Functional genomics identifies negative regulatory nodes controlling phagocyte oxidative burst

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The phagocyte oxidative burst, mediated by Nox2 NADPH oxidase-derived reactive oxygen species, confers host defense against a broad spectrum of bacterial and fungal pathogens. Loss-of-function mutations that impair function of the Nox2 complex result in a life-threatening immunodeficiency, and genetic variants of Nox2 subunits have been implicated in pathogenesis of inflammatory bowel disease (IBD). Thus, alterations in the oxidative burst can profoundly impact host defense, yet little is known about regulatory mechanisms that fine-tune this response. Here we report the discovery of regulatory nodes controlling oxidative burst by functional screening of genes within loci linked to human inflammatory disease. Implementing a multi-omics approach, we define transcriptional, metabolic and ubiquitin-cycling nodes controlled by Rbpj, Pfkl and Rnf145, respectively. Furthermore, we implicate Rnf145 in proteostasis of the Nox2 complex by endoplasmic reticulum-associated degradation. Consequently, ablation of Rnf145 in murine macrophages enhances bacterial clearance, and rescues the oxidative burst defects associated with Ncf4 haploinsufficiency.
Phagocytes such as neutrophils, macrophages and dendritic cells deploy multiple bactericidal mechanisms to kill microorganisms. During the earliest stages of pathogen encounter, phagocytes generate toxic superoxide and other reactive oxygen species (ROS) in phagosomes to kill microbes by oxidation of DNA, lipids and iron–sulfur centres within critical metabolic enzymes. Oxidative burst requires assembly of the Nox2 complex on the phagosomal membrane, which subsequently catalyses the conversion of di-atomic oxygen into superoxide radicals by utilizing NADPH as a cofactor for electron transfer. Recruitment of Nox2 NADPH oxidase regulatory subunits (p40phox, p47phox and p67phox) from the cytosol to the membrane-associated catalytic subunits (gp91phox and p22phox) requires signalling from receptors such as integrins, G-protein-coupled receptors or C-type lectins specialized in detection of pathogens and inflammatory mediators.

Many loss-of-function alleles have been described for Nox2 NADPH oxidase subunits, with X-linked CYBB (gp91phox) being the most common cause of chronic granulomatous disease (CGD), a life-threatening primary immunodeficiency associated with recurrent bacterial and fungal infections. More rare autosomal recessive forms of CGD have been attributed to loss-of-function mutations in additional Nox2 complex subunits such as NCF1 (p47phox), NCF2 (p67phox) and CYBA (p22phox). In one reported case, autosomal recessive inheritance of two NCF4 (p40phox) null alleles was associated with a unique clinical profile relative to other forms of CGD and manifested as severe colitis resembling IBD. In addition to the observation that all forms of CGD can be associated with inflammatory gastrointestinal disease, the notion that immunodeficiency can lead to pathological inflammation driven by commensal microorganisms is also supported by the recent discovery of hypomorphic alleles of Nox2 complex subunits linked to IBD.

Over several decades, a great deal of mechanistic insight into regulation of the Nox2 complex has been gained from a combination of characterizing cases of primary immunodeficiency in humans and in the study of knockout mice. As a result of these loss-of-function studies, many required genes have been discovered in the oxidative burst pathway, but few negative regulators have been identified. Among pathways that positively regulate oxidative burst, adhesion-dependent signalling through immunoreceptor tyrosine-based activation motifs (ITAMs) requires signalling from receptors such as integrins, G-protein-coupled receptors or C-type lectins specialized in detection of pathogens and inflammatory mediators. However, we describe a strategy for unbiased candidate selection and functional screening of candidates implicated in disorders of inflammation.

We further define the mechanistic basis of transcriptional, metabolic and ubiquitin-dependent regulation of the oxidative burst.

Results

Functional genetic screen for phagocyte oxidative burst.

