NALP3 inflammasome up-regulation and CASP1 cleavage of the glucocorticoid receptor causes glucocorticoid resistance in leukemia cells

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Abstract

Glucocorticoids are universally used in the treatment of acute lymphoblastic leukemia (ALL), and leukemia cell resistant to glucocorticoids confers a poor prognosis. To elucidate mechanisms of glucocorticoid resistance, we determined the sensitivity to prednisolone of primary leukemia cells from 444 newly diagnosed ALL patients, revealing significantly higher expression of *caspase 1 (CASP1)* and its activator *NLRP3* in glucocorticoid resistant leukemia cells, due to significantly lower somatic methylation of *CASP1* and *NLRP3* promoters. Over-expression of *CASP1* resulted in cleavage of the glucocorticoid receptor, diminished glucocorticoid-induced transcriptional response and increased glucocorticoid resistance. Knockdown or inhibition of *CASP1* significantly increased glucocorticoid receptor levels and mitigated glucocorticoid resistance in *CASP1* overexpressing ALL. Our findings establish a new mechanism by which the NLRP3/CASP1 inflammasome modulates cellular levels of the glucocorticoid receptor and diminishes cell sensitivity to glucocorticoids. The broad impact on glucocorticoid transcriptional response suggests this mechanism could also modify glucocorticoid effects in other diseases.

Glucocorticoids are steroid hormones that regulate multiple physiological processes involved in inflammation, immunity, metabolism and other homeostatic functions. They exert their effects by binding to the glucocorticoid receptor (GR, *NR3C1*), triggering its activation and translocation to the nucleus, leading to transcriptional changes responsible for diminished proliferative capacity and apoptosis of acute lymphoblastic leukemia (ALL) cells[1]. Synthetic glucocorticoids are widely prescribed medications used to treat a variety of human diseases with an inflammatory component (e.g., asthma and autoimmune disorders), and are an essential component of curative therapy of ALL and lymphomas. Advances in the treatment of children with ALL have led to five-year disease-free survival rates exceeding 85%[2]. However, children whose ALL cells show *in vitro* resistance to glucocorticoids have a significantly worse treatment outcome (disease-free survival) than patients whose ALL cells are sensitive to glucocorticoids[3–6]. Yet, relatively little is known about the mechanisms causing leukemia cells from some patients to exhibit *de novo* resistance to glucocorticoids or why leukemia cells are more resistant to glucocorticoids at the time of disease recurrence[5].

Here we report higher expression of two pro-inflammatory genes, *CASP1* and its activator *NLRP3* (NLR family, pyrin domain containing 3) in primary ALL cells that exhibited *de novo* resistance to glucocorticoids. We found that leukemia cells exhibiting higher expression of *CASP1* and *NLRP3* had significantly lower methylation of their promoter regions compared to glucocorticoid sensitive ALL. We show that *CASP1/NLRP3* overexpression induces glucocorticoid resistance via CASP1 cleavage of the glucocorticoid receptor in its transactivation domain, reducing cellular levels of functional glucocorticoid receptor and diminishing glucocorticoid transcriptional effects. We further show that enforced expression of a

glucocorticoid receptor that has been mutated to eliminate CASP1 cleavage sites mitigates glucocorticoid resistance due to CASP1 overexpression. Finally, we show that stably knocking down *CASP1* expression with shRNA or reducing CASP1 activity with an inhibitory protein (CrmA) in CASP1-overexpressing leukemia cells increases cellular glucocorticoid receptor levels and markedly increases sensitivity to glucocorticoids.

Results

Higher *CASP1, NLRP3* in glucocorticoid resistant leukemia

The *de novo* sensitivity of primary leukemia cells to prednisolone differed widely (>1000-fold) among patients in three independent cohorts of newly diagnosed children with ALL (Fig. 1A–C). We found that *CASP1* and *NLRP3,* both members of the NALP3 inflammasome, were the two most highly over-expressed genes sharing a common pathway in steroid resistant ALL cells (Fig. 1D–E, Supplementary Fig. 1). The mean expression of CASP1 in steroid resistant leukemia was 1.6-fold higher than in sensitive leukemia cells ($p = 3.2 \times 10^{-7}$; Fig. 1D), whereas the mean expression of *NLRP3* was 2.4-fold higher in prednisolone-resistant leukemia cells across all three cohorts of patients ($p = 3.5 \times 10^{-7}$; Fig. 1E).

Methylation of CASP1 and NLRP3 regulates their expression

To understand the basis for higher *CASP1* and *NLRP3* expression in glucocorticoid resistant leukemia cells, we assessed the relationship between *CASP1* and *NLRP3* mRNA expression and methylation of their promoter regions in leukemia cells. This revealed a highly significant relationship between the level of methylation of the *CASP1* promoter and *CASP1* mRNA expression in ALL cells ($p = 1.4 \times 10^{-22}$; Fig. 1F, Supplementary Fig. 2 panels A–C). In a subset of patients enrolled on St. Jude Protocol XVI where matching germline DNA from normal lymphocytes was available for methylation analysis (n = 55), *CASP1* promoter methylation did not differ significantly (Paired t-test p = 0.495, Supplementary Fig. 3) in lymphocyte germline DNA and leukemia cell DNA across the entire population. In contrast, of 10 patients with significantly lower *CASP1* promoter methylation in their ALL cells than their normal leukocytes, 70% were glucocorticoid resistant (n = 7), consistent with somatic *CASP1* demethylation in

glucocorticoid resistant ALL cells. Methylation of the promoter region of *NLRP3* was significantly greater in leukemia cell DNA than in germline leukocyte DNA (Paired t-test $p = 8.8 \times 10^{-11}$, Supplementary Fig. 3), and the level of *NLRP3* promoter methylation in leukemia cells correlated significantly with *NLRP3* expression in leukemia cells ($p = 6.7 \times 10^{-4}$; Fig. 1G, Supplementary Fig. 2 panels D–F). Categorization of patients using k-means clustering of *NLRP3* methylation and *CASP1* methylation levels significantly distinguished prednisolone-sensitive (blue symbols) from prednisolone-resistant (orange symbols) leukemia cells (Fisher's Exact test for count data $p = 4.7 \times 10^{-7}$; Fig. 1H).

