immune surveillance, and shuttle antigen to follicular dendritic cells 1, 2. Their localization to the MZ and their trafficking to and from the B cell follicle also contributes to their survival 2, 3, 4, 5. Upon antigen encounter, MZ B cells are poised to promote T cell activation by presenting antigen as well as differentiating into plasmablasts6. Compared to follicular B (Fo B) cells, MZ B cells express greater amounts of surface IgM, CD35, CD21 and CD1d. Together with elevated expression of Toll-like receptors (TLR), these features facilitate rapid responses against blood-borne pathogens such as encapsulated bacteria7, 8.

MZ B cells develop through an intermediary population of marginal zone precursor (MZP) cells which express CD939. Selection of MZ B cells depends upon signaling by membrane immunoglobulin, Notch2 and the BAFF-R7 9. MZ- and Fo-B cells can be separated on the basis of their gene expression profiles10, and the differences in their transcriptomes contribute to the differential development, localization and function of these cells. Transcriptional regulators have been shown to have specific roles in MZ- and Fo-B cells. Notch2 is necessary to promote MZ B cell fate11, 12. The transcription factor IRF4 and its paralog IRF8 limit the size of MZ B cell pool13, and IRF4 regulates the positioning of cells in the MZ. The transcription factor KLF2 enforces the Fo B cell phenotype. In its absence, the MZ B cell pool is expanded14, 15. By contrast, KLF3, which may antagonise KLF2 promotes the development of MZ B cells16. These factors are frequently mutated in splenic MZ lymphoma17, suggesting they may also form part of a network that sustains the survival and localization of MZ B cells in pathological situations. Thus the interrelationship between transcriptional regulation, signal transduction and cell positioning for the development and survival of MZ B cells needs to be further understood17.

The post-transcriptional control of RNA regulated by RNA binding proteins (RBP) and non-coding RNAs complements transcriptional control by adding robustness to gene regulatory networks18. Deletion of Dicer19, or miRNA-14220, led to increased proportions of B cells with a MZ phenotype, suggesting that microRNAs suppress MZ B cell formation or survival. Retroviral expression of lin28b in haematopoietic stem cells promoted the development of MZ B cells21, 22, but roles for other RBP in MZ B cells have not been found.

The ZFP36 family of RBP are characterised by tandem CCCH-type zinc fingers, which bind RNA. By interacting with AU-rich elements (AREs) in the 3’UTR of mRNAs, these RBP promote RNA decay23. ZFP36 has been best characterised as a suppressor of cytokine production in innate immune cells; its relatives ZFP36L1 and ZFP36L2 play redundant roles during T and B lymphocyte development24, 25. The function of the ZFP36 family during the development and maintenance of mature B-lymphocytes has not been studied.

Here we demonstrate the indispensible role of ZFP36L1 in the maintenance of MZ B cells. Through analysis of the transcriptomes of primary mouse B cells, and the identification of ZFP36L1 targets in B cells by individual-nucleotide resolution cross-linking and immunoprecipitation (iCLIP), we identified the direct and indirect targets of ZFP36L1 in B cells, and determined a network of factors under the control of ZFP36L1 that promote MZ B cell localisation and survival. This study demonstrates that a single RBP can determine the cellular identity and ultimately survival of MZ B cells.

Results

MZ B cells specifically require ZFP36L1

To identify the roles of the ZFP36 family during lymphocyte development we first generated *Zfp36*$^{fl/fl}$hCD2-iCre^{+} (Supplementary Fig. 1), *Zfp36l1*$^{fl/fl}$hCD2-iCre^{+} and *Zfp36l2*$^{fl/fl}$hCD2-iCre^{+} mice in which each RBP was deleted early in lymphoid ontogeny. CD21$^{hifl/fl}$

oral gavage induced the recombination and deletion of *Zfp36l1* in B cells, and a 1.3-fold decrease in MZ B cell numbers compared to *Zfp36l1*$^{+/+}$ERT2$^{cre/+}$ mice (Supplementary Fig. 3a, b). The recombination efficiency of *Zfp36l1* remained high at later time-points, and MZ B cells decreased 1.7-fold by day 10 and 3.2-fold by day 14 of tamoxifen treatment in *Zfp36l1*$^{fl/fl}$ERT2$^{cre/+}$ mice compared to *Zfp36l1*$^{+/+}$ERT2$^{cre/+}$ mice (Supplementary Fig. 3c-f). This on-going depletion of MZ B cells was selective, as Fo B cell numbers were not decreased at any of the three time-points tested (Supplementary Fig 3b, d, f). To exclude an effect of *Zfp36l1* deletion in non-haematopoietic cells in *Zfp36l1*$^{fl/fl}$ERT2$^{cre/+}$ mice27, 28, we reconstituted lethally irradiated CD45.1^{+} B6.SJL mice with bone marrow from CD45.2^{+} *Zfp36l1*$^{fl/fl}$ERT2$^{cre/+}$ or CD45.2^{+} *Zfp36l1*$^{+/+}$ERT2$^{cre/+}$ mice and eight weeks after reconstitution administered tamoxifen by oral gavage. Seven days following tamoxifen treatment the purified B cells from *Zfp36l1*$^{fl/fl}$ERT2$^{cre/+}$ showed more than 80% deletion of *Zfp36l1* (Fig. 3a). Chimeric mice reconstituted with *Zfp36l1*$^{fl/fl}$ERT2$^{cre/+}$ bone marrow had a selective loss of MZ and MZP B cells following seven days tamoxifen treatment (Fig. 3b), whilst Fo and transitional B cell subsets remained unchanged in number compared to the *Zfp36l1*$^{+/+}$ERT2$^{cre/+}$ chimeras (Fig. 3b; data not shown). Therefore *Zfp36l1* is dispensable for the maintenance of Fo B cells, but necessary for the persistence of MZ and MZP B cells.

