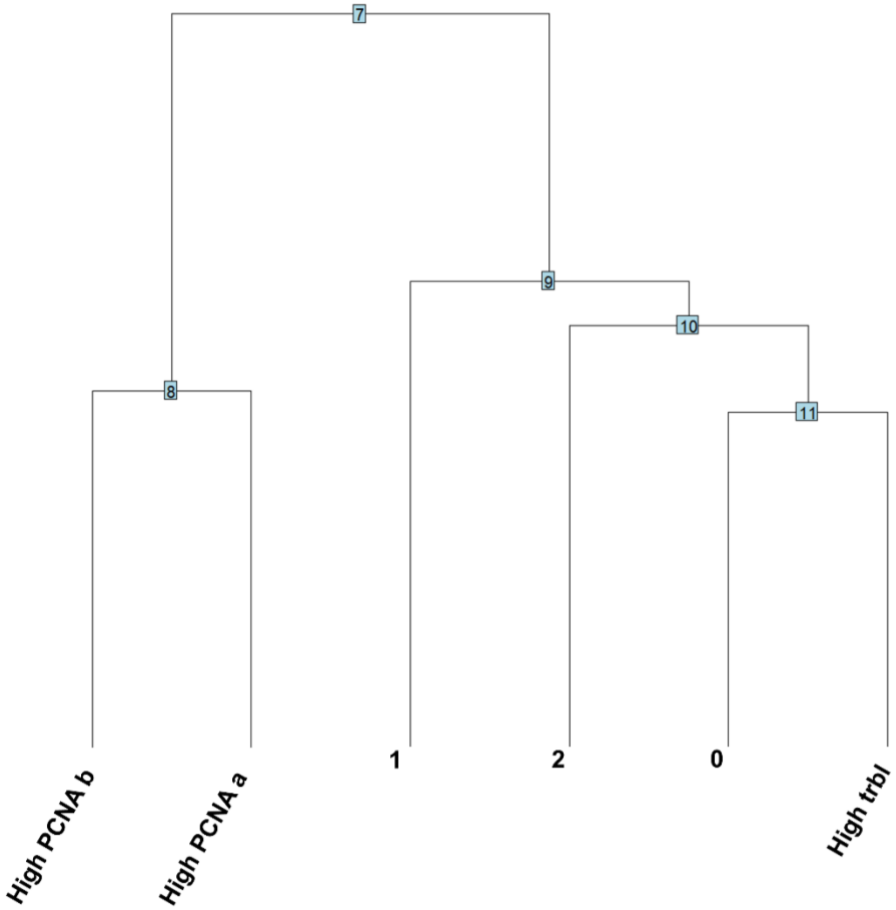
Figure 6. Visualization of expression changes using cell-cycle-based clustering **(A - A')** Cluster UMAPs of data processed and clustered on cell cycle genes at 0 rad **(A)** and 4000 rad **(A')**. **(B-C')** Expression in this UMAP object of *trbl* and *PCNA* at 0 rad **(B, C)** and 4000 rad **(B', C')**. **(D)** Heatmap showing average scaled expression of cell cycle marker genes in each cell cycle cluster. Numbers are the percent of cells expressing each gene in each cluster. **(E)** Bar plot showing the proportion of cells each cluster contributes to 0 rad (cream) and 4000 rad (teal) conditions. **(F-H')** High *trbl* cluster 4 (in orange) and high *PCNA* clusters 3 and 5 (in purple) from panels A-A' mapped onto the

standard UMAP at 0 rad **(F)** and 4000 rad **(F')**. **(G-H')** Standard expression UMAPs of *PCNA* and *trbl* at 0 rad **(G, H)** and 4000 rad **(G', H')**.


Resolution used
(0.5)
High PCNA b
High PCNA a
High trbl
Number of Cells in Cluster
3000
6000
9000
Proportion of Cells
Entering Cluster
0.00
0.25
0.50
0.75
1.00
Resolution
0.25
0.5
0.75
1
1.5
2
Number of Cells
Entering Custer
6000
4000
2000

Supplement S6_1. Cluster tree showing cluster stability at different clustering resolutions

Tree showing clusters generated using different Leiden clustering resolution parameters on the integrated dimensions derived from 175 cell cycle genes. Each node is a cluster (cluster numbers are in order of decreasing cluster size but otherwise arbitrary). Each row of clusters from top to bottom are derived from increasing resolutions. Arrows represent cells moving from one cluster identity at a lower resolution to a different cluster identity at the next resolution. Arrow color represents the number of cells making the transition, and arrow transparency represents the proportion contribution of those cells to the new cluster.