CASP1 cleaves the glucocorticoid receptor

Based on a prior report of CASP1 cleavage of the androgen receptor[7], we identified a putative CASP1 cleavage site (LLID) in the transactivation domain of the glucocorticoid receptor (Fig. 2A, upper panel), and we performed enzymatic assays that confirmed glucocorticoid receptor cleavage by recombinant CASP1 (Fig. 2A, lower panel). Site directed mutagenesis of the LLID motif ablated cleavage of the glucocorticoid receptor at this location (Fig. 2B) and led to the identification of a secondary CASP1 cleavage site (IKQE) (Fig. 2A, top). CASP1-induced cleavage of the glucocorticoid receptor was inhibited by both tetrapeptide (Fig. 2A, lower panel) and small molecule inhibitors (VX765 and VRT043198) of CASP1[8] (Fig. 2C).

CASP1 over-expression increases glucocorticoid resistance

We expressed full length CASP1 in a human leukemia cell line (NALM-6) to determine whether CASP1 over-expression increases resistance to glucocorticoids (Fig. 2D. Under standard conditions for activation of the NALP3 inflammasome and CASP1[9–13], cells transduced with

empty vector control virus did not show differences in their sensitivity to prednisolone or dexamethasone, whereas cells transduced with *CASP1*-expressing virus had markedly higher LC_{50} for prednisolone and dexamethasone (Fig. 2E). On average, *CASP1* over-expression increased resistance to prednisolone by 16.9-fold (Student's t-test $p = 5.7\times10^{-6}$) and dexamethasone by 5.3-fold (Student's t-test $p = 1.2\times10^{-9}$), with a 35% reduction in glucocorticoid receptor levels by 24 hours (Fig 2E). This was validated in a different human ALL cell line (697), revealing a similar increase in resistance to glucocorticoids (Supplementary Fig. 4).

CASP1 blunts transcriptional response to glucocorticoids

Because glucocorticoids regulate expression of target genes by binding to the glucocorticoid receptor, triggering its translocation into the nucleus and association with a glucocorticoid response element (GRE) to increase or decrease gene transcription, we assessed the ability of glucocorticoids to influence global gene expression in the presence or absence of *CASP1* enforced expression. This revealed markedly diminished glucocorticoid-induced changes in gene expression levels in cells with *CASP1* enforced expression compared to controls (Fig. 3A, 3B). Likewise, induction of Bim protein levels, encoded by *BCL2L11*, a known glucocorticoid response gene, was markedly blunted in *CASP1* overexpressing cells (Fig. 3A inset). *BCL2L11* upregulation was also significantly blunted at the mRNA level in CASP1 overexpressing cells as compared to control (fold change = 1.39 ± 0.12 versus fold change = 1.78 ± 0.17, $p = 0.024$). Genome wide assessment of changes in gene expression after glucocorticoid treatment (results of 4 replicate experiments) identified 93 genes that were induced by at least 3-fold following glucocorticoid treatment of control cells, 64 (68%) of which had lower (by at least 25%)

induction in *CASP1* overexpressing cells. Among these genes, the mean induction in control cells was 8.4-fold, compared to only 4.4-fold in *CASP1* overexpressing cells (Paired t-test p = 0.01). Analysis of the sequences of these genes (including 5 kilobases up and downstream of each), showed a significant enrichment (46/93, 49%) of either known GREs (glucocorticoid receptor binding motif, Supplementary Table 1, motifs 1–9,[14–16]) or a genomic region shown by ChIP-seq experiments to bind the glucocorticoid receptor[14], as compared to only 28% (1409/5000) of the randomly selected unchanged genes (Fisher's Exact Test $p = 2.2 \times 10^{-5}$). Inclusion of an additional motif containing the top two most frequent bases at each position of a previously reported motif[16] (motif 10, Supplementary Table 1) increased the percentage of upregulated genes with a GRE to 62% (58/93) of the transactivated genes.

Of the 119 genes whose expression was down-regulated by at least 50% following glucocorticoid treatment of control cells, 98 (82%) of these 119 genes were down-regulated to a lesser extent (by at least 25%) in *CASP1* overexpressing cells. Among these genes, the mean reduction in control cells was 58%, compared to only 33% in *CASP1* overexpressing cells (Paired t-test $p < 2.2 \times 10^{-16}$). Analysis of the sequences of these genes, including 5 kilobases up and downstream, revealed a significant enrichment (29/119, 24%) of NF-κB binding motifs (a transcription factor known to interact with an activated glucocorticoid receptor, Supplementary Table 1, motif 11) or a genomic region shown by ChIP-seq to bind to the glucocorticoid receptor[14], as compared to randomly selected unchanged genes (849/5000, 17%; Fisher's Exact Test p = 0.048). The top 50 up-regulated and down-regulated genes in control cells and their changes in cells with *CASP1* enforced expression are depicted in Fig. 3, with the complete gene lists provided in the supplement (Supplementary Tables 2–4).