To address whether ZFP36L1 affected B cell survival we used flow cytometry to measure the presence of active-caspase-3. There was a 2.5-fold increase in the proportion of MZ B cells positive for active-caspase-3^{+} in *fl/fl*

the number of reads mapped within the floxed region of *Zfp36l1* in MZ B cells from *Zfp36l1*$^{fl/fl}$ERT2$^{cre/+}$ when compared to *Zfp36l1*$^{+/+}$ERT2$^{cre/cre}$

As expected, BCR elicited calcium flux was enhanced in MZ B cells compared to Fo B cells in control *Zfp36l1*$^{fl/fl}$mb1$^{+/+}$ mice (Fig. 5e, f), while calcium flux in MZ B cells from *Zfp36l1*$^{fl/fl}$mb1$^{cre/+}$ was similar to that observed in Fo B cells (Fig. 5e). Calcium flux elicited by BCR crosslinking in CD93^{+} transitional B cell subsets (data not shown) or CD21^{+}CD23^{+} Fo B cells from *Zfp36l1*$^{fl/fl}$mb1$^{cre/+}$ mice was not different from that elicited in the same subsets from *Zfp36l1*$^{fl/fl}$mb1$^{+/+}$ mice (Fig. 5e).

Incubation of cells with EGTA chelates extracellular calcium and limits BCR stimulated calcium flux to that released from internal stores. Stimulation with anti-IgM elicited less calcium release in EGTA-treated MZ B cells from *Zfp36l1*$^{fl/fl}$mb1$^{cre/+}$ mice than from *Zfp36l1*$^{fl/fl}$mb1$^{+/+}$ mice (Fig. 5e). This indicates the defective release of calcium from the internal stores of *Zfp36l1*$^{fl/fl}$mb1$^{cre/+}$ MZ B cells. Thus, ZFP36L1 enhances BCR signaling in MZ B cells.

To investigate the function of the residual MZ B cells in the ZFP36L1-deficient mice, we immunized *Zfp36l1*$^{fl/fl}$CD23$^{cre/+}$, *Rosa26*^{GFPZFP36L1} CD23$^{cre/+}$ and CD23$^{+/+}$ littermate control mice with the CD1d-restricted antigen NP-α-GalCer32. Four days post-

Klf2 mRNA was increased 3.1 fold (Fig. 7a) and KLF2 protein was also increased as assessed by flow cytometry (Fig. 7b, c) when *Zfp36l1*$^{fl/fl}$ERT2$^{cre/+}$ and *Zfp36l1*$^{+/+}$ERT2$^{cre/cre}$ MZ B cells were compared. *Klf2* mRNA contains a TATTTATT ARE in its 3'UTR, which is conserved amongst mammalian species that have a *Klf2* ortholog (Fig. 7d). iCLIP analysis indicated that ZFP36L1 binds in this ARE (Fig. 7d); however the data did not reach statistical significance due to low KLF2 mRNA abundance in activated B cells15, 34. Thus, ZFP36L1 may directly limit expression of KLF2.

induce deletion of *Zfp36l1* and measured the localisation of CD1d+ cells by antibody staining of splenic tissue sections. We observed an increased proportion of $CD1d^{+}$

targets. Extensive further work is required to understand the molecular basis for the redundant and non-redundant functions of these RBPs.