Accumulating evidence demonstrates the importance of phagocyte oxidative burst in maintaining host defense and immune homeostasis in mucosal tissues. Specifically, coding variants in NCF4 and NCF2 are associated with IBD. Thus we reasoned that impaired oxidative burst mediated by the Nox2 complex represents a central pathway in mucosal immunity. Furthermore, we postulated that IBD GWAS loci would contain previously unknown regulators of the Nox2 NADPH oxidase complex. To identify novel regulatory nodes controlling oxidative burst, we performed a functional knockdown screen of genes present within IBD loci, those associated with Mendelian inheritance of IBD, and select genes involved in inflammatory pathways associated with autoimmunity. In total, we screened >400 candidate genes for functional effects on oxidative burst in RAW264.7 macrophages (Fig. 1a,b). Knockdown of candidate genes and controls (Ncf4 and Cat) was achieved by lentiviral delivery of shRNAs with an average coverage of 5 hairpins per gene, for a total of ~2,000 unique hairpins screened individually. After lentiviral delivery of shRNAs and selection, RAW cells were stimulated with zymosan yeast particles to engage dectin-1-dependent ITAM signalling and induce NADPH oxidase activation. Kinetics of ROS production was monitored over the course of 1 h with the chemiluminescent substrate luminol. In parallel, duplicate samples were assessed for cell viability by Alamar Blue and target gene knockdown by quantitative PCR (qPCR). Results from the primary screen demonstrate the sensitivity of the assay and ability to identify Ncf4 as a positive regulator and Cat as a negative regulator. Furthermore, known ROS activators such as Card9 scored as positive regulators in the screen. To identify negative regulatory nodes in the ROS pathway, we devised a hit-calling strategy using a structured Bayesian approach for control of false discovery rate (FDR; Fig. 1c). This approach incorporated the experimental structure into the FDR estimation to share strength among multiple shRNAs for the same gene, thus allowing genes with low but consistent support across shRNAs to be identified. A key strength of this approach is in integration of multiple data measurements from multiple hairpins per gene to minimize false positive calls due to off-target effects and/or inefficient target gene knockdown (Supplementary Data 1). Accordingly, we used Ncf4 to define the parameters of the activator class and Cat for the
inhibitor class. Cat was selected during assay development as a top performing control, presumably due to its effect on cellular hydrogen peroxide levels and/or peroxidase activity. By defining activator and inhibitor classes based on controls (Fig. 1c), we prioritized genes for further investigation and focused on three novel genes that negatively regulated phagocyte oxidative burst, each representing a putative regulatory node (Fig. 1d). We identified Dctn2 as a negative regulator, suggesting that perhaps vesicular trafficking and/or phagosomal fusion terminate Dectin-1 signalling. In addition, we identified a transcriptional node controlled by Rbpj, a Notch-dependent transcription factor; a metabolic node controlled by Pfkl, the rate-limiting enzyme in glycolysis, and a ubiquitin-cycling node controlled by Rnf145, a multipass transmembrane protein with a C-terminal RING domain.

Based on primary screening results, we proceeded with further functional characterization of hits. Accordingly, we selected two hairpins per gene based on knockdown efficiency and confirmed that ablation of Rbpj, Pfkl or Rnf145 resulted in augmented oxidative burst in response to zymosan or Staphylococcus aureus (Fig. 2a,b). To test the significance of increased ROS production on host defense, we monitored bacterial activity of macrophages after perturbation of candidate negative regulatory nodes. We chose S. aureus for these experiments, as this species is sensitive to superoxide and is frequently associated with chronic infection in CGD. After knockdown of candidate genes, macrophages were cultured with bacteria for 30 min to allow for phagocytosis. After washing away free bacteria, macrophages were cultured in the presence of gentamicin to kill any remaining extracellular bacteria. Bacterial killing was monitored by lysing macrophages and determining the number of colony-forming units (c.f.u.) of remaining live bacteria. As a control, Ncf4 knockdown resulted in impaired bacterial killing, as evidenced by increased bacterial burden (c.f.u. at 2 h; Fig. 2c). Consistent with augmented ROS production, knockdown of Rbpj, Pfkl and Rnf145 resulted in more effective bacterial killing relative to control shRNAs (Fig. 2c). Thus, perturbation of negative regulatory nodes within the ROS pathway can enhance bacterial killing.

Having identified three negative regulatory nodes in the oxidative burst response in RAW cells, we sought to determine if these nodes are functional in primary bone marrow-derived macrophages (BMDMs) and if this is affected by distinct priming stimuli. Thus we knocked down candidate genes in BMDMs and measured the oxidative burst response to zymosan in unprimed cells or those that had been primed with IFN-γ or IL-4. Our results indicate that Rbpj and Pfkl function as negative regulators of zymosan-induced oxidative burst regardless of the priming stimuli being tested (Fig. 3a,b). In contrast, Rnf145 represented a negative regulatory node on priming with IFN-γ or IL-4 but not in unprimed cells (Fig. 3c). We next addressed the possibility that these three regulatory nodes function in a cell type-dependent manner by performing the same experiment in bone marrow-derived dendritic cells (BMDCs). Although Rbpj and Pfkl knockdown trend towards enhanced zymosan-induced oxidative burst in primed BMDCs, Rnf145 knockdown did not (Supplementary Fig. 1). Thus, the oxidative burst may be controlled by distinct regulatory nodes in different cell types and under different stimulation conditions. To further dissect molecular regulation of these nodes in macrophages, we systematically evaluated Rbpj in the context of transcription, Pfkl in metabolism and then focused predominantly on Rnf145 because of its novel role in ubiquitin-dependent control of Nox2 function.

**Rbpj is a transcriptional node in the oxidative burst.** Enhanced ROS production and antibacterial activity after Rbpj knockdown suggests Notch regulation in macrophages, and we thus sought to determine if Notch signalling directly impacts ROS production in macrophages. In support of this model, ectopic expression of the constitutively active Notch1 intracellular domain (Notch-ICD)
inhibited oxidative burst, suggesting that Notch is a negative regulator in this context (Fig. 4a). To identify transcriptional programmes controlled by the Notch–Rbpj axis in myeloid cells, we knocked down Rbpj in BMDMs and performed transcriptional profiling. Towards this end, we designed a custom Fluidigm panel to measure expression of cytokines, inflammatory markers and transcription factors (Supplementary Data 2). Strikingly, Rbpj knockdown led to increased expression of the Notch target Hes1 (Fig. 4b). Given that Rbpj acts as a transcriptional repressor in the absence of Notch signalling and an activator in its presence, our results suggest that intrinsic Notch signalling is absent or low in cultured macrophages. Thus, Rbpj largely functions as a repressor in this context. Indeed, Rbpj knockdown dramatically upregulated the transcription factor Runx3 and the master regulator of gp91phox expression in myeloid cells Spf1 (PU.1)17 (Fig. 4b). Together, Runx3 and PU.1 function cooperatively to induce expression of myeloid-specific genes18. Consistent with this notion, our transcriptional profiling further demonstrated an upregulation (~20%) of gp91phox (Cybb) on knockdown of Rbpj. In addition, Rbpj knockdown in BMDMs resulted in enhanced expression of Ncf1, Ncf4, Cyba and Spf1 (Fig. 4b,c). Thus, our results demonstrate that repressive activity of Rbpj in macrophages inhibits a myeloid transcriptional programme associated with expression of Nox2 subunits.

