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Activation of the receptor tyrosine kinase, RET, improves long-term hematopoietic stem cell outgrowth and potency

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Expansion of Human Hematopoietic Stem Cells (HSCs) is a rapidly advancing field showing great promise for clinical applications. Recent evidence has implicated the nervous system and glial family ligands (GFLs) as potential drivers of hematopoietic survival and self-renewal in the bone marrow niche, but how to apply this to HSC maintenance and expansion is yet to be explored. We demonstrate a role for the GFL receptor, RET, at the cell surface of HSCs, in mediating sustained cellular growth, resistance to stress and improved cell survival throughout *in vitro* expansion. HSCs treated with the key RET ligand/co-receptor complex, GDNF/GFRa1, show improved progenitor function at primary transplantation and improved long-term HSC function at secondary transplantation. Finally, we demonstrate that RET drives a multi-faceted intracellular signalling pathway, including key signalling intermediates AKT, ERK1/2, NFkB and p53, responsible for a wide range of cellular and genetic responses which improve cell growth and survival under culture conditions.

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- RET cell surface expression and activity is enriched in HSCs.
- Activation of RET by GDNF/GFRα1 improves LT-HSC outgrowth *in vitro* and transplantation *in vivo*.

Abstract

Expansion of Human Hematopoietic Stem Cells (HSCs) is a rapidly advancing field showing great promise for clinical applications. Recent evidence has implicated the nervous system and glial family ligands (GFLs) as potential drivers of hematopoietic survival and self-renewal in the bone marrow niche, but how to apply this to HSC maintenance and expansion is yet to be explored. We demonstrate a role for the GFL receptor, RET, at the cell surface of HSCs, in mediating sustained cellular growth, resistance to stress and improved cell survival throughout *in vitro* expansion. HSCs treated with the key RET ligand/co-receptor complex, GDNF/GFRα1, show improved progenitor function at primary transplantation and improved long-term HSC function at secondary transplantation. Finally, we demonstrate that RET drives a multi-faceted intracellular signalling pathway, including key signalling intermediates AKT, ERK1/2, NFκB and p53, responsible for a wide range of cellular and genetic responses which improve cell growth and survival under culture conditions.

Introduction

Hematopoietic stem cells (HSCs) are highly potent stem cells of the blood system, known to reside in the bone marrow of adults and umbilical cord blood (UCB) during pregnancy. Whilst bone marrow biopsy is invasive and harsh, collection of UCB represents a less invasive, clinically important source of HSCs and progenitors (HSPCs) for treatment of a wide range of malignant and non-malignant disorders. UCB has a lower incidence of graft versus host disease, with less stringent donor cross-matching required compared to classical donor sources, increasing its value for both hematological and non-hematological malignancies[1]. Despite increasing UCB banking, limited progenitor cell dose[2], delay of engraftment and immune reconstitution[3] and the cost of double UCB transplantation in adults[4], underline a need to improve expansion and potency of these cells for the purposes of transplantation.

To address these limitations, critical advances have been made in both identification and successful outgrowth of HSCs from bone marrow and UCB sources[5–11]. Despite these advances, further expansion of HSCs is required to address clinical issues associated with delayed engraftment/immune reconstitution, and relative paucity of HSCs produced at the end of current culture protocols.

In recent years, there has been increasing evidence that the nervous system may be important for communication with, and influence over, the hematopoietic system. Central to this theory, the receptor tyrosine kinase, RET, has been demonstrated to be expressed in murine HSCs, playing an important role in their survival *in vivo*, and potentiating outgrowth *in vitro* when activated by glial derived neurotrophic factor (GDNF) family ligands and co-receptors, mediating *Bcl2* expression[12]. These findings indicate that neuronal signals are critically important for HSC efficacy, and may play a role in mitigating the stress response exerted on HSCs during *in vitro* expansion.

Here, we investigated the role of RET at the cell surface of UCB-derived HSCs and the effect of the RET ligand/co-receptor complex, GDNF/GFRα1, on outgrowth, initial *in vivo* potency, and long-term stem cell potential of UCB-derived HSPCs. We monitored key changes in protein signalling cascades, to understand the intracellular state governed by RET, and provide a mechanism by which activation of RET can be a positive addition to current culture methods for clinical purposes.

Methods

Primary Human Samples

Umbilical Cord Blood (UCB) was obtained from full term donors after informed consent at the Royal London Hospital (London, U.K.). Mononuclear cells were isolated by density centrifugation using Ficoll-Paque (GE Healthcare). Cells were depleted for lineage markers using an EasySep Human Progenitor Cell Enrichment Kit (Stem Cell Technologies) according to the manufacturer's instructions. Lineage depleted cells were stained with antibodies listed in the Key Resources Table and sorted using a BD FACS Aria Fusion.

In Vitro Culture Conditions

Human $CD34^+CD38^-$ cells were cultured in StemSpan SFEMII (Stem Cell Technologies) supplemented with Human SCF (150ng/ml), Human FLT3 ligand (150ng/ml) and Human TPO (20ng/ml; Peprotech) and when indicated GDNF/GFRα1 (100ng/ml, GDNF & GFRα1 mixed 1:1; R&D systems), SR1 (750nM; Stem Cell Technologies), UM171 (35nM; Stem Cell Technologies), or PZ1 (10nM, Sigma-Aldrich). Cells were incubated in a tissue culture incubator at 37°C, 5% CO_2 for seven days. For all culture experiments, independent pools of umbilical cord blood were used for treatments vs control.

Xenotransplantation Assays

Primary or cultured $CD34^+CD38^-$ HSPCs were injected in 8-10 weeks old unconditioned Female NBSGW mice intravenously (I.V.). Injected mice were euthanised after 12 weeks, in both primary and secondary transplantations, by cervical dislocation and 6 rear bones and spleen were collected. Bone marrow was flushed by centrifugation, spleens were crushed and passed through a 100μM strainer, and resulting cells were incubated in red blood cell lysis buffer (155mM NH_4Cl, 12mM $NaHCO_3$, 0.1mM EDTA) for 5 minutes at room temperature. Remaining cells were stained with antibodies listed in the Key Resources Table and sorted and analysed using a BD FACS Aria Fusion. Secondary transplantations were conducted as per primary transplantations using Human CD45 positive cells sorted from primary mice as donors.

See further methods description in supplementary information

Results

The receptor tyrosine kinase, RET, is more active in $CD34^+CD38^-$ HSPCs than $CD34^+CD38^+$ HPCs

In Human UCB, the $CD34^+CD38^-$ compartment (HSPCs) contains HSCs able to engraft long-term in immunodeficient mouse models. In comparison, the $CD34^+CD38^+$ compartment (HPCs) contains more

differentiated progenitor cells, and has no long-term HSC function in immunodeficient mice. We used PamGene kinome array technology to identify kinase activity differences, between the HSPC and HPC compartments (Supp. Fig. 1A).

Cell extracts from HSPCs and HPCs phosphorylated a range of peptides (Supp. Fig. 1B), and could be clearly separated by cell cycle phosphorylations (e.g. $RB^{pS807/S811}$, Supp. Fig. 1C) and classical hematopoietic signalling molecules (e.g. $AKT1^{pY326}$, $PRKDC^{pS2624/S2626}$; Supp. Fig. 1D-E). Upstream kinase analysis of the phosphorylations by HSPC extracts provides a functional annotation, assigning phosphorylation kinetics to kinase activities. This revealed an enrichment for well described kinases such as JAK1/2 and FLT1/3/4 in the HSPC compartment (Figure 1A).