We identified IRF8 and KLF2 as direct targets of ZFP36L1 that regulate a number of genes important for MZ B cell identity. The molecular basis for KLF2 regulation of the MZ B cell pool may also relate to its ability to control expression of adhesion receptors, while the mechanisms by which IRF8 limits the size of the MZ B cell compartment is unclear 13 41. Many, but not all, of the indirect changes in the transcriptome of *Zfp36l1*$^{+/+}$ERT2$^{cre/cre}$ MZ B cells appeared to stem from the increased expression of IRF8 or KLF2, as these transcription factors account for 18/54 differentially expressed Fo B cell “signature” genes. Many KLF2 targets, that were not ZFP36L1 targets, were increased in *Zfp36l1*$^{+/+}$ERT2$^{cre/cre}$ MZ B cells and a number of these were also suppressed in Fo B cells from *Rosa26*^{GFPZFP36L1}CD23$^{cre/+}$ mice. Thus the transcriptome of MZ B cells is regulated by network of factors, acting transcriptionally and post-transcriptionally, in which ZFP36L1 is a major hub. Additional ZFP36L1 targets identified in the iCLIP and increased in *Zfp36l1*$^{+/+}$ERT2$^{cre/+}$ MZ B cells may contribute to the abnormal localisation and survival of MZ B cells. Pim1 kinase42, 43; KIAA0101 (2810417H13Rik)44; Myadm (myeloid-associated differentiation marker) a putative adapter protein of uncertain function45, 46; Txnrd1 (thioredoxin reductase 1) an enzyme that catalyzes the reduction of thioredoxin47, 48; and Bhlhe4049; have all been implicated in adhesion and metastasis. ZFP36L1 may act to promote the interaction between MZ B cells and the unique extracellular matrix components that are enriched in the MZ and play an important role in promoting the survival of MZ B cells3. These mechanisms protect MZ B cells from apoptosis. It is therefore tempting to speculate that ZFP36L1-deficient MZ B cells are lacking these pro-survival

obtained from Jackson labs, USA. For bone marrow chimeras, B6.SJL recipient mice were irradiated and reconstituted with a total of $3x10^6$ donor bone marrow cells by i.v. injection. Reconstituted mice were fed neomycin sulphate (Sigma) in their drinking water for four weeks post-reconstitution, and were analysed after 8-10 weeks. In the case of μMT chimeras, of the $3x10^6$ donor BM cells, 80% were taken from μMT mice, and 20% from either *Zfp36l1*$^{fl/fl}$

with a FITC conjugated rabbit monoclonal antibody, which recognises active Caspase-3 (BD Biosciences).

Calcium flux analysis

Splenocytes were loaded for 30min at 37°C in the dark with 0.6μM Ca^{2+} indicator PBX (BD) in serum free DMEM (5-10x10^6 cells). Cells were then stained with surface antibodies at RT, and resuspended in serum free Hanks medium. Cells were pre-treated with 10mM EGTA for 1 minute if required, before being stimulated with 10μg/ml goat polyclonal α-IgM F'ab fragment (Jackson ImmunoResearch). Fluorescence emission (525nm) was measured using a 488nm laser and 530/30 filter on a BD LSRFortessa flow cytometer.

B cell purification and sorting

B cells from spleen or peripheral lymph nodes were isolated with a B cell Isolation Kit from Miltenyi Biotech. To purify specific B cell subsets, cells were subsequently sorted using a BD FACSAria III or a BD FACSAria Fusion, using staining described above.

DNA isolation, RNA extraction and RT-qPCR assays

Total RNA was extracted from purified B cells using TRIzol (LifeTech) or RNeasy Micro or Mini Kit (Qiagen). RNA was treated with DNase I before reverse transcription into cDNA. ZFP36 family expression was analysed using custom and commercially available TaqMan assays with specific primers (Supplementary Table 9). Expression of mRNA was calculated using a standard curve and normalised to the expression of β2M. Genomic DNA was extracted from purified B cells using Cell lysis solution (Qiagen) containing proteinase K (Roche). Protein was removed by salt precipitation, and the DNA was isolated using

Gene Expression Omnibus. The data the support the findings of this study are available from the corresponding author upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. Busslinger for the CD23-Cre expressing mice, M. Reth for the mb1-Cre expressing mice, T. Ludwig for the ERT2-cre expressing mice and D. Kioussis for the hCD2-iCre-expressing mice. This work was funded by a GSK-CASE studentship to RN and funding from the Biotechnology and Biological Sciences Research Council, The Medical Research Council and Bloodwise. We thank K. Bates, R. Walker, A.Davies and L. Waugh and members of the Biological Services Unit for technical support; D. Bell and P. Tolar for help with immunofluorescence analysis; members of the laboratory, L. Webb, M. Linterman and T. Arnon for helpful advice.