Supplement S6_2. Dendrogram of cell cycle based cluster relationships
Dendrogram showing the relationship of cell-cycle based clusters to one another in integrated PCA space. Shorter lines represent a closer relationship; Longer lines a more distant relationship.

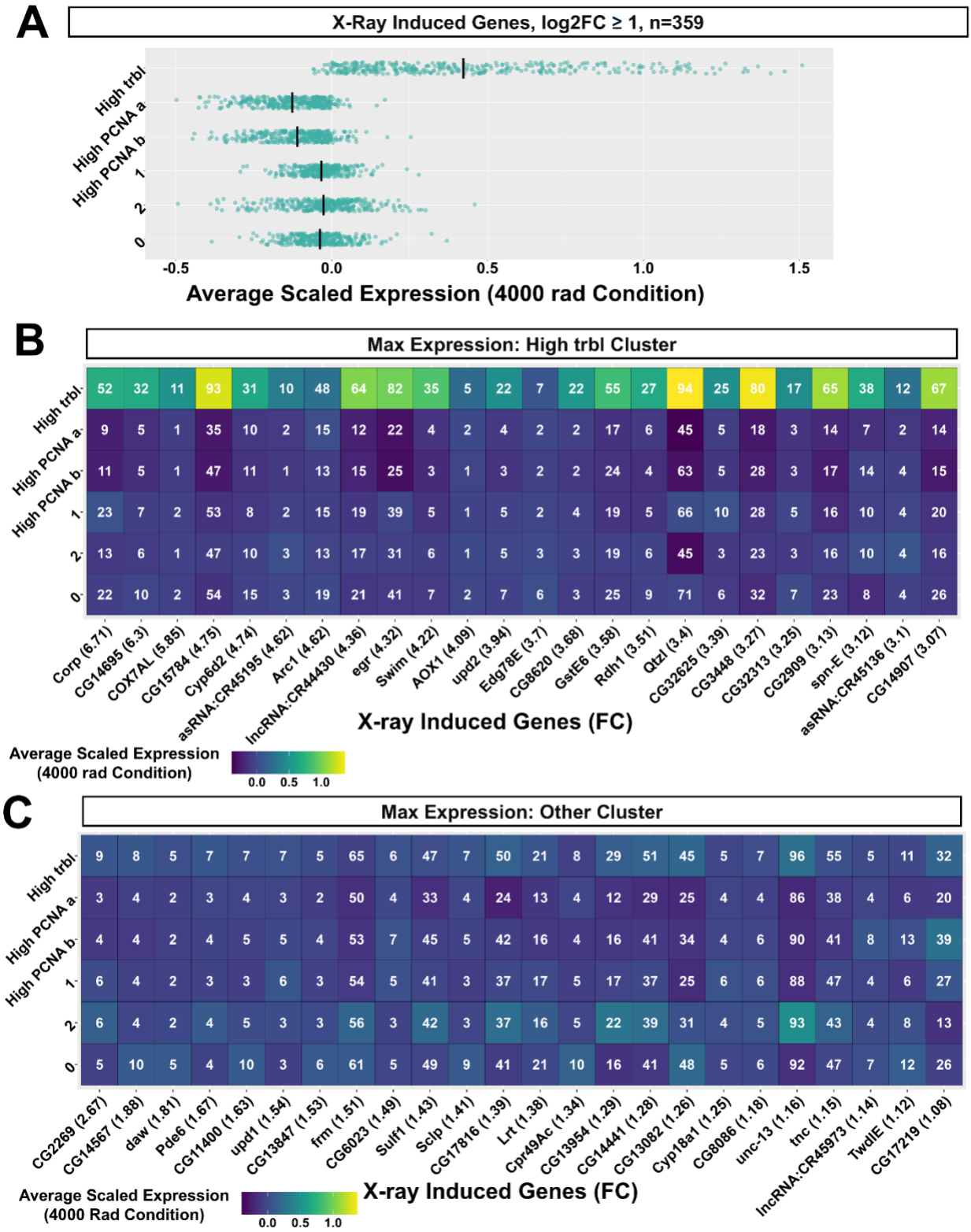

A
X-Ray Induced Genes, log2FC ≥ 1, n=359
High trbl
High PCNA a
High PCNA b
1
2
0
-0.5
0.0
0.5
1.0
1.5
Average Scaled Expression (4000 rad Condition)
B
Max Expression: High trbl Cluster
High trbl. 52 32 11 93 31 10 48 64 82 35 5 22 7 22 55 27 94 25 80 17 65 38 12 67
High PCNA a. 9 5 1 35 10 2 15 12 22 4 2 4 2 2 17 6 45 5 18 3 14 7 2 14
High PCNA b. 11 5 1 47 11 1 13 15 25 3 1 3 2 2 24 4 63 5 28 3 17 14 4 15
1. 23 7 2 53 8 2 15 19 39 5 1 5 2 4 19 5 66 10 28 5 16 10 4 20
2. 13 6 1 47 10 3 13 17 31 6 1 5 3 3 19 6 45 3 23 3 16 10 4 16
0. 22 10 2 54 15 3 19 21 41 7 2 7 6 3 25 9 71 6 32 7 23 8 4 26
Corp (6.71)
CG14695 (6.3)
COX7AL (5.85)
CG15784 (4.75)
Cyp6d2 (4.74)
asRNA:CR45195 (4.62)
Arc1 (4.62)
lncRNA:CR44430 (4.36)
egr (4.32)
Swim (4.22)
AOX1 (4.09)
upd2 (3.94)
Edg78E (3.7)
CG8620 (3.68)
GstE6 (3.58)
Rdh1 (3.51)
Qtzl (3.4)
CG32625 (3.39)
CG3448 (3.27)
CG32313 (3.25)
CG2909 (3.13)
spn-E (3.12)
asRNA:CR45136 (3.1)
CG14907 (3.07)
X-ray Induced Genes (FC)
Average Scaled Expression (4000 rad Condition)
0.0 0.5 1.0
C
Max Expression: Other Cluster
High trbl. 9 8 5 7 7 7 5 65 6 47 7 50 21 8 29 51 45 5 7 96 55 5 11 32