Additionally, CASP1 expression blunted genome-wide glucocorticoid receptor binding to DNA in response to prednisolone, as evidenced by ChIP-seq. The distribution of normalized read count ratios at all reproducible glucocorticoid receptor binding sites identified across control and CASP1 overexpressing cells showed a more than 4 fold enrichment of reads in control cells and 96% of all glucocorticoid receptor binding events displayed stronger read enrichments in control cells (Fig. 3C), in line with a lower level of functional glucocorticoid receptor from CASP1 over-expression. In support of these analyses, an assessment of identified binding sites revealed a marked overlap in glucocorticoid receptor binding events (Fig. 3E) between control and CASP1 overexpressing cells, and a 2.9 fold enrichment in the total number of binding sites was observed in control cells (10024 versus 3467 sites in control and CASP1 overexpressing cells, respectively). Notably, the top DNA-binding motif identified for control and CASP1 overexpressing cells (Fig. 3D) is consistent with the canonical glucocorticoid receptor binding motif. The extent of ChIP enrichment in control cells compared to CASP1 overexpressing cells is exemplified for four genes in Fig. 3F. Glucocorticoid receptor binding in CASP1 overexpressing cells consistently exhibit less ChIP enrichment compared to glucocorticoid receptor binding in control cells. Collectively, these data document a lower level of genome-wide glucocorticoid receptor occupancy in CASP1 overexpressing cells.

CASP1 and NLRP3 expression is higher in relapsed leukemia

To determine whether *CASP1* and *NLRP3* expression differed in leukemia cells at the time of disease recurrence (Fig. 4), we examined the mRNA expression levels of *CASP1* and *NLRP3* in paired ALL cells obtained at diagnosis and relapse from forty-nine patients[17]. This showed

significantly higher expression of both *CASP1* and *NLRP3* in ALL cells at the time of relapse when compared to the corresponding samples obtained from the same patients at diagnosis (Paired t-test $p = 3.2 \times 10^{-4}$ and 4.2×10^{-3} respectively). Likewise, in a separate cohort of patients, we found that ALL cells at the time of disease recurrence (relapse) were more resistant to glucocorticoids when compared to sensitivity at the time of initial diagnosis (Fig. 4C; $p = 9.72 \times 10^{-7}$).

CASP1 inhibition reverses glucocorticoid resistance

To determine whether inhibition of CASP1 could diminish CASP1-induced glucocorticoid receptor cleavage and increase sensitivity to glucocorticoid treatment, we first used shRNA to knockdown over-expressed CASP1. This revealed that knockdown of CASP1 expression by ~50% (Fig. 5A, shCASP1) reduced CASP1-induced glucocorticoid receptor cleavage (Fig. 5A, shCASP1), and markedly enhanced glucocorticoid sensitivity (prednisolone $LC_{50} = 12.8 \pm 4.7$ μM vs. 570 ± 423.2 μM, mean ± S.E.M., 44-fold reduction, t-test $p = 0.028$) (Fig. 5C, shCASP1) in CASP1 overexpressing ALL cells when compared to cells transduced with scrambled non-targeting shRNA (Fig. 5C, shNT).

Likewise when we transduced CASP1 overexpressing NALM-6 cells with the gene encoding CrmA (cytokine response modifier A), a known inhibitor of CASP1 catalytic activity[18–20], this blocked CASP1 induced glucocorticoid receptor cleavage (Fig. 5B) and markedly increased sensitivity to glucocorticoids (3.2 ± 0.2 μM vs. 137.3 ± 24.3 μM, mean ± S.E.M., 43-fold reduction in LC_{50}, t-test $p = 0.011$, Fig. 5D) when compared to GFP transfected controls.

We also over-expressed CrmA + RFP or RFP alone in glucocorticoid resistant primary leukemia cells that had a high level of CASP1 expression compared to several other glucocorticoid sensitive primary leukemia xenografts (not shown). This led to a diminution of the *de novo* glucocorticoid resistance (Supplementary Fig. 6A, dexamethasone LC_{50} = 0.14 μM (95% confidence interval = 0.1426×10^{-2} – 0.2773 μM versus > 10 μM, $p < 1 \times 10^{-14}$) and partial restoration of glucocorticoid receptor protein levels (Supplementary Fig. 6B). The levels of CASP1 expression in glucocorticoid-resistant primary ALL cells isolated from two patients (prednisolone LC_{50} = 1387 μM and 206.4 μM) were comparable to that of the glucocorticoid-resistant NALM-6 ALL cells in which heterologous CASP1 was over-expressed to recapitulate this mechanism of glucocorticoid-resistance (Supplementary Fig. 7).

Removal of CASP1 cleavage sites mitigates resistance

We overexpressed full length wild-type glucocorticoid receptor or a glucocorticoid receptor that had been mutated to eliminate the CASP1 cleavage sites (i.e., alanines substituted for the LLID and IKQE motifs identified in *in vitro* enzymatic assays), in leukemia cells overexpressing CASP1 in combination with either shRNA-based knockdown of overexpressed *CASP1* or non-targeting shRNA control. Enforced expression of wild-type glucocorticoid receptor was unable to reverse CASP1 induced glucocorticoid resistance, whereas expression of a glucocorticoid receptor without the CASP1 cleavage sites markedly attenuated CASP1 induced glucocorticoid resistance (9.4-fold reduction in LC_{50}), restoring sensitivity to levels similar to cells with shRNA-based knockdown of overexpressed CASP1 (Fig. 6).