References

1. Cinamon G, Zachariah MA, Lam OM, Foss FW Jr, Cyster JG. Follicular shuttling of marginal zone B cells facilitates antigen transport. Nature immunology. 2008; 9:54–62. [PubMed: 18037889]
2. Arnon TI, Horton RM, Grigorova IL, Cyster JG. Visualization of splenic marginal zone B-cell shuttling and follicular B-cell egress. Nature. 2013; 493:684–688. [PubMed: 23263181]
3. Song J, et al. Extracellular matrix of secondary lymphoid organs impacts on B-cell fate and survival. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110:E2915–E2924. [PubMed: 23847204]
4. Simonetti G, et al. IRF4 controls the positioning of mature B cells in the lymphoid microenvironments by regulating NOTCH2 expression and activity. J Exp Med. 2013; 210:2887–2902. [PubMed: 24323359]
5. Fasnacht N, et al. Specific fibroblastic niches in secondary lymphoid organs orchestrate distinct Notch-regulated immune responses. J Exp Med. 2014; 211:2265–2279. [PubMed: 25311507]
6. Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. Nature reviews. Immunology. 2013; 13:118–132.
7. Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. Nature reviews. Immunology. 2009; 9:767–777.
8. Martin F, Kearney JF. Marginal-zone B cells. Nature reviews. Immunology. 2002; 2:323–335.
9. Srivastava B, Quinn WJ, Hazard K, Eriks 120nEfdlman D. Characterization of marginal zone B cell precursors. J Exp Med. 2005; 202:1225–1234. [PubMed: 16260487]
10. Kleiman E, et al. Distinct Transcriptomic Features are Associated with Transitional and Mature B-Cell Populations in the Mouse Spleen. Frontiers in immunology. 2015; 6:30. [PubMed: 25717326]
11. Ta120B, et al. Lunatic and manic fringe cooperatively enhance marginal zone B cell precursor competition for delta-like 1 in splenic endothelial niches. Immunity. 2009; 30:254–263. [PubMed: 19217325]
12. Witt CM, Won WJ, Hurez V, Klug CA. Notch2 haploinsufficiency results in diminished B1 B cells and a severe reduction in marginal zone B cells. J Immunol. 2003; 171:2783–2788. [PubMed: 12960298]
13. Feng J, et al. IFN regulatory factor 8 restricts the size of the marginal zone and follicular B cell pools. J Immunol. 2011; 186:1458–1466. [PubMed: 21178004]
14. Hart GT, Wang X, Hogquist KA, James 12SC. Krüppel-like factor 2 (KLF2) regulates B-cell reactivity, subset differentiation, and trafficking molecule expression. PNAS. 2010; 2:1–6.
15. Winkelmann R, et al. B cell homeostasis and plasma cell homing controlled by Kruppel-like factor 2. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108:710–715. [PubMed: 21187409]
16. Vu TT, et al. Impaired B Cell Development in the Absence of Kruppel-like Factor 3. Journal of immunology. 2011; 187:5032–5042.

17. Clipson A, et al. KLF2 mutation is the most frequent somatic change in splenic marginal zone lymphoma and identifies a subset with distinct genotype. Leukemia. 2015; 29:1177–1185. [PubMed: 25428260]

38. Kaymak E, Ryder SP. RNA recognition by the Caenorhabditis elegans oocyte maturation determinant OMA-1. The Journal of biological chemistry. 2013; 288:30463–30472. [PubMed: 24014033]
39. Farley BM, Ryder SP. POS-1 and GLD-1 repress glp-1 translation through a conserved binding-site cluster. Molecular biology of the cell. 2012; 23:4473–4483. [PubMed: 23034181]
40. Tamburino AM, Ryder SP, Walhout AJM. A Compendium of Caenorhabditis elegans RNA Binding Proteins Predicts Extensive Regulation at Multiple Levels. G3-Genes Genom Genet. 2013; 3:297–304.
41. Hoek KL, et al. Follicular B cell trafficking within the spleen actively restricts humoral immune responses. Immunity. 2010; 33:254–265. [PubMed: 20691614]
42. Santio NM, et al. Pim Kinases Promote Migration and Metastatic Growth of Prostate Cancer Xenografts. PLoS One. 2015; 10:e0130340. [PubMed: 26075720]
43. Grundler R, et al. Dissection of PIM serine/threonine kinases in FLT3-ITD-induced leukemogenesis reveals PIM1 as regulator of CXCL12-CXCR4-mediated homing and migration. J Exp Med. 2009; 206:1957–1970. [PubMed: 19687226]
44. Jain M, Zhang L, Patterson EE, Kebebew E. KIAA0101 is overexpressed, and promotes growth and invasion in adrenal cancer. PLoS One. 2011; 6:e26866. [PubMed: 22096502]
45. Aranda JF, et al. MYADM regulates Rac1 targeting to ordered membranes required for cell spreading and migration. Molecular biology of the cell. 2011; 22:1252–1262. [PubMed: 21325632]
46. Aranda JF, et al. MYADM controls endothelial barrier function through ERM-dependent regulation of ICAM-1 expression. Molecular biology of the cell. 2013; 24:483–494. [PubMed: 23264465]
47. Devis L, et al. Activated leukocyte cell adhesion molecule (ALCAM) is a marker of recurrence and promotes cell migration, invasion and metastasis in early stage endometrioid endometrial cancer. J Pathol. 2016
48. Nalvarte I, Damdimopoulos AE, Ruegg J, Spyrou G. The regulation299en adrenal cancer. PLoS Okm1(gg s /P xa, et4g 6 [(re

60. Chen J, Bardes EE, Aronow BJ, Jegga AG. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. Nucleic acids research. 2009; 37:W305–311. [PubMed: 19465376]
61. Supek F, Bosnjak M, Skunca N, Smuc T. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One. 2011; 6:e21800. [PubMed: 21789182]
62. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics. 2004; 20:307–315. [PubMed: 14960456]
63. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome biology. 2009; 10:R25. [PubMed: 19261174]
64. Zhang Y, et al. Model-based analysis of ChIP-Seq (MACS). Genome biology. 2008; 9:R137. [PubMed: 18798982]