High PCNA a. 3 4 2 3 4 3 2 50 4 33 4 24 13 4 12 29 25 4 4 86 38 4 6 20
High PCNA b. 4 4 2 4 5 5 4 53 7 45 5 42 16 4 16 41 34 4 6 90 41 8 13 39
1. 6 4 2 3 3 6 3 54 5 41 3 37 17 5 17 37 25 6 6 88 47 4 6 27
2. 6 4 2 4 5 3 3 56 3 42 3 37 16 5 22 39 31 4 5 93 43 4 8 13
0. 5 10 5 4 10 3 6 61 5 49 9 41 21 10 16 41 48 5 6 92 47 7 12 26
CG2269 (2.67)
CG14567 (1.88)
daw (1.81)
Pde6 (1.67)
CG11400 (1.63)
upd1 (1.54)
CG13847 (1.53)
frm (1.51)
CG6023 (1.49)
Sulf1 (1.43)
Sclp (1.41)
CG17816 (1.39)
Lrt (1.38)
Cpr49Ac (1.34)
CG13954 (1.29)
CG14441 (1.28)
CG13082 (1.26)
Cyp18a1 (1.25)
CG8086 (1.18)
unc-13 (1.16)
tnc (1.15)
lncRNA:CR45973 (1.14)
TwdlE (1.12)
CG17219 (1.08)
X-ray Induced Genes (FC)
Average Scaled Expression (4000 Rad Condition)
0.0 0.5 1.0

Figure 7. Differences in gene expression between clusters in cell cycle UMAP
(A) Mean scaled expression of X-ray induced genes ≥ avg $\log_2$FC 1 between conditions. Values calculated using the 4000 rad condition on cell-cycle-based clusters. Each row contains the same genes. **(B)** Heatmap showing average scaled expression of the top 24 X-ray-induced genes with highest expression in the *trbl* cluster. **(C)** 24 of the genes expressed maximally in one of the other clusters. Numbers are the percent of cells expressing each gene in each cluster. Only genes with ≥ 5% expression in any cluster at 4000 rad were selected from the initial 359.