Figure 2 | Targeting negative regulatory nodes controlling oxidative burst enhances bactericidal activity in macrophages. (a) Hits from the primary screen were validated and functionally characterized using the top two hairpins per gene, as defined by target gene knockdown efficiency in the primary screen (see Supplementary Data 1). Accordingly, RAW264.7 cells were subjected to shRNA knockdown followed by IFN-γ priming and zymosan or Staphylococcus aureus stimulation to induce oxidative burst. Data represent ROS production as relative luminescence units (RLU) measured with luminol substrate. (b) Target gene knockdown was measured by qPCR and normalized to Actb housekeeping gene or by western blot. Data represent mean ± s.d. of biological triplicates. (c) The indicated genes were knocked down by shRNA in RAW264.7 cells. After IFN-γ priming, macrophages were infected with Staphylococcus aureus for 30 min, washed and cultured in gentamicin media to kill any bacteria that had not been phagocytosed. After the indicated time points, cells were lysed, and remaining live bacteria were enumerated as colony-forming units (c.f.u.). Data represent the mean of triplicates ± s.d. *P ≤ 0.05 as determined by Student’s t-test.
Pfkl regulates a metabolic node in the oxidative burst. To specifically delineate the role of Pfkl in the oxidative burst response, we tested the hypothesis that Pfkl controls a regulatory node functioning at the level of metabolism. Given that Pfkl is the rate-limiting enzyme in glycolysis, we determined the metabolic consequences of Pfkl knockdown in BMDMs stimulated over the course of 20 h with IFN-γ and zymosan. We evaluated the levels of ~200 polar metabolites extracted from macrophages and quantified by mass spectrometry (MS). Consistent with the notion that phagocyte activation is associated with a metabolic switch from mitochondrial oxidative phosphorylation to glycolysis\(^\text{19,20}\), we observed increased lactate and decreased TCA intermediates (isocitrate) in control macrophages. However, Pfkl knockdown reduced glycolytic flux as evidenced by diminished lactate (Fig. 5a and Supplementary Data 3). Moreover, the bottleneck in glycolysis imposed by Pfkl knockdown resulted in diversion of glucose into the pentose phosphate pathway (PPP). In this context, we observed accumulation of early PPP intermediates (6-phosphogluconate and erythrose-4-phosphate) and depletion of late-stage metabolites (purines and pyrimidines) (Fig. 5a,b), which is consistent with increased flux through the pathway to compensate for impaired glycolysis and to meet the energy demands of the cell. Importantly, PPP is the major cellular source of NADPH, which functions as a critical cofactor for NADPH oxidase by providing reducing equivalents to generate superoxide radicals. Indeed, Pfkl knockdown resulted in an increase in intracellular NADPH relative to control knockdown (Fig. 5c). Thus, Pfkl controls a metabolic regulatory node that modulates oxidative burst by fine-tuning NADPH levels within macrophages. Consistent with our findings in macrophages, Pfkb has been shown to control glycolytic flux in T cells\(^\text{21}\). In T cells derived from patients with rheumatoid arthritis, Pfkb3 expression was reduced, glycolysis was impaired and PPP flux was increased, resulting in impaired energy generation and senescence\(^\text{21}\). Thus, inhibition of glycolysis shunts glucose metabolism towards the PPP and increases cellular levels of NADPH, which functions as a cofactor for NADPH oxidase catalysis and superoxide production.

Rnf145 is a ubiquitin-cycling node in the oxidative burst. To identify the function of Rnf145 in the oxidative burst response, we tested the hypothesis that Rnf145 controls a regulatory node functioning in the ubiquitin cycle. Given that Rnf145 contains a C-terminal RING domain and shares structural homology with several transmembrane-anchored E3 ubiquitin ligases involved in endoplasmic reticulum-associated degradation (ERAD), we sought to determine the effects of Rnf145 depletion on the ubiquitin landscape in macrophages. Towards this end, we knocked down Rnf145 in RAW cells and compared the ubiquitin proteome to that in control cells with and without stimulation by IFN-γ and zymosan. After treatment, cells were lysed and subjected to trypsin digest prior to immunoprecipitation with a monoclonal antibody specific for the ubiquitin remnant (KGG) (Lys-Gly-Gly) present on trypsin-cleaved ubiquitin substrates. Ubiquitin substrates were then identified and quantified by MS. We observed several thousand ubiquitinated peptides in macrophages and ~100 of these increased in abundance after zymosan stimulation.
stimulation. Among these were Irap2 and Ikk (Supplementary Data 4). However, given that Rnf145 is a transmembrane protein, we next compared ubiquitin levels in all transmembrane proteins from RAW cells with and without Rnf145 knockdown. Strikingly, ubiquitinated peptides derived from gp91phox and p22phox were significantly less abundant in cells with Rnf145 knockdown (Fig. 6a,b). Moreover, 11 distinct peptides from these Nox2 complex subunits were differentially ubiquitinated after Rnf145 knockdown, and we defined specific lysine residues that were ubiquitinated (Fig. 6b,c). Notably, zymosan stimulation did not significantly alter ubiquitin levels of Nox2 complex subunits, suggesting that it is not likely to be ubiquitinated in phagosomes.