Discussion

The present study has revealed a novel mechanism by which CASP1 and its activator NLRP3 modulate the biological and pharmacological effects of glucocorticoids via cleavage of the glucocorticoid receptor. Glucocorticoids mediate their effects by binding to the glucocorticoid receptor, causing it to translocate into the nucleus where it modulates the expression of genes that contain a GRE. Prior work has shown that low cellular levels of functional glucocorticoid receptor, due either to siRNA knockdown[14,21], rare mutations in the human *NR3C1* gene encoding the glucocorticoid receptor[22] or heterogeneity in the cellular levels of glucocorticoid receptor in leukemia cells by undefined mechanisms[23], decrease sensitivity to glucocorticoids. However, it was previously not known that CASP1 can cleave the glucocorticoid receptor and thereby reduce functional receptor levels and modulate cellular response to glucocorticoids.

We have demonstrated that recombinant CASP1 cleaves the glucocorticoid receptor in its transactivation domain, and that forced over-expression of *CASP1* coupled with its activation via the NLRP3 inflammasome causes human leukemia cells to become more resistant to glucocorticoids. Overexpression of CASP1 without its activation via the NLRP3 inflammasome did not alter glucocorticoid sensitivity of leukemia cells, demonstrating that catalytically active CASP1 is required for loss of glucocorticoid sensitivity. Further, overexpression of a glucocorticoid receptor lacking CASP1 cleavage sites mitigated the effects of CASP1 overexpression on leukemia cell sensitivity to glucocorticoids. Activation of the NALP3 inflammasome in the absence of CASP1 overexpression had only a small effect on glucocorticoid resistance, possibly due to activation of endogenous CASP1. Furthermore, our finding of higher expression of *CASP1* and *NLRP3* in leukemia cells at the time of disease

relapse provides a plausible mechanism for prior observations that leukemia cells at relapse are more resistant to prednisolone compared to leukemia cells at initial diagnosis[19]. In support of this, we observed markedly greater resistance to dexamethasone in ALL cells obtained from patients at the time of relapse. Likewise, using publically available data, we found that many glucocorticoid-resistant ALL cell lines[24,25] and primary ALL xenografts[26,27] express relatively high levels of CASP1 (supplemental Figure 8). Collectively, this indicates that although there are multiple mechanisms via which ALL cells can become resistant to glucocorticoids[28–30], CASP1 over-expression in glucocorticoid resistant ALL is not uncommon.

Importantly, when we knocked down CASP1 expression in CASP1 overexpressing cells, we were able to reverse CASP1-induced glucocorticoid receptor cleavage and markedly increase sensitivity to glucocorticoids, suggesting CASP1 inhibition as a potential new therapeutic strategy to mitigate glucocorticoid resistance. There have been small molecule CASP1 inhibitors in clinical development[31], although phase 2 clinical trials for epilepsy failed to show therapeutic benefit and were terminated[31]. The lack of clinical efficacy of this class of agents is consistent with their inability to reverse glucocorticoid resistance in our CASP1 overexpressing cells, which may be due to their poor penetration into cells (not shown). Our experiments showing that CrmA inhibition of CASP1 catalytic activity restores sensitivity to glucocorticoids in CASP1 overexpressing leukemia cells provide a proof of principle for this strategy and a strong rationale for future studies to identify small molecule CASP1 inhibitors that can achieve sufficient cellular concentrations to reverse glucocorticoid resistance in CASP1 overexpressing leukemia cells.

CASP1 belongs to a family of cysteine proteases that cleave proteins following an aspartic acid residue. Produced as a pro-enzyme, CASP1 requires removal of its caspase activation and recruitment domain (CARD) before it becomes an active enzyme[32]. CARD cleavage is mediated by the formation of inflammasomes, of which the most extensively characterized is the NALP3 inflammasome. NALP3 (encoded by *NLRP3*) can be activated by exposure to pathogen associated molecular pattern or damage associated molecular pattern molecules, or by whole pathogens or environmental irritants[32]. There is also emerging evidence that the NALP3 inflammasome can form in response to host-derived molecules, including extracellular ATP, glucose or monosodium urate crystals[10,32–34]. *In vivo* induction of the NALP3 inflammasome typically results in self-oligomerization, recruitment of the ASC (PYCARD) adaptor protein, and clustering and auto-activation of CASP1. CASP1 is known to have pro-inflammatory effects, including the activation of inflammatory cytokines (e.g. interleukin 1β and interleukin 18). Our findings raise the possibility that during inflammatory processes, CASP1 negatively regulates anti-inflammatory glucocorticoid signaling to further amplify the pro-inflammatory effects of the NALP3 inflammasome.

The significant effects we observed on glucocorticoid sensitivity by partial reduction of cellular glucocorticoid receptor levels is consistent with prior studies showing that glucocorticoid receptor (NR3C1) haploinsufficiency produces a number of phenotypes including familial glucocorticoid resistance[35] and adrenal hyperplasia[36], alters blood pressure, glucose and lipid homeostasis in glucocorticoid receptor heterozygous mice[37] and causes glucocorticoid resistance in Jurkat leukemia cells[38].