Differential phosphorylation events (Supp. Fig. 1A) and kinase activities (Figure 1A) between HSPCs and HPCs showed strong enrichment in anti-apoptosis signalling, both by PI3K/AKT (FDR = 3.74E-13, 18 proteins) and MAPK/JAK/STAT (FDR = 1.896E-11, 15 proteins), erythropoietin signalling (FDR = 1.167E-10, 13 proteins) and inflammatory pathways including; IL-2 signalling (FDR = 4.045E-07, 9 proteins), TREM1 signalling (FDR = 4.726E-07, 10 proteins) and IFN-gamma signalling (FDR = 4.726E-07, 9 proteins; Figure 1B).

Interestingly, the receptor tyrosine kinase, RET, was specifically enriched in the HSPC fraction, with a mean final score of 2.3 based on 17 peptide phosphorylations (Figure 1A). RET is a transmembrane receptor tyrosine kinase, with well-defined ligand/co-receptor interactions, and publicly available datasets indicate that within the HSPC compartment, the *RET* gene is expressed at significantly higher levels in HSCs than more differentiated progenitor cells (Supp. Fig. 1F). RET signalling, at the cell surface, shows a diverse array of responses in different cell types, and considering the well-defined ligand/co-receptor activation interaction[13], evidence of GFL support from the niche[14], and bio-available stimulating factors *in vitro*[15], provided an excellent candidate for further investigation.

RET cell surface expression functionally enriches for stem cell activity in the HSPC compartment

The RET protein must be at the cell surface for ligand/co-receptor-dependent transduction of signals across the membrane[16]. When probing for RET at the cell surface, immunophenotypic HSCs ($CD34^+CD38^-CD45RA^-CD90^+CD49f^+$) typically show higher RET cell surface expression than MPPs (Multipotent progenitors; $CD34^+CD38^-CD45RA^-CD90^-CD49f^-$; Figure 1C & Supp. Fig. 1G-J; gating as per Notta et al. 2011[17]). Multiple markers have been proposed to further purify HSCs within the $CD34^+CD38^-$ compartment, and we sought to investigate the stem/progenitor cell frequency of cells expressing RET at the cell surface after 12 weeks *in vivo*. Selection of $CD34^+CD38^-$ cells solely classified for cell surface expression of RET enriches for HSPC stem cell activity in an *in vivo* limiting dilution assay, with high RET HSPCs (RET^{hi}) showing a stem cell frequency of ~1 in 135 cells and RET^{low} HSPCs showing an almost 4-fold reduction in stem cell frequency of ~1 in 531 cells ($p = 0.026$, Figure 1D&E, Supp. Fig. 2A). In addition, RET^{hi} HSPCs show much more classical lineage balance in immunodeficient mice, whereas RET^{low} HSPCs are more myeloid biased (Supp. Fig. 2B).

Activation of RET by GDNF/GFRα1 improves survival and expansion of HSPCs

A key question in hematopoietic stem cell biology remains how to grow HSCs *in vitro* for both engineering and expansion purposes[18]. Currently, $CD34^+CD38^-$ HSPCs can be grown in culture for 7 days with a minimal

cocktail of cytokines, including: SCF, FLT3L and TPO, retaining enough functional HSCs to engraft immunodeficient mice[19]. To understand the role of RET at the surface of HSPCs and whether this could be a target for HSC maintenance and expansion, we added its primary ligand/co-receptor combination, GDNF/GFRα1 to the culture medium in addition to SCF/FLT3L/TPO and cultured 5,000 HSPCs for 7 days (Figure 2A). HSPCs expand up to 40-fold in minimal serum free, SCF/FLT3/TPO supplemented conditions over 7 days. The addition of GDNF/GFRα1 significantly increased the number of HSPCs by 71-fold at day 7 compared to input cells (Figure 2B).

It has previously been reported that EPCR expression marks expanded $CD34^+$ cord blood stem cells in culture[20], and we used this marker in combination with CD90 to estimate the number of expanded HSCs in control and GDNF/GFRα1 treated conditions. The frequency of immunophenotypic HSCs within the cultures ($CD34^+CD90^+EPCR^+$) was significantly enriched by GDNF/GFRα1 treatment at both day 3 (Figure 2C) and day 7 (Figure 2D & Supp. Fig. 3A-C).

GDNF/GFRα1 cultured HSPCs have improved long-term in vivo engraftment

The gold standard for Human HSC functionality under laboratory conditions is engraftment in immunodeficient mouse models to reveal stem/progenitor (primary engraftment for 12 weeks) and long-term self-renewing HSC (secondary engraftment for 12 weeks) function. The observed increase in cell numbers in the GDNF/GFRα1 cultures at day 7 may correlate with outgrowth of functional stem cells in this system, or may be due to another factor such as increased progenitor cell proliferation[21]. To test the stem cell potency of cultured HSPCs in the presence of GDNF/GFRα1, we retrieved all cells from culture replicates at day 7 and transplanted them into immunodeficient mice harbouring the $cKit^{W41}$ mutation (1well:1mouse; NBSGW). Bone marrow and splenic engraftment was significantly higher after GDNF/GFRα1 treatment compared to control. The enhanced engraftment resulting from RET activation was comparable to the previously published combination of SR1/UM171, and the combination of SR1/UM171/GDNF/GFRα1 further improved engraftment (Figure 2E, Supp. Fig. 3D-F). These data indicate that activation of RET can improve progenitor activity for colonising primary recipients as a single addition to classical SCF/FTL3L/TPO cytokines, similar to that of SR1/UM171. Analysis of the immunophenotypic HSC compartment within the $HuCD45^+$ cells from the bone marrow of primary recipient mice revealed a significant enrichment in all treatment cases (GDNF/GFRα1, SR1/UM171 and SR1/UM171/GDNF/GFRα1) compared to controls (Figure 2F). Together, these data indicate improved expansion of stem/progenitor cells treated with GDNF/GFRα1, and expansion *in vivo* of phenotypic long-term HSCs.

To test the long-term self-renewal HSC function and frequency of GDNF/GFRα1 treated cells, we engrafted $HuCD45^+$ cells obtained from the bone marrow of primary mice into secondary recipients in a limiting dilution fashion. Primary cells from GDNF/GFRα1, SR1/UM171 and SR1/UM171/GDNF/GFRα1 treatments, engrafted secondary mice significantly better than controls at the highest dose tested (Figure 2G; Supp. Fig. 3G-I; $2x10^5$ $hCD45^+$ injected). The estimation of stem cell frequency by ELDA (Extreme Limiting Dilution Analysis) indicated that control cells have very low long-term stem cell frequency (~1 in 1,500,000). GDNF/GFRα1 treatment significantly improved long-term stem cell frequency by more than 75-fold (~1 in 20,000). This was also improved in the SR1/UM171 treated cells (~1 in 41,000), and the combination of

SR1/UM171/GDNF/GFRα1 treatment was similar to GDNF/GFRα1 treatment alone with a moderate improvement (~1 in 13,000; Figure 2H, Supp. Fig. 3H&J), indicating that GDNF/GFRα1 provides significant improvement in LT-HSC production. Considering initial cell expansion, total engraftment in primary mice, percentage of total bone marrow represented by rear leg long bones (~20%) and long-term stem cell frequency in secondary recipients, GDNF/GFRα1 treatment increases HSC outgrowth over the experimental course compared to control conditions by approximately 148-fold (~742 versus ~5 stem cells produced, respectively), compared to SR1/UM171 by approximately 1.3-fold (~565 stem cells produced) and is further improved by the triple combination (~1,275 stem cells produced, Supp. Fig. 3K).