Europe PMC Funders Author Manuscripts

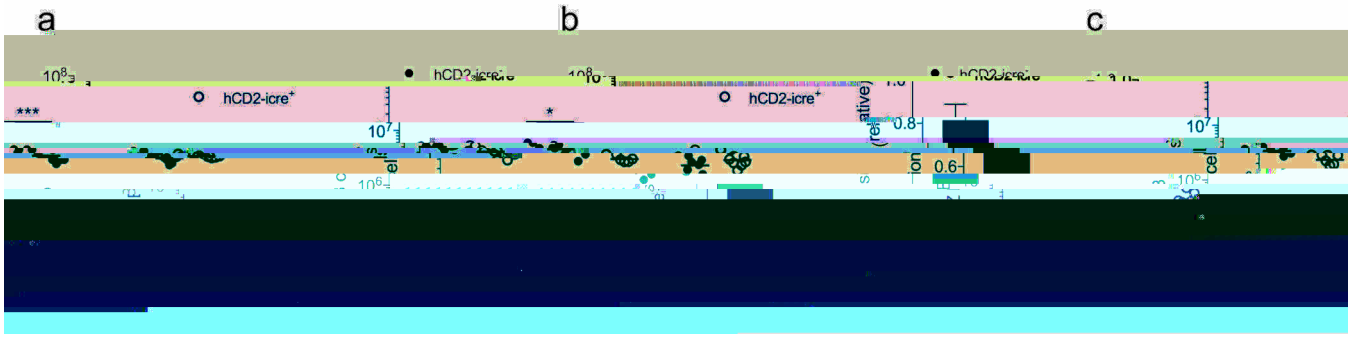

Figure 1.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Figure 2.

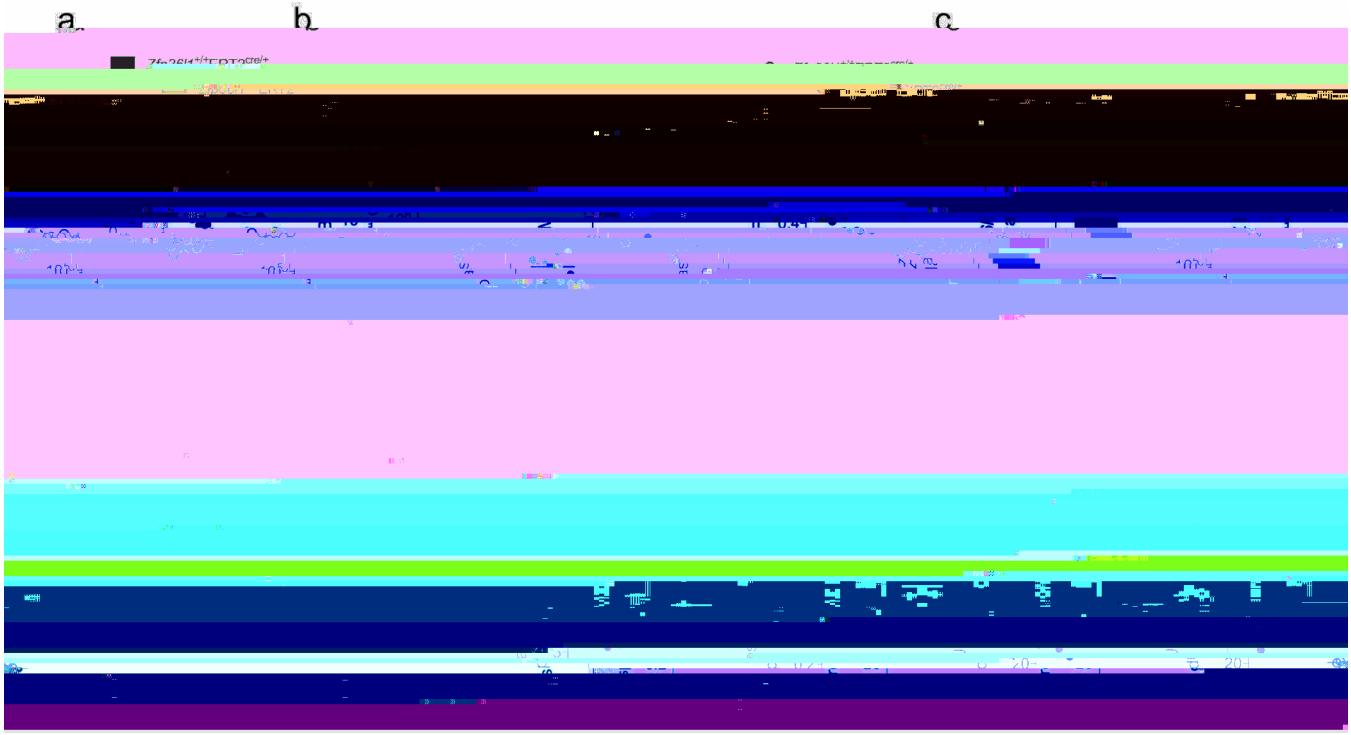

Figure 3.