The fact that not all glucocorticoid responsive genes were affected by CASP1 overexpression likely reflects differences among genes in their sensitivity to activated glucocorticoid receptor as a transcription factor. Whether glucocorticoids cause transactivation or transrepression of a given gene is determined in part by the presence or absence of either a positive GRE, leading to transactivation or a negative GRE leading to transrepression. Glucocorticoids may also repress gene expression via binding of the activated glucocorticoid receptor to transcription factors such as NF-κB and AP1[39,40]. Although we found a significant enrichment of positive GREs among transactivated genes and negative GREs in transrepressed genes, failure to find GREs in all glucocorticoid-responsive genes likely reflects the current incomplete definition of GREs. Our ChIP-seq experiments showed that DNA binding by the glucocorticoid receptor is markedly diminished in CASP1 overexpressing cells. The broad spectrum of genes affected in CASP1 overexpressing cells indicates that our findings likely have broader implications, beyond the modulation of the anti-leukemic effects of glucocorticoids.

Taken together, our findings demonstrate a novel mechanism whereby leukemia cells develop resistance to glucocorticoids via somatic epigenetic changes that cause overexpression of *CASP1* and *NLRP3*, leading to enhanced CASP1-mediated cleavage of the glucocorticoid receptor and diminished cellular response to glucocorticoids. These findings provide the foundation for future efforts to identify small molecule inhibitors of CASP1 that can reverse this mechanism of glucocorticoid resistance and thereby improve the treatment of acute lymphoblastic leukemia and potentially other illnesses for which glucocorticoids are used therapeutically.

URLs

William E. Evans lab website: http://www.stjuderesearch.org/site/lab/evans/

Pediatric Preclinical Testing Program website: http://pptp.nchresearch.org/

NCI60 ALL cell line expression data from Chiron Pharmaceuticals:
https://wiki.nci.nih.gov/download/attachments/155845004/WEB_DATA_CHIRON.zip

Accession codes

DNA methylation, gene expression and ChiP-seq data available at GEO accession: GSE66708. Primary and relapse expression data available at GEO accession: GSE28460. NALM-6 expression data available at GEO accession: GSM852997.

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Author Contributions

S.W.P., E.J.B., D.S., L.B.R., W.E.T., P.G., R.K.S.M., M.A., A.M., J.M., D.R.C., L.T.L., Y.F., R.K.G., T.D.K., M.V.R. and W.E.E. designed experiments. C.H.P., S.J., M.V.R. and W.E.E. designed clinical trials. S.W.P., E.J.B., D.S., L.B.R., W.E.T., P.G., R.K.S.M., M.A., A.M., D.R.C., L.T.L., Y.F., A.Z., A.G., D.C., J.J.B., L.H. performed experiments. S.W.P., E.J.B., D.S., L.B.R., W.E.T. and W.E.E wrote the manuscript (reviewed by all authors). S.W.P., E.J.B., D.S., L.B.R., W.E.T., D.R.C., L.T.L., J.C.P., J.R.M., Y.F., K.R.C., G.S., M.R.W., A.M.F., C.C., W.Y., S.E.K., C.A.F., B.D., C.S., J.K.H., A.Z., A.G., D.C., J.J.B. L.H., C.G.M., M.L.d.B., R.P., S.J., T.L.D., F.L., D.B., W.L.C., C.H.P., R.M.M., R.K.G., T.D.K., M.V.R. and W.E.E. analyzed data.

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Figure Legends

Figure 1

Glucocorticoid resistant leukemia cells have higher expression and hypo-methylation of *CASP1* and *NLRP3* genes

Primary leukemia cells were obtained from 444 patients (B and T cell leukemia) with newly diagnosed acute lymphoblastic leukemia and analyzed for their sensitivity to prednisolone using the MTT assay (see Methods)[28]. Distributions of measured LC_{50} values are shown for the three independent cohorts of patients; sensitive and resistant leukemias are highlighted in blue and orange, respectively (panels A–C). *CASP1* (panel D) and *NLRP3* (panel E) expression was significantly higher in glucocorticoid resistant leukemia cells from these three cohorts of newly diagnosed patients with B-lineage leukemia. In both patient cohorts for whom DNA was available for DNA methylation analysis (St. Jude Protocols XV and XVI), significantly lower levels of *CASP1* (panel F) and *NLRP3* (panel G) methylation were found in leukemia cells (from patients with B lineage leukemia) with higher expression of *CASP1* and *NLRP3*. k-means clustering analysis (▲ represents k-means identified group A, ● represents k-means identified group B, pink and red squares represent k-means identified centers for group A and B respectively) utilizing only *CASP1* and *NLRP3* methylation status significantly discriminated sensitive leukemias (blue symbols, higher methylation) from resistant leukemias (orange symbols, lower methylation) in both St. Jude Protocol XV and XVI (panel H and supplementary Fig. 2) patients. Welch's t-test p-values are shown for panels 1D–G and Fisher's Exact test p-value is shown for panel H. Boxes and whiskers are as defined in Online Methods.