To determine if steady-state levels of the Nox2 complex are controlled by Rnf145 and ubiquitination, we depleted Rnf145 in RAW cells and observed elevated levels of gp91phox as measured by western blot (Fig. 6d and Supplementary Fig. 1a). Importantly, zymosan stimulation did not appear to alter steady-state levels of gp91phox, suggesting that the role of Rnf145 in regulating Nox2 complex levels is independent of stimulation. Consistent with this notion, depletion of Rnf145 had no effect on zymosan-induced signalling events such as Erk phosphorylation (Fig. 6d), which is a requisite event for activation of the oxidative burst. Having shown increased levels of gp91phox after Rnf145 knockdown, we performed the converse experiment by overexpressing Rnf145 in RAW cells and measuring gp91phox expression by western blot. Indeed, Rnf145 expression reduced protein levels of gp91phox compared with the mock-transduced RAW cells (Fig. 6e). Moreover, we observed increased ubiquitin K48 linkages on gp91phox after ectopic expression of Rnf145 (Fig. 6e and Supplementary Fig. 1b), suggesting that these conjugates are destined for proteosomal destruction through ERAD. In fact, the close parologue of Rnf145, AMFR, has been shown to participate in ERAD22. Similarly, we observed that Rnf145 localizes to the ER along with the core ERAD protein Derlin 1 (Fig. 6f). While Rnf145 localized to the ER, we found that gp91phox was widely distributed throughout the cell, with its primary address in vesicles and the plasma membrane (Fig. 6g). Thus, these data indicate that Rnf145 does not colocalize with mature gp91phox in vesicular structures, but instead may interact with nascent gp91phox protein translated on the rough ER.

Having identified novel regulatory nodes in the oxidative burst pathway, we hypothesized that perturbation of these nodes could compensate for genetic lesions associated with IBD. We first deleted Rnf145 in RAW cells using RNA-guided Cas9 nuclease23,24. Two independent CRISPR lines, each containing an in-del mutation rate of ~80% within the Rnf145 coding sequence, led to augmented oxidative burst induced by zymosan (Fig. 7a). As a specificity control, we demonstrated that ectopic expression of human Rnf145 (but not green fluorescent protein (GFP)) in murine RAW cells reduced elevated ROS production in CRISPR-targeted cells (Fig. 7a). We then employed CRISPR technology in primary BMDMs (Fig. 7b) to determine if perturbation of the Rnf145 node can rescue defective oxidative burst associated with Ncf4 deficiency. Like in human subjects, deletion of Ncf4 in mice resulted in a severe impairment of Nox2 NADPH oxidase-derived phagosomal ROS (Fig. 7c). Moreover, haploinsufficiency of Ncf4, which may more accurately simulate IBD-associated Ncf4 variants, led to a partial defect in oxidative burst (Fig. 7c). We reasoned that Ncf4 haploinsufficiency decreases NADPH oxidase activity by altering the stoichiometry of regulatory subunits (p40phox and indirectly p67phox) and decreases efficiency of their assembly. We hypothesized that increasing gp91phox expression by CRISPR targeting of Rnf145 may promote assembly of the complex even if the regulatory subunits are present at suboptimal levels. However, this strategy was less effective than Rnf145 deletion in restoring functional burst through the endogenous Ncf4 gene (Fig. 7d).
levels. Strikingly, we found that CRISPR-mediated deletion of Rnf145 in Ncf4+/− macrophages rescued oxidative burst and restored the response to levels akin to wild type (Fig. 7c,d). Taken together, we leverage functional genomics to discover regulatory nodes controlling oxidative burst and show that perturbation at these nodes can bypass or compensate for genetic lesions associated with IBD.

Discussion

Here we demonstrate the utility of human genomics as a source of candidate genes controlling critical regulatory nodes in immune pathways. Given genetic and functional evidence implicating phagocyte oxidative burst in mucosal immunity, we performed a functional genetic screen of genes within IBD risk loci. We based this strategy on the notion that IBD candidates will be enriched for regulators of key immune pathways, irrespective of their putative relationship with IBD. Thus, our objectives were threefold: (1) to assign function to incompletely annotated genes, (2) to identify critical regulatory nodes constraining oxidative burst and (3) to define the mechanistic basis of Nox2 NADPH oxidase regulation. While the connection between genetic variation and IBD pathophysiology is complex, our data are consistent with the notion that alterations in oxidative burst (either positive or negative) can impose selective pressure on microbial communities in the gut, and that this can precipitate dysbiosis resulting in maladaptive inflammatory pathology.