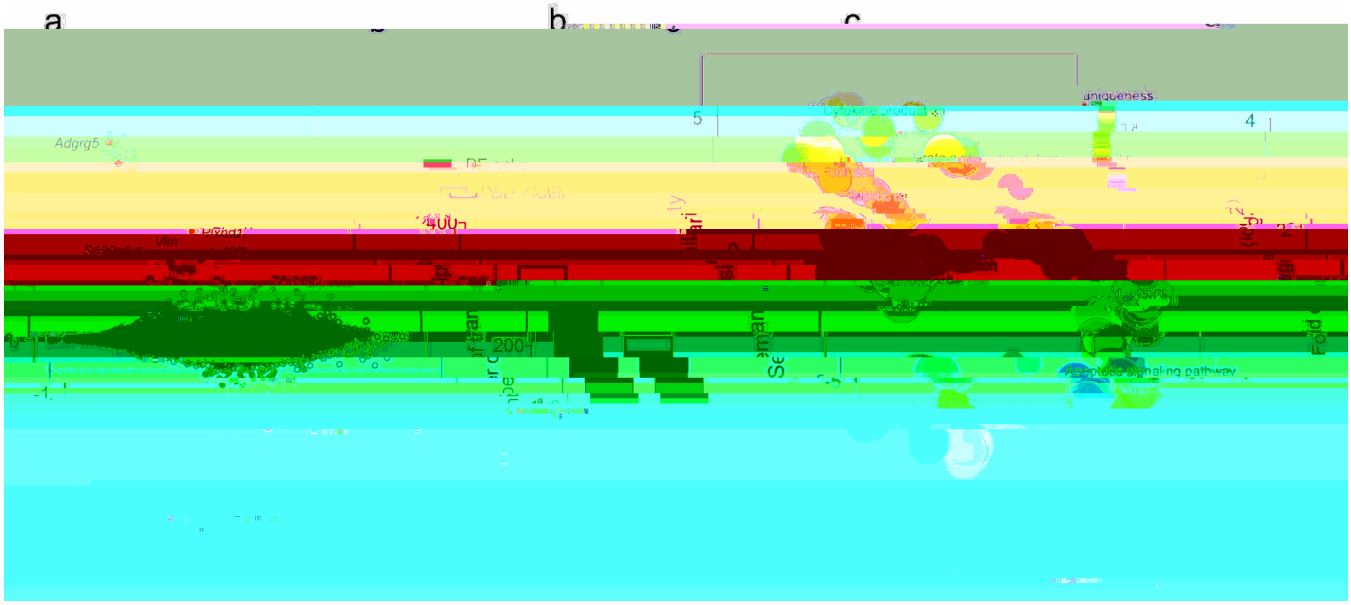

Figure 4.