Figure 2

CASP1 cleaves the glucocorticoid receptor and increases resistance to glucocorticoids

Bioinformatic analysis of the glucocorticoid receptor amino acid sequence (panel A, top) revealed a putative CASP1 cleavage site (LLID) in the glucocorticoid receptor (NR3C1) transactivation domain that is similar to a previously reported CASP1 cleavage site in the androgen receptor. Enzymatic assays revealed that recombinant CASP1 cleaves the glucocorticoid receptor and that this cleavage was inhibited by a CASP1 tetrapeptide inhibitor (panel A, bottom). Site directed mutagenesis of NR3C1 at the putative cleavage site (LLID motif) blocked CASP1 cleavage at this location and revealed a secondary CASP1 cleavage site more proximal to the carboxy terminus of the protein, as evidenced by a smaller enzymatic product (panel B). Further inhibition studies showed that small molecule inhibitors VX-765 and VRT-043198 (panel C) can also inhibit recombinant CASP1 cleavage of NR3C1, with VRT-043198 having higher inhibitory activity. Enforced expression of CASP1 in a human leukemia cell line (NALM-6) increased resistance to prednisolone and dexamethasone after activation of the NALP3 inflammasome (by the addition of LPS and ATP). NALM-6 cells were transduced with a lentivirus containing full length CASP1 and puromycin *N*-acetyl-transferase or puromycin *N*-acetyl-transferase alone (Control). Cells were puromycin selected and then their sensitivity to prednisolone and dexamethasone (panel E, data is representative of four independent experiments with standard deviation error bars) was measured using the MTT assay, in the presence(+) and absence(-) of inflammasome activation (LPS/ATP). The panel E inset is a western blot depicting the lower glucocorticoid receptor protein levels in cells over expressing *CASP1* (24 hours after activation of CASP1).

Figure 3

Transcriptional modulation induced by glucocorticoids is diminished by CASP1

Genome-wide gene expression was measured (n = 4) with median values shown (panels A and B, error bars depict median absolute deviation) and fold-differences (with or without prednisolone treatment) were compared between control cells treated with prednisolone and the same cells not treated with prednisolone or in cells with enforced expression and activation of CASP1 treated with prednisolone or not. The top 50 genes activated (blue bars) and repressed (green bars) by prednisolone in untreated control cells are shown, with the fold-change in *CASP1* overexpressing cells shown by the adjacent gold bars. Additionally, control and *CASP1* overexpressing cells were treated with and without prednisolone and LPS/ATP and protein lysates were collected for determination of Bim protein levels by western blotting, a known glucocorticoid response gene (panel A inset). The histogram (panel C) depicts the distribution of average normalized read count ratios at all reproducible glucocorticoid receptor binding sites identified across control and CASP1 overexpressing cells after prednisolone treatment. Binding sites with values above 0 highlight stronger enrichment in control cells while ratio values less than 0 depict sites with stronger enrichment in CASP1 overexpressing cells. The top binding motif identified (Panel D) is consistent with the canonical glucocorticoid receptor motif in both control and CASP1 overexpressing cells. The number and the extent of overlap of glucocorticoid receptor binding sites found is given in Panel E. ChIP enrichments near prednisolone-responsive genes are shown for 4 genes in Panel F. The images display ChIP enrichment in control cells (upper) and CASP1 overexpressing cells (lower).

Figure 4

***CASP1* and *NLRP3* expression is higher and glucocorticoid resistance is greater in leukemia cells at the time of disease relapse**

Expression levels of *CASP1* (panel A) and *NLRP3* (panel B) in leukemia cells obtained from 49 patients at diagnosis and at the time of disease relapse. Quantile normalized measures of gene expression revealed significantly higher expression of *NLRP3* and *CASP1* at relapse. Paired t-test values are shown for panels A and B. Panel C depicts dexamethasone sensitivity (LC_{50}) for 7 paired leukemia (ALL) cells obtained at diagnosis and at relapse from the same patients (connecting lines). Boxes and whiskers are as defined in Online Methods.

Figure 5

Knockdown or inhibition of CASP1 reverses glucocorticoid receptor cleavage and prednisolone resistance

NALM-6 cells overexpressing CASP1 were transduced with lentiviral vectors containing non-targeting scrambled hairpin (shNT) or shRNA hairpins targeting *CASP1* (shCASP1). The two cell lines were cultured for 48 hours in the presence and absence of LPS/ATP, and analyzed on Western blot for the levels of CASP1 and glucocorticoid receptor (Panel A). The intensities of the glucocorticoid receptor and active CASP1 normalized against HSP90, are shown in Panel A. After activation of CASP1 (Panel A, lane 2 and 4), glucocorticoid receptor is approximately 2-fold lower in scrambled hairpin cells (Panel A, lane 4) whereas shCASP1 cells showed minimal degradation of the glucocorticoid receptor (Panel A, lane 2).

The prednisolone sensitivity (LC_{50}) of shCASP1 and shNT cells was determined by MTT assays (Panel C). In the absence of CASP1 activation (– LPS/ATP), the PRED-LC_{50} of shCASP1 and shNT cells were comparable (Panel B). Upon activation of CASP1, shCASP1 cells were 44-fold

more sensitive to prednisolone compared to shNT cells (Panel C). Error bars represent S.E.M, n = 4. Transduction of CrmA in *CASP1* overexpressing cells showed effects similar to knockdown of *CASP1* by shRNA. Without CASP1 activation, the PRED-LC_{50} for CrmA-expressing cells and GFP-expressing cells was comparable (Panel D). However, upon activation of CASP1, CrmA blocked CASP1 induced glucocorticoid receptor cleavage (Panel B) and increased glucocorticoid sensitivity by 43-fold compared to the GFP expressing control cells (Panel D). Error bars are as defined for Panel C.