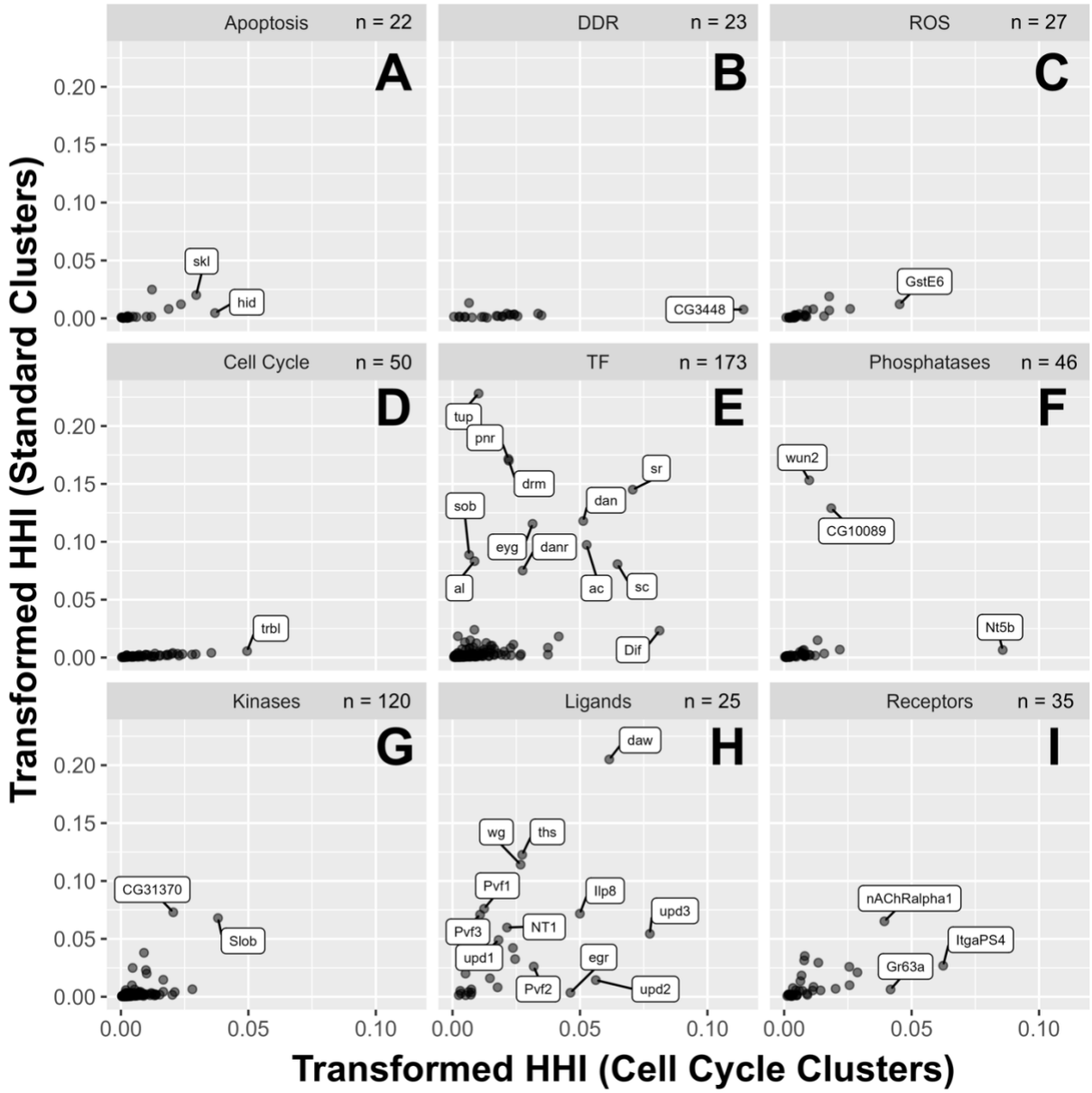


Figure 8. HHI scoring of categorical genes in standard clusters vs cell cycle clusters.