RET activation induces a dynamic change in the kinome of HSPCs

To understand the specific changes governed by RET activation in HSPCs, we investigated functional changes in the kinome after GDNF/GFRα1 treatment using PamGene kinase profiling. We compared functional changes in both Serine/Threonine (Figure 3A) and Tyrosine (Figure 3B) kinases in HSPCs at days 0, 1 and 3 post GDNF/GFRα1 treatment. As GDNF/GFRα1 is rapidly used and turned over *in vitro*, day 1 changes represent the acute early events, and day 3 changes represent longer-reaching changes in the kinome of treated HSPCs.

At the early time point after GDNF/GFRα1 treatment (day 1), significant phosphorylations on chip (Figure 3C) were predominantly representative of Tyrosine kinase activity (Figure 3D, Supp. Fig. 4A). At the late time point after GDNF/GFRα1 treatment (day 3), significant phosphorylations on chip (Figure 3E) were predominantly representative of Serine/Threonine kinase activity (Figure 3F, Supp. Fig. 4B).

The early changes at day 1 were enriched in process networks for anti-apoptotic PI3K/AKT signalling (p=1.8e-7), anti-inflammatory IL2 signalling (p=1.9e-6), anti-apoptotic MAPK/JAK/STAT signalling (p=5.3e-5) and Notch signalling (p=1.4e-4; Figure 4A). At day 3 changes were enriched for the same process networks seen at day 1 (Figure 4A), indicating that fundamental pathways are sustained beyond the immediate GDNF/GFRα1 downstream signalling, converging on anti-apoptosis and anti-inflammation.

Differential phosphorylation events exclusively at the early time point (day 1; Figure 4B-C) include: cell cycle components $CDK2^{pY15}$ and RB^{pT356} (indicative of an exit from mitosis and progression through the G1/S boundary; Figure 4D&E), interleukin signalling components (e.g. $JAK3^{pY980/981}$, Supp. Fig. 5B) and the p53 anti-apoptotic phosphorylations at $p53^{pT18}$ and $p53^{pS315}$ (Figure 4F&G). These phosphorylation events indicate that cells treated with GDNF/GFRα1 at early time points are more positively cycling, have an earlier anti-inflammatory response and increased anti-apoptotic activity.

In normoxic cultures, anti-inflammatory and anti-apoptotic signalling are important for HSC maintenance, expansion and survival, and phosphorylation networks in day 3 GDNF/GFRα1 treated cells represent a convergence on these key pathways (Figure 4J). For example, the phosphorylation of BAD^{pS99}, which is hyper-phosphorylated when cells are under stress and are resisting apoptosis[22], is reduced under GDNF/GFRα1 treatment (Figure 4K). Upstream, FOXO3, the transcription factor responsible for expression of another pro-apoptotic factor, *BIM*, also shows reduced phosphorylation at S30/T32 in GDNF/GFRα1 treated cells, indicating there is a block in expression of pro-apoptotic genes such as *BIM* (Figure 4L). In addition, RB phosphorylation switches, and there is a significant reduction in $RB^{pS807/811}$, resulting in less potential for BAX binding and further indication that anti-apoptotic functions are no longer required (Figure 4M). This switch in phosphorylation events between early and late time points coincides with the emergence of kinase activity by

IKK complex members (IKKα, IKKβ and IKKε; Figure 3F & Supp. Fig. 4B), a pathway known to be downstream of RET induced AKT/ERK activity[23]. These pathways indicate that a mechanism of protection by GDNF/GFRα1 treatment at later time points is due to protection against apoptosis through RET-induced AKT/ERK activity and downstream via NFκB signalling.

Next, we sought to understand how GDNF/GFRα1 treatment mitigates changes from input cells over time compared to controls. Whilst there is clear concordance between phosphorylation changes from input cells to day 1 controls and GDNF/GFRα1 treatment ($R = 0.56$, $p < 2.2e\text{-}16$; Supp. Fig. 4C), and from input cells to day 3 controls and GDNF/GFRα1 treatment ($R = 0.75$, $p < 2.2e\text{-}16$; Supp. Fig. 4D), there are key peptide changes seen exclusively in control cells or in GDNF/GFRα1 treated cells at each time point (Supp. Fig. 4E). The most highly changed phosphorylation site in day 1 control cultures compared to input cells is, DSP^{pS2849}, which remains unchanged throughout all other conditions (Supp. Fig. 5A). The DSP^{pS2849} phospho-site is dependent on GSK3ß and PKACA activity, which are important kinases involved in normal and malignant hematopoiesis, and phosphorylation at this site reduces desmoplakin-mediated adhesion to extracellular matrices (Supp. Fig. 5A)[24]. At day 3, control cells uniquely lack: $ADDB^{pS697/S701}$, phospho-sites associated with induction of cell growth, notably a site that is better maintained throughout by GDNF/GFRα1 supplementation (Supp. Fig. 5D).

Conversely, at day 1 culture with GDNF/GFRα1, the $p53^{pS315}$ phospho-site is significantly increased (Figure 4G), a site known to be phosphorylated by CDK1 and important for anti-apoptotic functions. In addition to improved survival phosphorylation events at day 1, by day 3, GDNF/GFRα1 treated cultures also display major reductions when compared to controls in phosphorylation of $IF4E^{pS209/T210}$ (Supp. Fig. 5E) and $RB^{pS807/S811}$, indicative of cell cycle alterations and anti-apoptotic functions (Figure 4M).

These profiles indicate that overlapping and independent phosphorylation changes between control and GDNF/GFRα1 treated cultures lead to diverse pathway activation. These signalling alterations are likely to be responsible for the differences in functional output of HSPCs.

GDNF/GFRα1 treatment sustains an integrated cell survival and proliferation program in cultured HSPCs

Despite the wide-scale dynamic changes in the kinome, key regulatory phosphorylation cascades surrounding an NFκB/p53/BCL2 cell survival and proliferation program were consistently affected at early and late time points. We sought to utilise mass cytometry to investigate the dynamics of these phosphorylation steps and protein abundance in $CD34^+$ cells after initial isolation, at early (day 3) and late (day 7) expansion time points (Figure 5A&B). RET is hyper-phosphorylated after GDNF/GFRα1 treatment at day 3 compared to controls and reduces over time as GDNF/GFRα1 depletion occurs. In contrast, total RET abundance increased early and continued to increase at day 7 (Figure 5A&B).

Many of the key factors identified throughout our kinome analysis are downstream of RET, mediated by one of two key signalling cascade partners, AKT and ERK. Interestingly, both AKT^{pS473} and $ERK1/2^{pT202/Y204}$ mirror RET phosphorylation, and are activated early. ERK phosphorylation was sustained over time, whereas AKT increased further at day 7 (Figure 5A&B).

Downstream of AKT/ERK activity, we observed increased $p53^{pS392}$, which induces interaction with NFκB, and in addition we observed increased NFκB transcriptional activity (Figure 5A-C & Supp. Fig. 6A&B). This

NFκB/p53 axis is an important regulator of the cell survival and growth characteristics we observed in our *in vitro* cultures.