Figure 6

Expression of glucocorticoid receptor with both CASP1 cleavage sites mutated to AAAA mitigates CASP1 induced prednisolone resistance

NALM-6 cells overexpressing CASP1 co-transfected with either non-targeting scrambled hairpin (shNT) or shRNA hairpins targeting CASP1 (shCASP1) were stably transduced with lentiviral vectors containing a wild-type glucocorticoid receptor or a glucocorticoid receptor in which both CASP1 cleavage sites had been eliminated (double mutagenized glucocorticoid receptor, DM-GR). The sensitivity (LC_{50}) of these cells to prednisolone was determined by MTT assays, revealing that cells overexpressing a glucocorticoid receptor without CASP1 cleavage sites remained sensitive to glucocorticoids when CASP1 was over-expressed. The inset is a western blot depicting recombinant glucocorticoid receptor protein levels (wild-type, or double mutagenized), in cells at time of MTT drug sensitivity assays. Data are representative of three independent experiments with standard deviation bars.

Online Methods

Patients

A total of 444 children aged 18 years or younger with newly diagnosed ALL were included in this study. Two hundred seventy one were enrolled on the St. Jude Total Therapy XV or XVI protocols; 173 were enrolled on the 9th ALL Dutch Childhood Oncology Group protocol at Erasmus Medical Center, Sophia Children's Hospital, in Rotterdam, The Netherlands or on treatment protocols 92 and 97 of the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia in Hamburg, Germany. Patients were enrolled in these studies if they had a diagnosis of ALL, were younger than 18 years of age, and had not been previously treated for ALL. In an additional cohort of 49 pediatric patients with relapsed B-precursor ALL, cryopreserved bone marrow samples from diagnosis and relapse from the Children's Oncology Group (COG) were subjected to gene expression analysis as previously described.[17] All patients in the relapsed cohort had a bone marrow or bone marrow/central nervous system combined relapse and had been initially treated on COG protocols for newly diagnosed ALL[17]. In a separate cohort of patients enrolled at St. Jude Children's Research Hospital, dexamethasone LC_{50} was measured at time of diagnosis and relapse. Written informed consent was obtained from all patients or their parents/guardians, and assent from the patients, as appropriate. The research and use of these samples were approved by the institutional review board at the host institution.

Gene expression analyses

Total RNA was extracted with TriReagent (Molecular Research Center, Inc.) from freshly isolated or cryopreserved mononuclear cell suspensions from patient bone marrow aspirates

obtained at diagnosis. In the COG relapsed cohort, an RNA sample was extracted from leukemia cells from patient bone marrow aspirates obtained at the time of original diagnosis and again at the time of disease recurrence. All gene expression microarrays were performed by the St. Jude Children's Research Hospital, Hartwell Center for Bioinformatics & Biotechnology. High-quality RNA was hybridized to the HG-U133A (GPL96) or HG-U133 Plus 2.0 (GPL570) oligonucleotide microarrays in accordance with the manufacturer's protocol (Affymetrix). These microarrays contain 22,283 or 54,675 gene probe sets, representing approximately 18,400 or 47,400 human transcripts, respectively. Gene expression data were MAS5 [41] processed using the affy[42] Bioconductor[43] R-project package or using Affymetrix Microarray Suite version 5.0[44,45] as previously described[28].

DNA methylation analyses

DNA was isolated at the time of diagnosis from leukemia cells obtained from patients via bone marrow aspirates. Genome-wide DNA methylation status was determined using either an Infinium HumanMethylation27 BeadChip Kit or Infinium HumanMethylation450 BeadChip Kit in accordance with the manufacturer's protocol (Illumina). HumanMethylation27 BeadChip experiments were performed at either Emory Integrated Genomics Core (EIGC) or Wellcome Trust Centre for Human Genetics Genomics Lab, Oxford, UK. HumanMethylation450 BeadChip experiments were performed at the Heflin Center for Genomic Science at the University of Alabama at Birmingham. DNA methylation status was classified as low if the Beta value[46] was less than or equal to 0.25 and high if greater than 0.25.

Statistical analyses

Analyses were performed using R software unless otherwise specified. Exact Wilcoxon Mann-Whitney Rank Sum tests were used for analyses of differential gene expression and differential DNA methylation and Stouffer's Z-score method was used for meta-analysis47. For boxplots, the upper and lower value of each box depicts the 75th and 25th percentile respectively, the solid line depicts the group median and the top and bottom of each dashed vertical line depicts the most extreme data point which is no more than 1.5 times the interquartile range (75th percentile – 25th percentile) from the box. K-means clustering analysis with a k = 2 was used for DNA methylation data and either untransformed or log-transformed gene expression data, with Fisher's exact test for determining clustering significance. Fisher's exact test was used to assess the significance of enrichment of known GREs.

CASP1 enzymatic assays

Recombinant human CASP1 (100–200 U, where U = 1 pmol/min at 30°C, 200 μM YVAD-pNA) from a CASP1 assay kit for drug discovery (Enzo Life Sciences, catalog number BML-AK701-0001) was incubated at 30°C with wild-type or mutated glucocorticoid receptor in the presence (10 μM unless otherwise indicated) or absence of inhibitors, (Ac-YVAD-CHO, Enzo Life Sciences, catalog number BML-P403-9090), VX765, VRT-043198. Substrates for enzyme assays were prepared in CASP1 assay buffer (Enzo Life Sciences, catalog number KI-111) consisting of 50 mM HEPES, pH7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA and 10% glycerol.