Transcriptional regulation of oxidative burst was first described with the discovery that IFN-γ primes phagocytes to massively enhance antibacterial function and oxidative burst. Further investigation then revealed the mechanistic basis of Stat1-dependent transcriptional activation of Nox2 subunits. In fact these studies motivated clinical trials demonstrating benefit among CGD patients from IFN-γ therapy, although, as it turns out, efficacy was independent of effects on Nox2 subunit expression. While Nox2 NADPH oxidase transcriptional...
regulation is potently induced by IFN-γ and Stat1, we report that Rbpj acts conversely to negatively regulate expression of the Nox2 complex components Ncf1, Ncf4, Cybb and Cyba and their master regulator Sfpi1 (PU.1). Depending on the transcriptional cofactors with which it interacts, Rbpj functions as a transcriptional activator or repressor downstream of Notch. Although Notch functions as an early lineage commitment factor in T lymphocyte development, it shapes myeloid differentiation in more nuanced ways. Mice congenitally deficient in nicastrin, a nonredundant Notch-activating protease, develop a malignant myeloproliferative disease dominated by cells expressing elevated myeloid populations. Mice congenitally deficient in nicastrin, a nonredundant Notch-activating protease, develop a malignant myeloproliferative disease dominated by cells expressing elevated myeloid populations.

**Figure 6 | Rnf145 knockdown reduces ubiquitination of the Nox2 complex.** (a) RAW264.7 cells were transduced with lentivirus-encoding shRNA targeting Rnf145 or control (LacZ). Cells were then left untreated or stimulated with zymosan for 30 min. Cells were then lysed, and ubiquitinated peptides were immunoprecipitated and enumerated by UbiScan (see methods). Differential abundance of each ubiquitinated peptide was compared between control and Rnf145 knockdown. The network was constructed by anchoring on hit components (>2.5-fold difference between Rnf145 knockdown and control). Hits are represented by nodes and the network was extended by iteratively incorporating interacting partners using protein–protein interaction data derived from the curated Human Protein Reference Database and genome-wide interactome screens. The network was then pruned to include only first-order interactions (represented by edges) that directly connect the hits from UbiScan (enrichment $P = 2 \times 10^{-13}$). (b) Relative abundance of ubiquitinated peptides derived from transmembrane proteins. Values represent fold difference between Rnf145 knockdown and control. (c) Peptide sequences derived from p22phox and gp91phox showing ubiquitination sites (*), fold difference in Rnf145 knockdown relative to control and relative abundance (max intensity). (d) Knockdown of Rnf145 was performed in RAW264.7 macrophages (as in Fig. 2a), followed by stimulation with zymosan and western blot for the indicated proteins. (e) Rnf145 was expressed in RAW264.7 cells by lentiviral transduction. Cells were then lysed and analysed by western blot for gp91phox and ERK2 as a loading control. In addition, gp91phox was immunoprecipitated to detect K48-specific ubiquitin linkages by western blot. (f) RAW264.7 cells were transduced with lentivirus encoding Rnf145 with a C-terminal V5 epitope tag. Confocal microscopy was performed to visualize cellular localization of Rnf145 and Derlin 1. Scale bar, 10 μm. Arrows indicate colocalization of Rnf145 and Derlin 1 (f) or gp91phox-positive vesicles (g).
such as Ncf1, Fms, and FcgR. Thus, Notch appears to constrain the myeloid compartment by repressing late-stage differentiation factors controlling phagocyte effector function. In fact, Notch2 is required to enforce late-stage differentiation of CD11b+ mucosal dendritic cells and CD8+ splenic dendritic cells. Moreover, Notch signalling influences myeloid effector function by converging with Toll-like receptor pathways. In this context, intrinsic Notch signalling in macrophages restrains cytokine production in an Rbpj-dependent manner. Our results suggest that intrinsic Notch signalling is low in cultured macrophages, as evidenced by the observation that Rbpj knockdown enhanced expression of the Notch target Hes1, and that Rbpj is known to function as a repressor in the absence of Notch signalling.

Of note, we also found that Rbpj knockdown leads to increased expression of Runx3, which has been shown by ChIP-seq to physically bind the Cybb promoter. Given that Runx3 can function together with PU.1 to promote transcriptional activation of myeloid-specific genes in dendritic cells, our data are consistent with a model in which Rbpj-mediated suppression of Runx3 limits PU.1-dependent transcription of Nox2 subunits. While the complexity of Notch–Rbpj transcriptional regulation in macrophages will require further investigation, our findings collectively highlight the role of Rbpj in restraining the oxidative burst by repressing expression of Nox2 complex subunits.