When assessing the downstream genetic targets of these key proteins, we observed significant down-regulation of the FOXO3, pro-apoptotic target, *BIM*, and significant up-regulation of anti-apoptotic NFκB target genes *BCL2* and *TP53*, but not consistent changes in NFκB pro-inflammatory target genes *TNF-alpha* and *IL1-beta* (Figure 5C, Supp. Fig. 6A&B). To further confirm that the changes we see are caused acutely by phosphorylation cascades downstream of GDNF/GFRα1 treatment, and not secondary to transcriptomic adaptions, we monitored RNA levels of key components of this pathway, altered at the protein level, including; *FOXO3A*, *RELA, ELK1* and *IKBKB* (Figure 5D). Indeed, *FOXO3A, ELK1* and *IKBKB* remain similar to controls until the late time point (day 7), at which, *FOXO3A* and *IKBKB* are upregulated (*ELK1* remained constant throughout), presumably as feedback in response to their inactivity at the protein level. In contrast, *RELA* is initially downregulated early (day 1) and increases over time. Therefore, activation of RET induced changes at the protein phosphorylation and total abundance levels are the predominant effectors of the response observed, with input from transcriptional changes contributing a smaller part of the downstream effectors mediating the phenotypic response.

These data provide a two-pronged mechanism, by which RET activation induces the activity of AKT and ERK as key signalling hubs to drive a cell survival and proliferation program in HSPCs *in vitro*. The NFκB/p53/BCL2 axis provides a stable platform for HSPCs to survive and expand in culture before transplantation *in vivo* (Figure 5E).

HSCs have a specific response mechanism to GDNF/GFRα1 in culture

Protein changes responsive to GDNF/GFRα1 treatment, monitored in $CD34^+$ cells during culture, were consistent within the immunophenotypic HSC compartment of cultured cells ($CD34^+CD38^-CD45RA^-CD90^+$), but less responsive in the MPP compartment ($CD34^+CD38^-CD45RA^-CD90^-$), indicating a specific response mechanism in HSCs (Supp. Fig. 7A&B). In addition, at day 0 HSCs have higher total RET than MPPs (but not bulk $CD34^+CD38^-$), and HSCs show the strongest RET^{pY905} signal of all compartments (data not shown), indicating that RET signalling is already primed in HSCs pre-culture.

In comparison to control cultures, HSCs show a strong response at day 3 to GDNF/GFRα1 by increases in RET^{pY905}, AKT^{pS473} and $ERK1/2^{pT202/Y204}$ (Figure 6A&B). In addition, $NFκB^{pS529}$ and $p53^{pS392}$ are upregulated at day 3 by GDNF/GFRα1 treatment, indicating the cell survival and oxidative stress response network discovered in bulk HSPCs (Figure 5A&B) is similarly stimulated in HSCs (Figure 6A&B). Interestingly, GDNF/GFRα1 treatment also suppresses the abundance of the differentiation pioneer factor, PU.1, at later stages (day 7) whilst inducing GATA1 expression at early stages (day 3; Figure 6A&B). The changes induced at day 3 by GDNF/GFRα1, are generally spikes in signalling, lost upon the exhaustion of ligand/co-receptor. Only 4 proteins remain more abundant in GDNF/GFRa1 treated culture ($STAT5^{pY694}$, $ERK1/2^{pT202/Y204}$, $S6^{pS235/S236}$, cREL and Ki67), indicating that the spike in activity early is enough to induce a survival and expansion program in HSCs in culture (Figure 6A&B, Supp. Fig. 7A&B).

In agreement with our earlier findings of anti-apoptotic and anti-inflammatory signatures (Figure 4A), HSCs show a specific spike in $p53^{pS392}$ at day 3, but no upregulation of $NFkB^{pS529}$ (Figure 6A&B). *In vitro* this leads to a reduction in intracellular reactive oxygen species (ROS) for both bulk $CD34^+$ cells, and specifically HSCs

(Figure 6C&D, Supp. Fig. 8 C&D). When inhibiting RET signalling, with the pan-RET/VEGFR2 inhibitor PZ1 (Supp. Fig. 8A), the reduction in intracellular ROS is abolished, and the number of $CD34^+$ cells, and more importantly HSCs, in culture is lost (Figure 6E, Supp. Fig. 8E), with $CD34^+$ cells showing a significant increase in apoptosis in response to PZ1 at day 7 (Supp. Fig. 8B). Together, these data indicate that the tailored response in HSCs is critically dependent on RET signalling maintaining fundamental stress response pathways during *in vitro* outgrowth.

Discussion

The use of UCB for hematopoietic stem cell transplantation is a rapidly increasing treatment option for both hematological and non-hematological malignancies, as well as new gene therapy and regenerative medicine approaches. The current outcomes from cord blood transplantation are limited primarily by low stem cell dose and delayed hematopoietic recovery[4]. Early strategies to grow HSCs *in vitro* induce a large amount of differentiation in culture[19], but recent improvements in expansion of HSCs, such as those conferred by SR1, UM171[25,26] and here, GDNF/GFRα1, *in vitro,* provide a positive platform for improvement of UCB-derived HSCs *in vivo*.

Our finding of higher RET activity in HSPCs derived from UCB may be due to cell-intrinsic mechanisms/ autocrine signalling loops or from specific niche components. Indeed, there is evidence of enervation of the HSC bone marrow niche, and recent high dimensional analysis of niche components reveal expression of GFLs from $COL2.3^+$ osteoblasts[14]. Therefore, the provision of GDNF/GFRα1 may be a key component, already provided by the bone marrow niche, for HSCs to maintain their potential *in vitro*. Regardless of the source of activation, the increased phosphorylation of RET in phenotypic HSCs from UCB indicates an active RET signalling pathway *in vivo*, specifically tailored to HSCs.

We provide a mechanism by which RET can govern an anti-apoptotic and anti-inflammatory program, due to diverging and exclusive contributions to the same goal, to improve survival and expansion of HSCs for regenerative and engineering purposes. A key issue when expanding HSCs *in vitro* is the need to grow them in normoxic conditions for maximum expansion. The induction of oxidative stress under these conditions can lead to a loss in stem cell activity[27,28]. The stimulation of RET signalling can reduce the accumulation of ROS in HSCs and maintain their potency, whilst providing further signals to expand *in vitro*. Interestingly, the basic complement of cytokines used to grow HSPCs in culture (SCF/FLT3L/TPO) is known to activate ERK/AKT signalling[29]. Our findings that this is strongly enhanced by the activation of RET indicates that there is both capacity to increase these signalling cascades (strength and time of response), and improve the diversity of the response (in our case the IκBα arm, Figure 5E), ultimately leading to improvement in HSC function over the experimental course. The addition of UM171 to SCF/FLT3L/TPO when culturing HSPCs has also been shown to re-tune NFκB pro- and anti-inflammatory activity, through EPCR, ultimately reducing the ROS burden in HSCs *in vitro*[30]. Although it is unknown what the direct target of UM171 is, it is possible that association with EPCR function may activate AKT/ERK signalling and even stimulate RET activity to some extent. Yet, the reduction of estimated stem cells produced by SR1/UM171 compared to GDNF/GFRα1 (Supp. Fig. 3H)

indicates that classical stimulation of RET activity (by GFLs) has a stronger effect than UM171 if this is the case.

In addition to potential improvements in patient outcome, improved outgrowth of UCB-derived HSCs can begin to address the issue of double cord blood transplantation and associated costs, increasing the practicality of using UCB banks in frontline treatment[4]. These benefits could potentially provide an immediate improvement to clinical outcomes, but also, with the rapidly increasing promise of gene therapy, improvements in survival during expansion may provide a critical edge to genetic engineering protocols for future therapies.