Europe PMC Funders Author Manuscripts

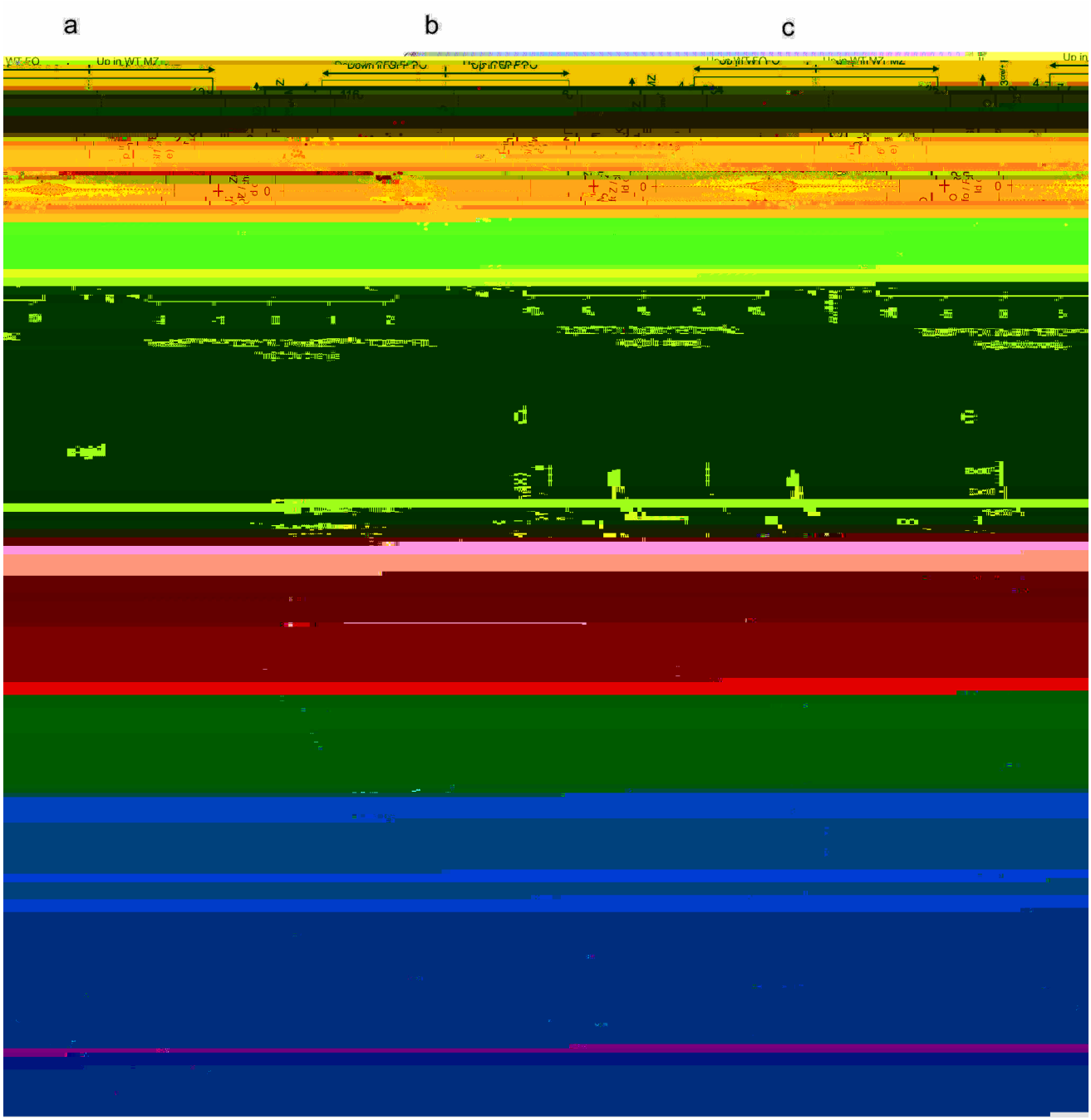

Figure 5.

Europe PMC Funders Author Manuscripts

Figure 6.