Post-transcriptional regulation of the Nox2 complex occurs at multiple levels. After assembly of the cytosolic regulatory subunits with membrane-associated gp91phox and p22phox, catalytic activity of NADPH oxidase commences. Superoxide generation involves a two-step electron transfer reaction through cofactors bound to gp91phox. First, gp91phox binds NADPH, which donates an electron to FAD. In turn, FAD transfers an electron to haem and subsequently to molecular oxygen for production of superoxide radicals. Thus, metabolic regulation of cellular redox state and NADPH levels form a restriction point that controls the catalytic rate of the oxidative burst. Our metabolomic analyses reveal that the rate-limiting glycolytic enzyme Pfkl regulates NADPH oxidase activity by affecting the PPP and NADPH levels. In the absence of Pfkl, glucose is diverted towards the PPP, which along with controlling purine metabolism is the primary source for cellular NADPH. We observed increased flux through the PPP and augmented oxidative burst in macrophages lacking Pfkl. Consistent with this notion, phagocyte activation by pathogen-associated adaptations induced by pathogen exposure reinforces innate effector functions such as oxidative burst.

In addition to identifying mechanisms controlling oxidative burst at the level of Nox2 subunit transcription (Rbpj) and
catalytic function (Pfk), we identify Rnf145 as a novel E3 ubiquitin ligase that regulates gp91phox steady-state protein levels. This is the only E3 ligase that scored among more than a dozen that we screened. Very little is known about regulation and turnover of proteins within the Nox2 complex. Recent work identified gp91phox as a caspase 1 substrate that is cleaved after inflammasome activation; however, this was an inductive event that controlled the duration and amplitude of oxidative burst and had no effect on baseline proteostasis. More recently, gp91phox protein levels were shown to be regulated by Lrrc33, although a specific ubiquitin ligase targeting the Nox2 complex was not identified. Here we identify Rnf145 as required for ubiquitination of the Nox2 complex and precisely define ubiquitinated lysine residues in gp91phox and p22phox. Our data support a model in which Rnf145 controls steady-state levels of the Nox2 complex by ubiquitination. Importantly, the function of Rnf145 as a central ubiquitin ligase controlling oxidative burst has relevant implications for host defense. In fact, perturbation of this ubiquitin-cycling node by ablation of Rnf145 in Ncf4−/− macrophages restores oxidative burst to levels in Ncf4−/−-replete cells. This example of epistatic compensation highlights the potential value of identifying regulatory nodes as therapeutic targets.

Taken together, we report key insights from mining genes within GWAS candidate loci and performing genetic screens followed by deep functional analysis. In particular, this approach has proven fruitful in defining central regulatory nodes in the oxidative burst pathway.

**Methods**

**Primary screen and ROS assay.** The primary screen was performed in murine RAW264.7 cells (ATCC) that were seeded at a density of 1 × 10⁴ per 100 μl per well in 96-well, clear bottom, black wall plates. After 24 h, each well was transduced with 20 μl of shRNA lentivirus (The RNAi Consortium, Broad Institute) supplemented with 8μg ml⁻¹ polybrene (Sigma) and centrifuged at 1,150g 25°C for 30 min. The following day, media was exchanged and supplemented with 5μg ml⁻¹ puromycin (InvivoGen). Three days after selection, cell viability was approximated by Alamar Blue assay according to the manufacturer’s recommendation (Life Technologies). Cells were primed overnight with 10 ng ml⁻¹ murine IFNγ (Peprotech) prior to assaying oxidative burst. Media was exchanged for assay buffer (PBS, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 20 mM dextrose, 200 μM l-lysine, 25 mM L-arginine, 100 μg ml⁻¹ zymosan or heat killed S. aureus) and plates were briefly centrifuged to settle zymosan particles. Luminol-derived chemiluminescence was then monitored kinetic in a BioTek SynergyH4 microplate reader every 3 min for 30 min with temperature control at 37°C. Duplicate plates were designated for RNA extraction and validation of knockdown efficiency by qPCR. Where indicated, knockdown validation was performed by western blot with rabbit anti-Pfk1 (AV5774, Sigma, dilution 1:1,000) or rabbit anti-Rbpj (5313S, Cell Signaling Technology, dilution 1:1,000). Where indicated, RAW264.7 cells were transduced with lentivirus encoding murine Rnf145 (C-terminal V5 epitope tag), GFP or human NOTCH1 ICD within bicistronic IRES-GFP cassette of the lentiviral backbone CSCGW.

**Primary macrophage assays.** Murine BMDMs were seeded at 3.5 × 10⁵ cells per 50 μl per well in 96-well, clear bottom, black wall plates. Following 24–48 h in culture with granulocyte-macrophage colony-stimulating factor, cells were transduced with 50 μl of the indicated lentiviral shRNA and cultured for an additional 3 days, at which point media was supplemented with puromycin at a concentration of 4 μg ml⁻¹. After 2 days in selection, cells were primed with IFN-γ (25 ng ml⁻¹) or IL-4 (25 ng ml⁻¹) and stimulated with zymosan as described for the RAW264.7 screen.