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Author contributions

W.G. designed and carried out experiments, analysed the data and wrote the manuscript. R.C., M.P., H.H.E & M.G-A. carried out experiments. N.Q.M. supervised the project. D.B. supervised the project and wrote the manuscript. All authors provided critical feedback on the manuscript.

Conflict of Interest

The authors declare no relevant conflicts of interest.

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Figure Legends

Figure 1. RET is functionally active in CD34$^+$CD38$^-$ HSPCs and cell surface expression enriches for HSC function. A. Kinase activity alterations between CD34$^+$CD38$^-$ HSPCs (green) and CD34$^+$CD38$^+$ HPCs (lilac). **B**. Process network enrichment for significantly altered kinases and phosphorylation events from **A**. **C**. *z*-normalized geometric mean fluorescence intensity of cell surface RET within the indicated populations. Significance was tested using a paired Student's *t*-test for individual cord blood donors tested (N=9). **D**. Plot depicting frequencies and confidence interval for RET^{hi}(red) and RET^{low}(grey) CD34$^+$CD38$^-$ cell *in vivo* engraftment at limiting dilution after 12 weeks (N=3 mice per dose tested). **E**. Table of 1/stem cell frequency numerical data calculated from the *in vivo* LDA presented in **D**, including: estimated stem cell frequency, upper and lower intervals of estimation, Chi-squared test and estimated p-value.

Figure 2. GDNF/GFRα1 treatment stimulates growth of transplantable HSCs. A. Experimental design for GDNF/GFRα1 supplemented outgrowth of HSCs and transplantation ability. 1^{o}TP and 2^{o}TP represents the first and second transplantation respectively. **B**. Live cell count of *in vitro* cultured HSPCs (N=5). Proportion of expanded HSCs (CD34$^+$CD90$^+$EPCR$^+$) at day 3 (**C**) and day 7 (**D**) during *in vitro* culture (N=5). **E**. Percentage of Human CD45 positive cells of total CD45 positive bone marrow cells in primary transplantation mice (Ctrl N=12, GDNF/GFRα1 N=10, SR1/UM171 N=7, SR1/UM171/GDNF/GFRα1 N=6). **F**. Percentage of immunophenotypic HSCs (CD34$^+$CD38$^-$CD45RA$^-$CD90$^+$CD49f$^+$) retained in Human CD45 bone marrow cells in primary transplantation mice. **G**. Percentage of Human CD45 positive cells of total CD45 positive bone marrow cells in secondary transplantation mice ($2x10^5$ hCD45 cells transplanted shown, N=5 for all conditions). **H**. Boxplot indicating 1/Stem Cell Frequency of secondary transplanted Human CD45 positive cells. Estimates with upper and lower intervals are shown (N=5 for top dose, N=3 for all other doses). For all graphs, A Student's *t*-test was used to calculated significant differences (* = $p < 0.05$ vs Ctrl, ** = $p < 0.005$ vs Ctrl).

Figure 3. Activation of RET by GDNF/GFRα1 alters kinome dynamics during HSPC outgrowth. Heatmaps depicting **A**. Serine/Threonine and **B.** Tyrosine containing row *z*-normalized peptide

phosphorylations supervised by day and treatment. Rows are clustered by correlation. **C**. Fold change differential phosphorylation of GDNF/GFRα1 treated $CD34^+CD38^-$ cells compared to control after 1 day of culture. **D**. Upstream kinases calculated as responsible for phosphorylations in **C**. **E**. Fold change differential phosphorylation of GDNF/GFRα1 treated $CD34^+CD38^-$ cells compared to control after 3 days of culture. **F**. Upstream kinases calculated as responsible for phosphorylations in **G**. **C-F:** Red dots indicate significantly upregulated peptides or kinases, Blue dots represent significantly downregulated peptides or kinases in response to GDNF/GFRα1 treatment.

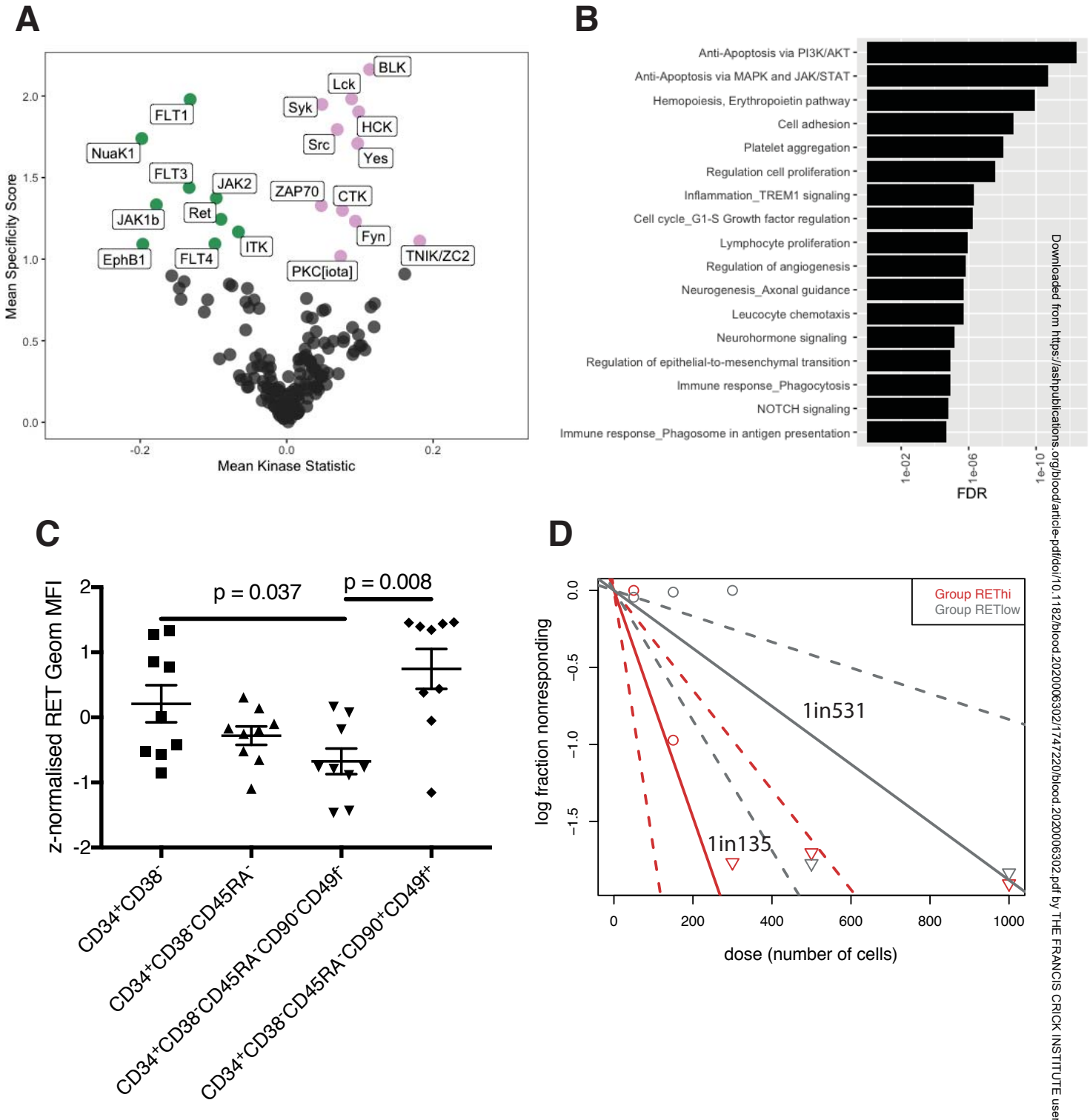
Figure 4. GDNF/GFRα1 treatment induces anti-apoptotic and anti-inflammatory processes in cultured HSPCs. A. Enriched process networks from significantly changed peptides in GDNF/GFRα1 versus control cultures after 1 day (left, light red) or 3 days (right, dark red). **B**. Venn diagram depicting overlap of significantly altered peptides between day 1 (light red) and day 3 (dark red) from GDNF/GFRα1 versus control cultures. **C**. String protein network for differential phosphorylation events at day 1. Lines indicate reported interactions. **D-I**. Key differential phosphorylations induced by GDNF/GFRα1 treatment at day 1, represented as relative phosphorylation. A Student's *t*-test was used to measure significant differences. Day 0 $CD34^+CD38^-$ input cells (white), control (black) and GDNF/GFRα1 (red) treatments at days 1 and 3 are presented. **J**. String protein network for differential phosphorylation events at day 3. Lines indicate reported interactions. **K-M**. Key differential phosphorylations induced by GDNF/GFRα1 treatment at day 3, represented as relative phosphorylation. A Student's *t*-test was used to measure significant differences. Day 0 $CD34^+CD38^-$ input cells (white), control (black) and GDNF/GFRα1 (red) treatments at days 1 and 3 are presented.