(A-I) Transformed HHI scores calculated using the 35 standard clusters (y-axis) and the 6 cell cycle based clusters (x-axis). Genes used were the 521 categorical genes used previously in Fig. 3A. Scores were calculated using the 4000 rad condition only. Genes displayed encode for proteins involved in apoptosis **(A)**, DDR **(B)**, ROS **(C)**, cell cycle **(D)**, TFs **(E)**, Phosphatases **(F)**, Kinases **(G)**, Ligands **(H)**, and Receptors **(I)**. Genes are labeled where space permits, but were otherwise chosen arbitrarily.

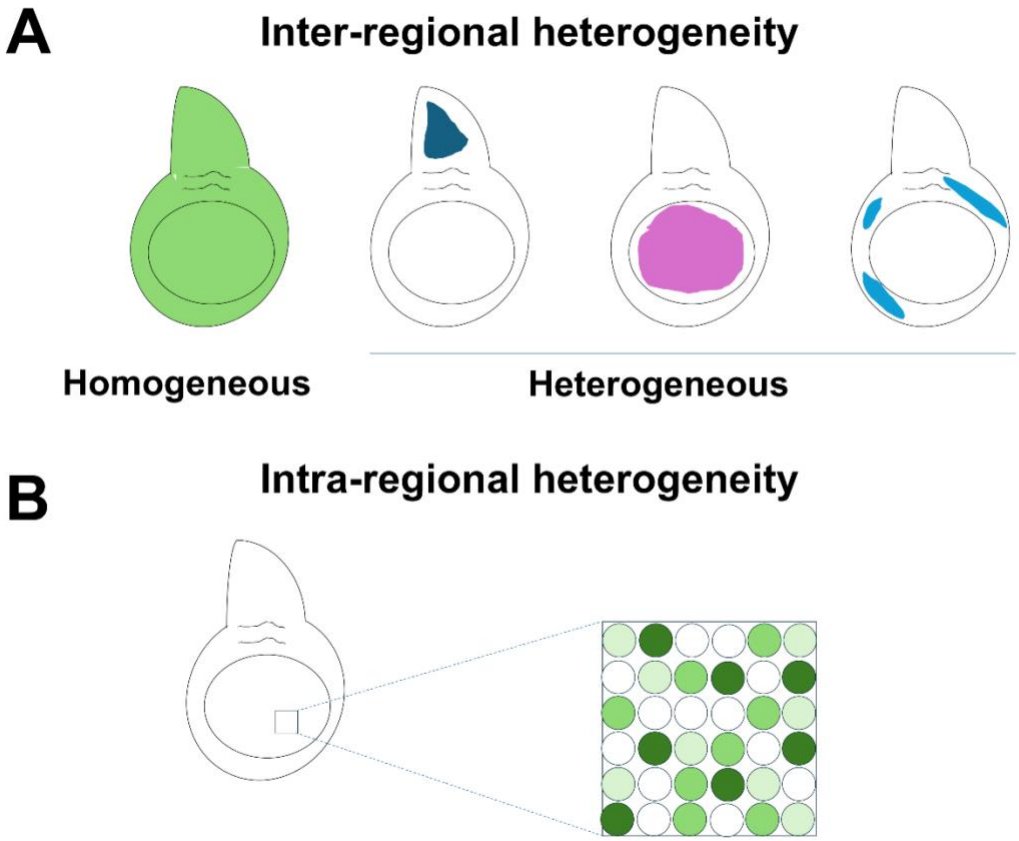


Figure 9. Two types of heterogeneity following X-ray irradiation
(**A**) Inter-regional heterogeneity. Many genes are expressed uniformly while some are expressed in specific territories following irradiation.
(**B**) Intra-regional heterogeneity. In the example shown, a gene is expressed at high levels in some cells, intermediate levels in some, and no expression in others. Cells with different levels of expression are interspersed.