**Bacterial killing assay.** RAW264.7 cells were plated at 5 × 10⁴ per well in 96-well plates in DMEM with 10% FBS overnight. S. aureus (Reynolds strain capsular serotype 5) was grown overnight at 37°C in Columbia media supplemented with 2% NaCl and sub-cultured the next day to the mid-exponential phase (OD₅₅₀ = 0.8 – 0.9). One hour before infection, media was replaced with DMEM with 1% FCS. S. aureus bacterial particles were disrupted by passing 10 to 15 times through a 30-G needle with a 1-ml syringe and were then added at a multiplicity of infection of 5. The plate was centrifuged for 5 min at 515g, 4°C and incubated at 37°C for 30 min to allow internalization of S. aureus by macrophages. Cells were washed three times with 200 μl Dulbecco’s phosphate-buffered saline (DPBS) to remove non-adherent cells and 200 μl of fresh media containing gentamicin (final concentration of 300 μg ml⁻¹) was added to killed extracellular bacteria. After 1 h of incubation, media was replaced with fresh DMEM containing 100 μg ml⁻¹ gentamicin. At indicated time points (time after internalization), supernatants were removed and cells were washed three times in 200 μl DPBS. Supematants in 200 μl of 0.02% Triton X-100, serial dilutions of the bacterial suspensions were plated on blood agar (Remel) and colonies were counted after overnight incubation at 37°C.

**Transcriptional readouts.** Knockdown of the indicated target genes in murine BMDMs was achieved as described above. Following selection and stimulation, total RNA was extracted (Qagen RNeasy Mini kit) and subjected to transcriptional profiling with the BioMark HD system (Fluidigm). In brief, primary mouse mRNA was reverse transcribed (qScript Supermix, VWR) prior to target-specific pre-amplification by standard PCR. Custom primers (Supplementary Data 2) specific for inflammatory genes were utilized for pre-amplification followed by qPCR ( EvaGreen Supermix, Bio-Rad).

**Metabolomics.** Endogenous, polar metabolite profiles were measured using two separate tri-phoric interaction liquid chromatography (HILIC) tandem MS methods. 199. Polar metabolites were extracted in the positive ion mode using a liquid chromatography (LC) system comprised of a 1,200 Series pump (Agilent Technologies) and an HTS PAL autosampler (Leap Technologies) that was coupled to a 4,000 QTRAP mass spectrometer (AB SCIEX) equipped with an electrospray ion source. Samples were prepared by drying 100 μl of cell extract under nitrogen and resuspending the residue in 10μl of 10/67.4/22.4/0.2 v/v/v/v water/acetonitrile/methanol/foric acid containing stable isotope-labelled internal standards (valine-d8, Sigma-Aldrich; and phenylalanine-d8, Cambridge Isotope Laboratories). The samples were centrifuged (10 min, 10,000 r.p.m., 4°C) and the supernatants were injected directly onto a 130 × 2.1 mm Atlantis HILIC column (Waters Corp.). The column was eluted isocratically with 5% mobile phase A (10 mM ammonium formate/0.1% formic acid) for 1 min followed by a linear gradient to 40% mobile phase B (acetonitrile/0.1% formic acid) over 10 min. The ion spray voltage was 4.5 kV and the source temperature was 425°C. All metabolites were measured using multiple reaction monitoring scans. MS settings, including declustering potentials and collision energies, for each metabolite were optimized by infusion of reference standards prior to sample analyses.

Analyses of polar metabolites using negative ion mode MS were performed using an ACQUITY UPLC (Waters Corp.) coupled to a 5,500 QTRAP triple quadrupole mass spectrometer. Cells extracts in 80% methanol were directly injected onto a 150 × 2.0 mm Luna NHZ column (Phenomenex) that was eluted at a flow rate of 400 μl min⁻¹ with initial conditions of 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide in water) and 90% mobile phase B (10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol) followed by a 10 min linear gradient to 100% mobile phase B. The ion spray voltage was 4.5kV and the source temperature was 500°C. All data were acquired using multiple reaction monitoring scanning.

MultiQuant software (version 2.1; AB SCIEX) was used to process all raw LC-MS data and integrate chromatographic peaks. The processed data were manually reviewed for quality of integration and compared against known standards to confirm metabolite identities.

Measurement of NADPH was performed by enzymatic cycling as described by the manufacturer (Abcam).

**Statistical analysis of metabolomics data.** Fold-change levels in metabolites were analysed by a two-tailed t-test assuming equal variance between two samples. Metabolites with fold change > 10% and Benjamini–Hochberg adjusted P value < 0.05 were considered significant for differential expression across wild-type and knockdown cells at baseline; each of the two knockdown samples were normalized to the two control samples independently and then assessed for...
differences in fold change. Differences at 4 h and 20 h were analysed separately.

**Ubiquitin proteomics.** Rb145 knockdown in RAW264.7 cells was performed as described above, with scale-up for 10 cm dishes. Cells were primed with IFN-γ (10 ng ml⁻¹ overnight, and where indicated stimulated with zymosan (50 μg ml⁻¹) for 30 min. Samples were processed at Cell Signaling Technology, Inc. using the Ubiscan proteomic platform as previously described40,41. Samples were washed once with cold PBS and lysed in urea lysis buffer (10 mM HEPES pH 8.0, 9.0 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate). Lysates were snap frozen on dry ice/ethanol until further processing. Cells were sonicated at 15 W output power twice for 20 s, and once for 15 s. The resulting sonicated lysates were centrifuged at 20,000 g for 15 min to remove insoluble material. The yielded protein extracts were reduced and carboxamidomethylated.