Figure 5. RET activation by GDNF/GFRα1 sustains an NFκB/p53/BCL2 anti-apoptotic program in HSPCs during *in vitro* culture. A. Bar graphs depict median intensity of signal from histograms below showing the profiles of key protein changes in $CD34^+$ cells at day 0 (blue), day 3 control (orange) day 3 GDNF/GFRα1 (green), day 7 control (red) and day 7 GDNF/GFRα1 (purple, a.u. = arbitrary units). **B** z-normalized heatmap of data in **A**, illustrating differences in $CD34^+CD38^-$ cells at input, and $CD34^+$ cells at day 3 and day 7 culture with or without GDNF/GFRα1 treatment assayed by mass cytometry, supervised by treatment condition. **C**. Fold change RNA expression of key NFκB target genes in GDNF/GFRα1 treated $CD34^+CD38^-$ cells compared to controls at days 1, 3 and 7. Gene names are noted under bar labels. A Student's *t*-test was used to calculate significant differences (* = $p < 0.05$, ** = $p < 0.005$, N=3 per condition and day tested). **D**. Fold change RNA expression of key genes altered at the protein level in GDNF/GFRα1 treated $CD34^+CD38^-$ cells compared to controls at days 1, 3 and 7. Gene names are noted under bar labels. A Student's *t*-test was used to calculate significant differences (* = $p < 0.05$, ** = $p < 0.005$, N=3 per condition and day tested). **E.** Illustrated pathway identified through kinome, mass cytometry and RNA changes, defining activating (green) and inhibiting (red) phosphorylations, protein levels or RNA levels and proposed modes of action.

Figure 6. HSCs exhibit specific responses to GDNF/GFRα1 resulting in reduced accumulation of intracellular ROS. A. Bar graphs depict median intensity of signal from histograms below illustrating profiles

of key protein changes in HSCs at day 0 (blue), day 3 control (orange) day 3 GDNF/GFRα1 (green), day 7 control (red) and day 7 GDNF/GFRα1 (purple, a.u. = arbitrary units). **B** z-normalized heatmap illustrating differences in HSC clusters at input, day 3 and day 7 culture with or without GDNF/GFRα1 treatment assayed by mass cytometry, supervised by treatment condition. **C**. Mean fluorescence intensity of intracellular ROS in HSCs at day 7± GDNF/GFRα1/PZ1 (* = $p < 0.05$, N=4). **D**. Histograms illustrating changes in intracellular ROS at day 7. **E**. Percentage of HSCs in cultured cells at day 7± GDNF/GFRα1/PZ1 (* = $p < 0.05$, N=4).