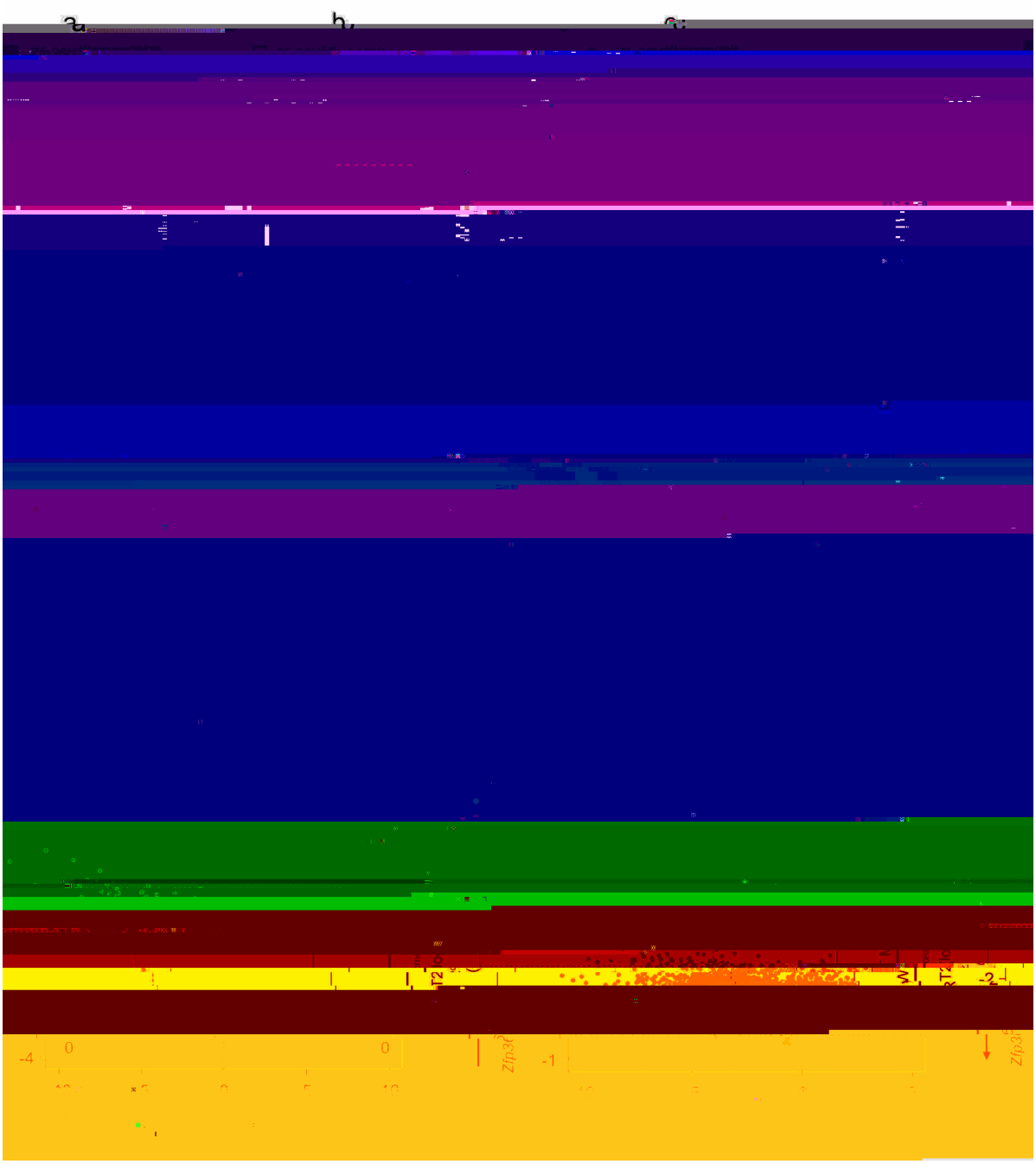

Figure 7.

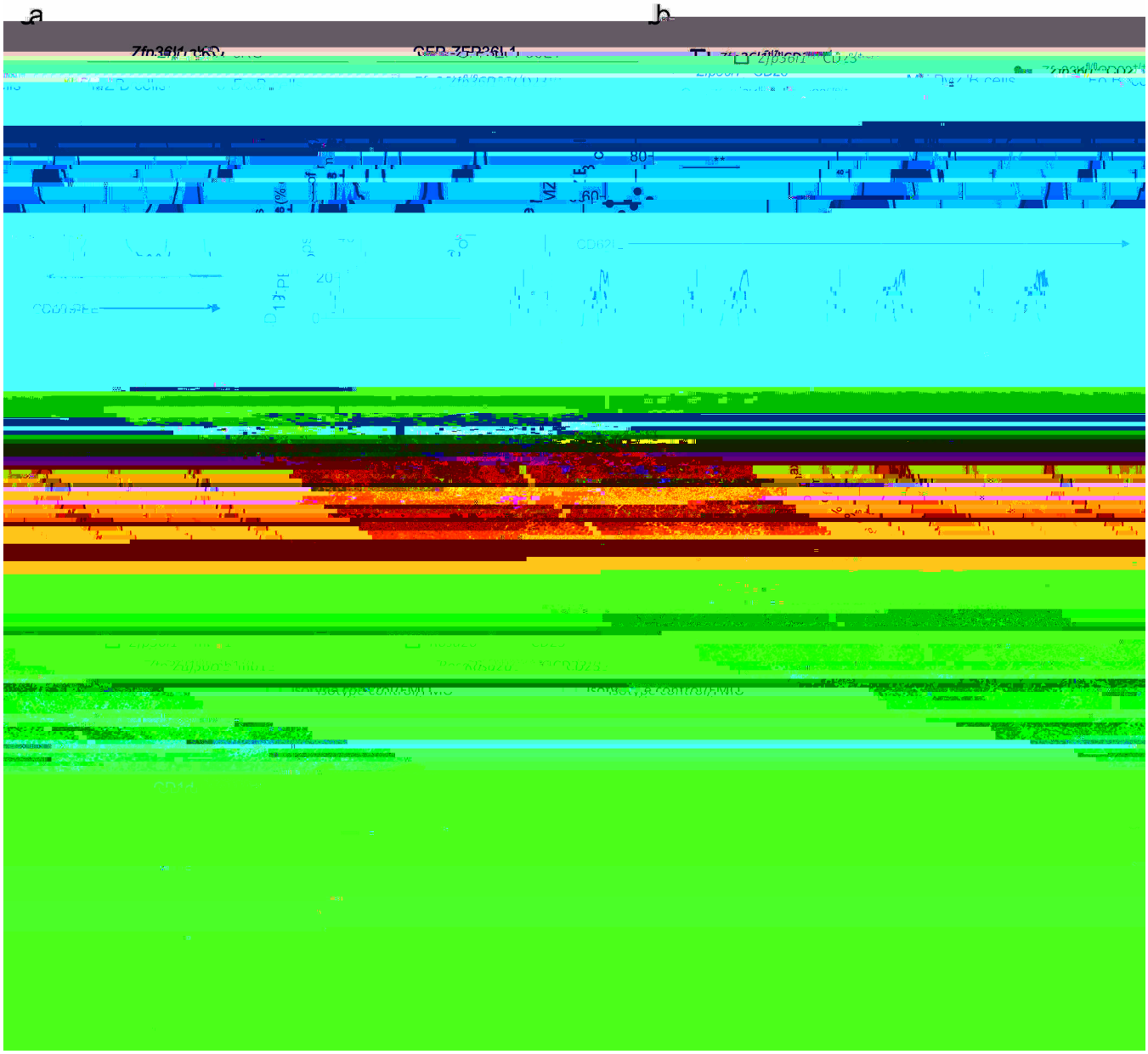

Figure 8.