After normalizing total protein for each cell line (15 mg of total protein each), proteins were digested overnight with trypsin (Worthington). Resulting peptides were separated from non-peptide material by solid-phase extraction with Sep-Pak Classic C18 cartridges (Waters Corp.). Purified peptides were lyophilized for 3 days at 30 mtorr at −65 °C (Virtis). Lyophilized peptides were redissolved in IAP buffer (50 mM MOPS pH 7.2, 10 mM NaH₂PO₄, 50 mM NaCl), and ubiquitinated peptides were enriched by immunoaffinity purification using the Ubiquitin Remnant Motif antibody (#5562, Cell Signaling Technology) immobilized on Protein A agarose beads (Roche) following the standard protocol for immunoaffinity enrichment (http://www.cellsignal.com/services/pdfs/PTScan_Ubiquitomecks_System_Protocol.pdf). Peptides were eluted from antibody-resin into a total volume of 100 μl in 0.1% trifluoroacetic acid (TFA), and concentrated with C18 STAGE tips40.

**Mass spectrometric analysis.** The samples were run in duplicate to generate analytical replicates and increase the number of MS/MS identifications from each sample. Peptides were loaded directly onto a 10 cm × 75 μm PicoFrit capillary column packed with Magic C18 AQ reversed-phase resin. The column was developed with a 90-min linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nL min⁻¹. Tandem mass spectra were collected with an LTQ-Orbitrap VELOS hybrid mass spectrometer running XCalibur 2.0.7 SP1 using a top 20 method, a dynamic exclusion repeat count of 1 and a repeat duration of 30 s. Real-time recalibration of mass error was performed using lock mass with a singly charged polysiloxane ion (m/z = 371.101237). MS spectra were collected in the Orbitrap component of the mass spectrometer, and MS/MS spectra were collected in the LTQ.

**Data analysis.** Data processing was performed as described previously42. MS/MS spectra were processed using SEQUEST 3G and the SORCERER 2 platform from Sage-N Research (v4.0). Searches were performed against the Mus musculus NCBI database (6/28/2011). Reverse decoy databases were included for all searches to estimate false positive rates, and peptide assignments were obtained using a 5% false positive discovery rate in the Peptide Prophet module of SORCERER 2. Results were filtered using a mass accuracy of ± 5 p.p.m. for precursor ions and 1 Da for product ions. Enzyme specificity was limited to trypsin, with at least one K or R terminus required per peptide and up to four mis-cleavages allowed. Cysteine carboxamidomethylation was specified as a static modification, and methionine oxidation and di-glycine (lysine epsilon-gly-gly modification) was allowed. Results were further narrowed to peptides containing the di-glycine modification. The final false positive discovery rate on motif-containing peptides ranged from 2.0 to 2.6%.

**Label-free quantitation.** Label-free quantitation was performed using Progenesis V4.1 (Nonlinear Dynamics) combined with previously described peak detection software44,47–49. MS1 peak intensities for ubiquitinated peptides were analysed using a top 20 method, a dynamic exclusion repeat count of 1 and a repeat duration of 30 s. Retention time warping (or chromatographic alignment) was performed on Protein A agarose beads (Pierce) coupled to rabbit anti-gp91phox (ab129068, Abcam, 5 μg per sample). Western blots were performed as described above with anti-Ubiquitin K48 (128055, Cell Signaling Technology, dilution 1:1000).

**Microscopy.** IFN-γ-primed RAW264.7 cells were allowed to adhere to coverslips, then fixed in 4% paraformaldehyde for 15 min at 37 °C. Cells were then stained with mouse monoclonal anti-V5 (R960-25, Invitrogen dilution 1:250), polyclonal rabbit anti-ERK1/2 (ab292, dilution 1:250) and polyclonal rabbit anti-derlin-1 (8897S, Cell Signaling Technology, dilution 1:250) in diluting buffer (3% BSA, 0.1% saponin, PBS). Secondary staining was performed with goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen) and goat anti-mouse IgG Alexa Fluor 594 (Invitrogen). After thorough washing, cells were visualized using a laser-scanning confocal microscope (Leica SP5X) and analysed with Image software (NIH).

**CRISPR.** Guide RNAs targeting the mouse genome were selected from the CRISPR design tool and cloned into a pPHO lentiviral vector (a gift from B. Ebert, Harvard Medical School and Broad Institute)50. RAW264.7 or BMDMs were transduced with CRISPR lentivirus as described above, but with concentrated viral supernatant produced by centrifugal filtration (Millipore). NiF91/2 mice were a gift from P.T. Hawkins (Babraham Institute, Cambridge, UK).

**Animals.** All animal studies were conducted under protocols approved by the Subcommitte on Research Animal Care (SAC) at Massachusetts General Hospital. All mice have been backcrossed to the C57BL/6 background, and both sexes were utilized for bone marrow harvest at 8–12 weeks of age.

**References.**


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Author contribution
D.B.G., C.E.B., A.D., E.J.V. and A.M. performed the experiments. G.G., D.K. and A.C.Y.N. performed the computational analyses. J.G.D., D.E.R. and C.B.C. provided novel reagents and contributed to experimental design. D.B.G. and R.I.X. conceived the experiments, interpreted the data and wrote the manuscript.

Additional information
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