Figure 1



E

	Lower	Estimate	Upper
RET^{Hi}	308	135	59
RET^{Low}	1193	531	236

Chisq 4.93194 on 1 DF

p-value: 0.02636

Figure 2

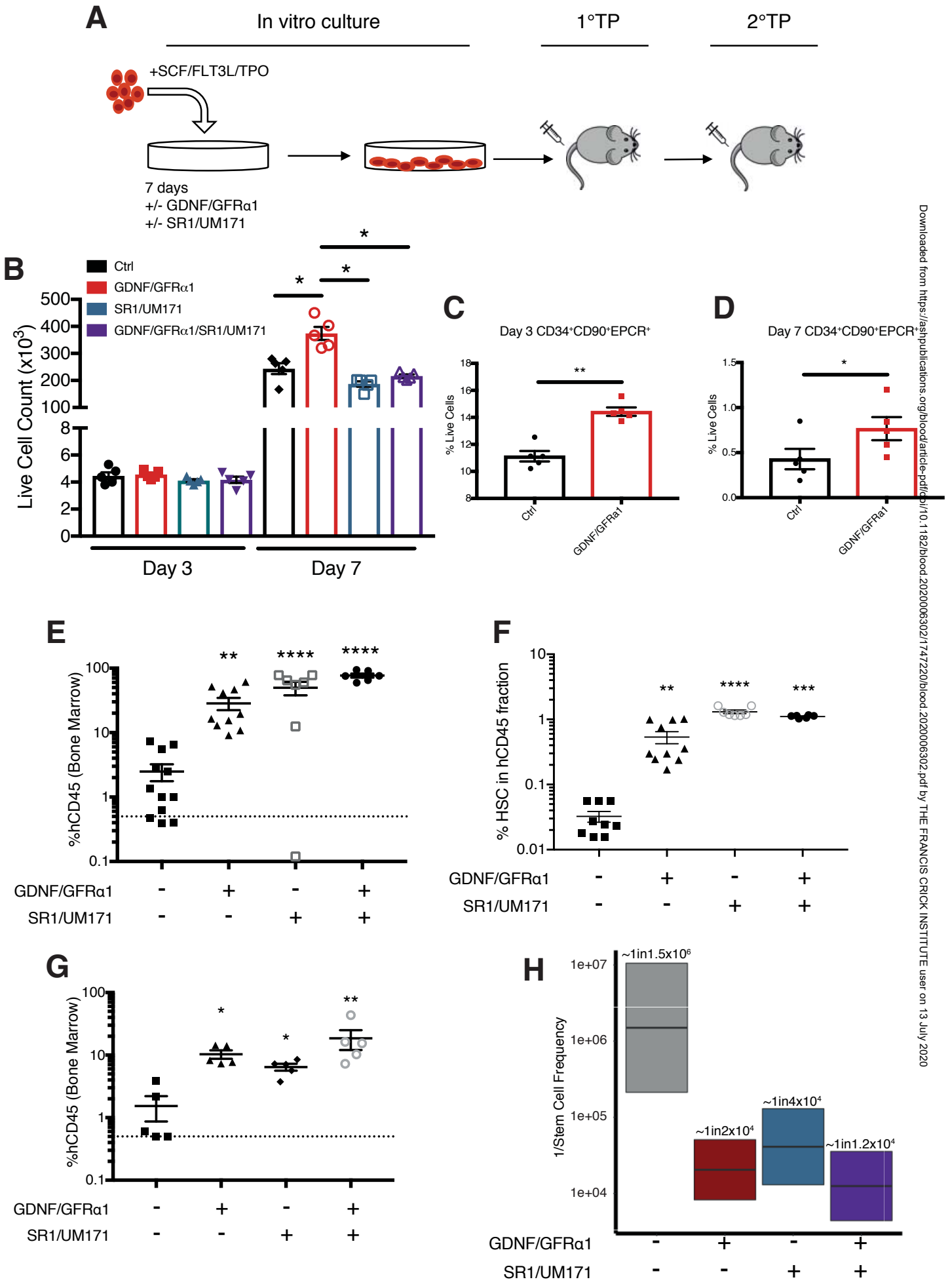


Figure 3

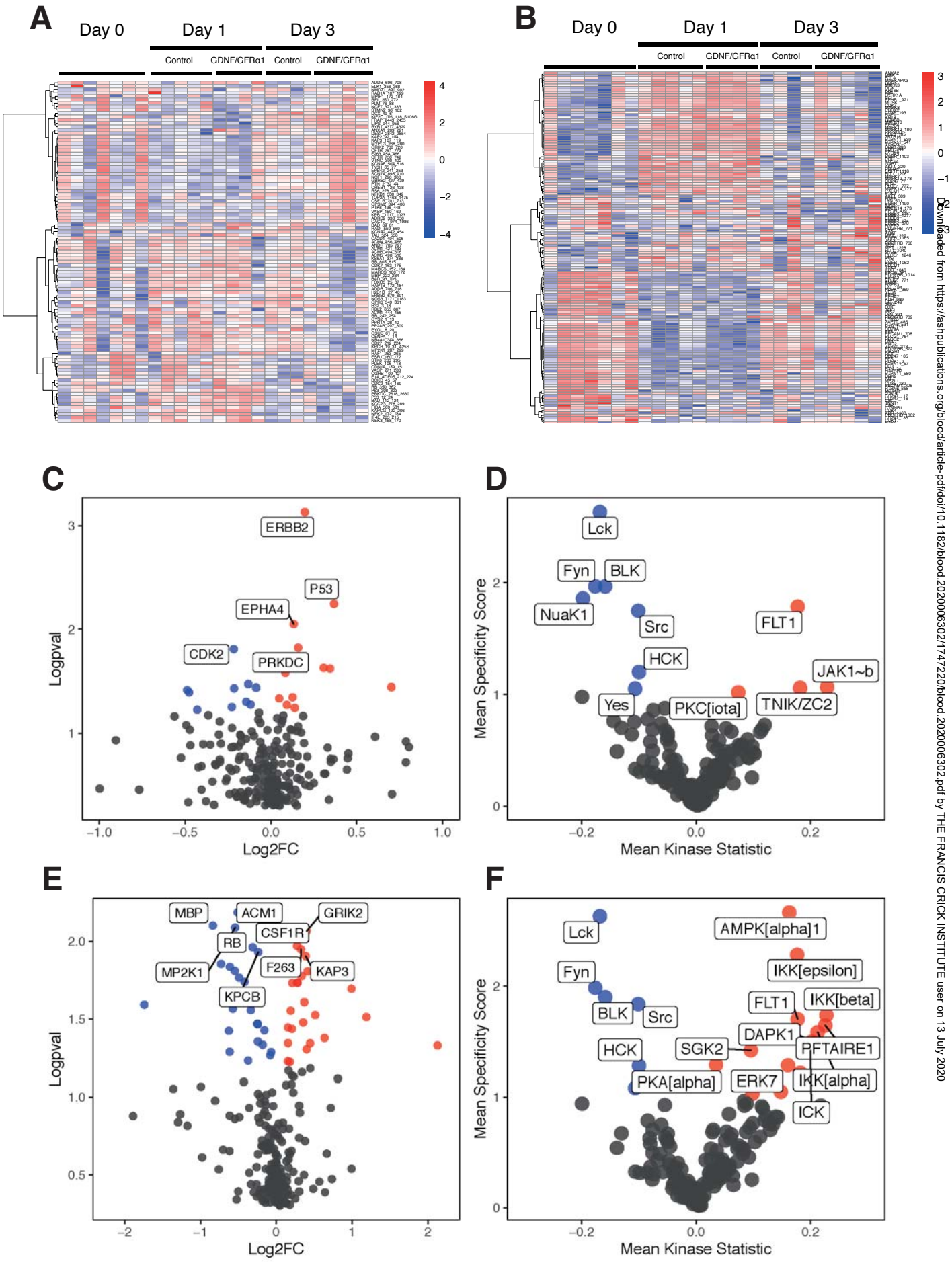
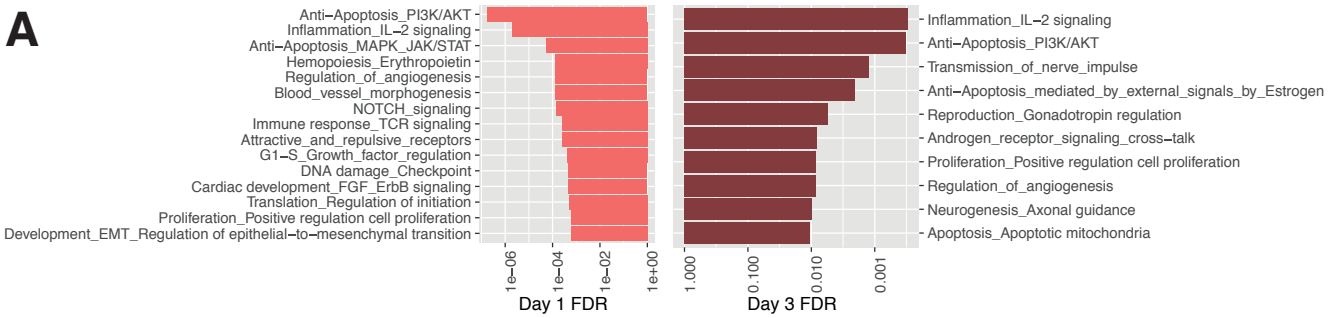
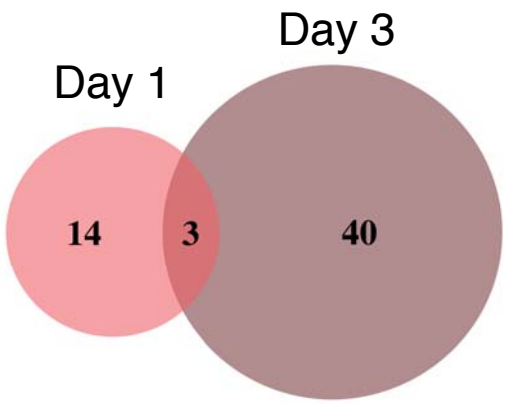
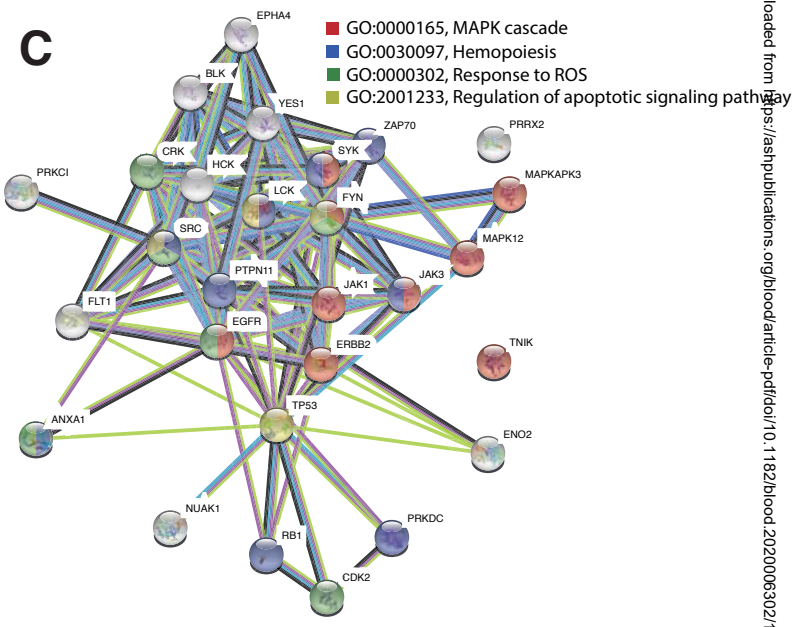
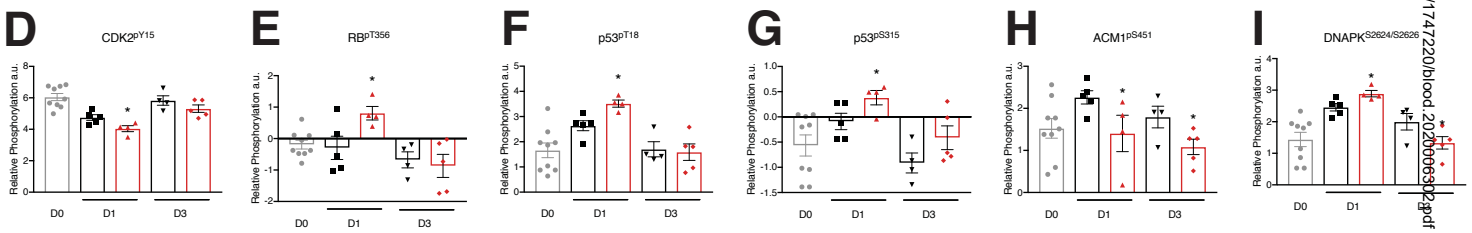


Figure 4









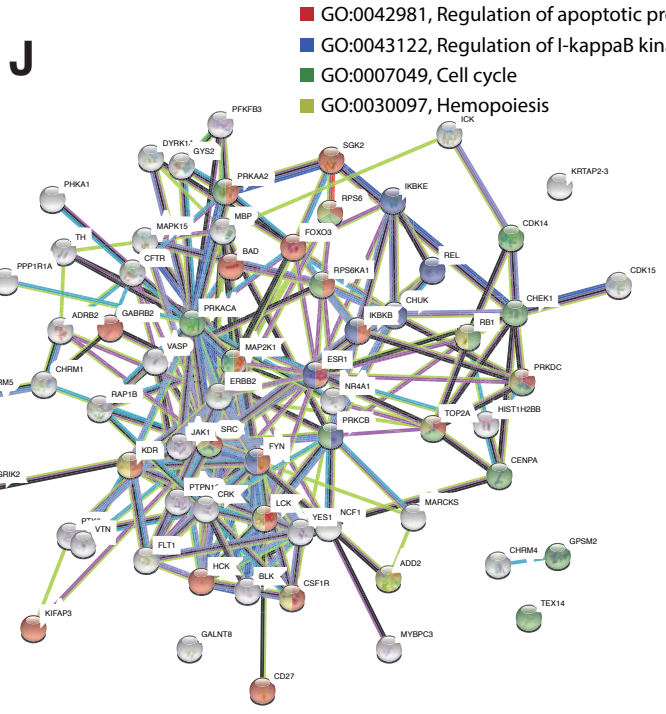


Figure 5

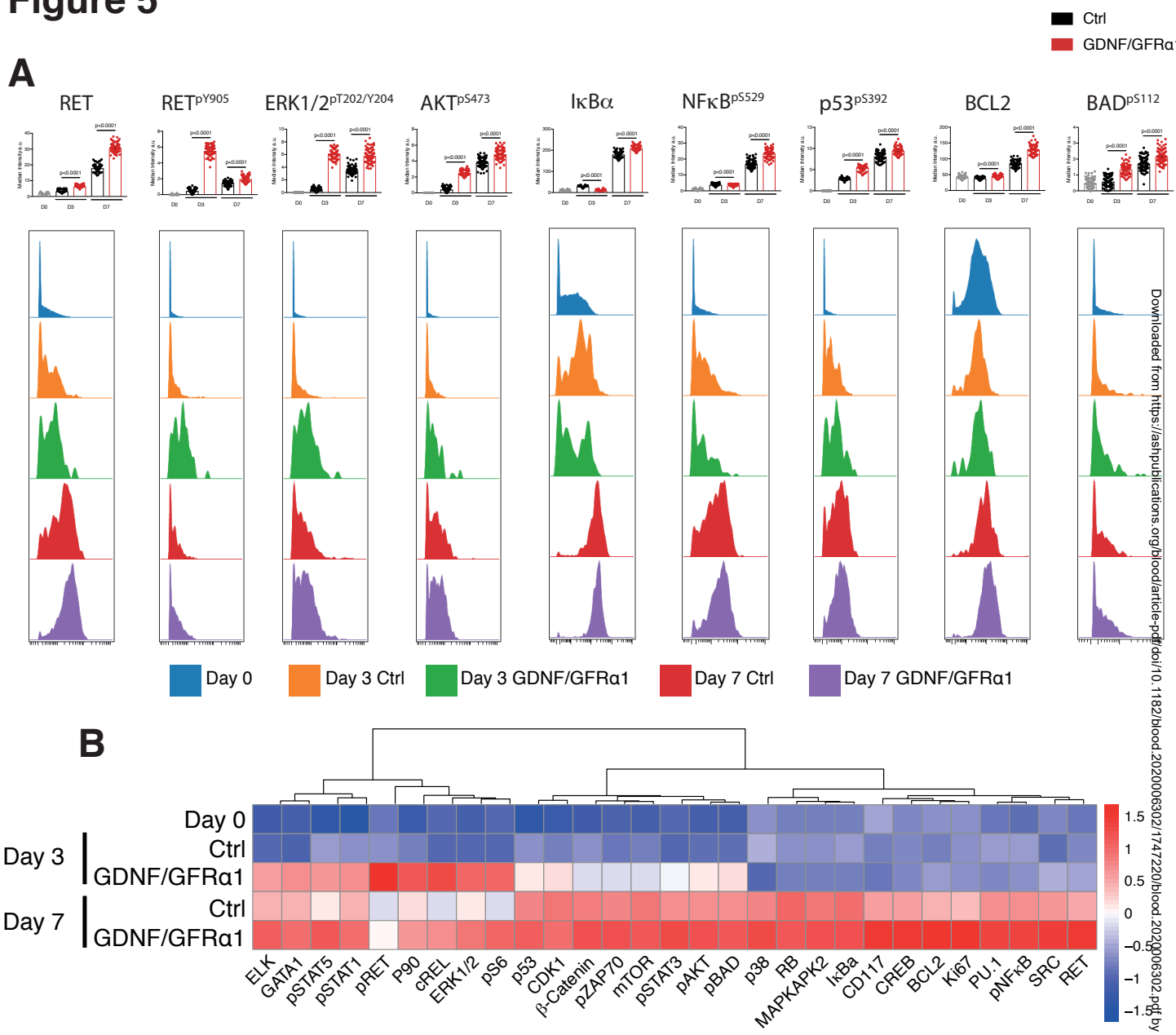


Figure 6