Site-directed mutagenesis

Site-directed mutagenesis was performed on *NR3C1* using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutagenesis of the LLID motif was performed in 4 sequential mutagenesis reactions from Myc-DDK tagged *NR3C1* (Origene, Rockville, MD, catalog # RC220189). The following mutagenesis primers were used: MutF1-4-NR3C1 and MutR1-4-NR3C1 (supplemental table 5). Mutagenesis of the IKQE motif was performed in 2 sequential mutagenesis reactions from the LLID mutated NR3C1. The following mutagenesis primers were used: MutF5-6-NR3C1 and MutR5-6-NR3C1 (supplemental table 5).

Successful mutagenesis was confirmed by Sanger sequencing.

Sensitivity of leukemia cells to glucocorticoids

Leukemia cells were isolated at diagnosis from patient bone marrow aspirates. If the leukemia cell percentage from diagnostic bone marrow samples was less than ninety percent, magnetic activated cell sorting (Miltenyl Biotec) was performed to further enrich leukemia cells. If red cell contamination was greater than thirty percent, red blood cell lysis was performed. Cells were centrifuged at 300g for five minutes and resuspended in RPMI-1640 with 2 mM L-glutamine, 20% heat-inactivated fetal bovine serum, 1X Antibiotic-Antimycotic solution (Life Technologies) and 1X ITS solution (Life Technologies) at a concentration of two million cells per milliliter. Eighty microliters of this leukemia cell suspension was then plated into each experimental well of round-bottom 96-well plates. Twenty microliters of decreasing concentrations of prednisolone were added and the plates were incubated for ninety-six hours in a humidified incubator containing 5% CO_2 at 37°C. For the final six hours, ten microliters of 5 milligram per milliliter MTT (3-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) was

added to each experimental well. Drug resistance assays in NALM-6 and 697 cell lines (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were performed using between 0.25 and 2.5 million cells per milliliter. Assays were developed and performed as described for primary ALL cells. LC_{50} values were determined for patients at diagnosis or replapse as previously described[28], and consistent with prior analyses, glucocorticoid resistant ALL was defined as having an LC_{50} of 64 μM or greater, whereas glucocorticoid sensitive cases were defined as having an LC_{50} less than 0.1 μM. Among patients treated on the Total XV protocol (the only uniformly treated cohort with sufficient follow-up to assess event free survival), those patients whose samples were classified as sensitive to prednisolone (LC_{50} < 0.1 μM) had a significantly better treatment outcome than the other patients, (5 year event free survival 95.6% vs. 82.7%, $p = 0.01$ logrank test).

CASP1 enforced expression

Full length *CASP1* cDNA (Origene, catalog # RC218364) was subcloned into a lentiviral backbone (System Biosciences, catalog number CD527A-1), in frame with a T2A linked puromycin resistance gene. Lentivirus was produced in 293T cells and the NALM-6 leukemia cell line was transduced. Seventy-two hours post transduction, cells were selected with 2.5 micrograms per milliliter puromycin. Where indicated, cells were treated with 10 μg/mL LPS (InvivoGen) followed 2 hours later by 5 mM ATP (Roche). NALM-6 and 697 cell lines were routinely authenticated and screened for mycoplasma.

Expression of mutant glucocorticoid receptor without CASP1 cleavage sites

Doubly mutated *NR3C1* (LLID and IKQE) was subcloned into the pLX304[48] lentiviral backbone (Addgene plasmid 25890). Lentivirus was produced in 293T cells and NALM-6 leukemia cell lines with *CASP1* overexpression and shRNA directed towards *CASP1* or a non-targeting shRNA were transduced. Seventy-two hours post transduction, cells were selected with blasticidin.

Expression of GFP and CrmA

Green fluorescent protein (Addgene plasmid 15301)[49] or CrmA encoding cDNAs (Addgene plasmid 11832)[50] was subcloned into the pLX304[48] lentiviral backbone (Addgene plasmid 25890). Lentivirus was produced in 293T cells and NALM-6 leukemia cell lines with CASP1 overexpression and shRNA directed towards CASP1 or a non-targeting shRNA were transduced. Seventy-two hours post transduction, cells were selected with blasticidin.

Western blotting

In brief, cells were pelleted by centrifugation, washed once with PBS and lysed with RIPA buffer or CASP1 assay buffer (Enzo Life Sciences, catalog number KI-111), equal amounts of proteins (1–20 μg) separated by 4–12% Novex Bis-Tris gels (Life Technologies), and then transblotted to PVDF membranes (Life Technologies). Anti-glucocorticoid receptor (1:1000–1:10,000, BD catalog number 611227), anti-CASP1 p20 (Adipogen catalog number AG-20B-0048), anti-DDK tubulin (1:1000, Origene catalog number TA50011), anti-Bim (1:1000, Cell Signaling catalog number 2819) anti-Tubulin (1:1000, Santa Cruz, sc-8035), or anti-HSP90 (1:1000, Cell Signaling catalog number 4874) were used as primary antibodies followed by appropriate secondary HRP-conjugated IgG (1:1000, Dako) and immunocomplexes were visualized by chemiluminescence using a Chemidoc Imager (BioRad). Signal intensities of

CASP1 and glucocorticoid receptor where quantified using Quantity One Software (BioRad) and normalized for the signal intensity of the loading control in the same lane.

Glucocorticoid induced changes in gene expression

NALM-6 transduced with either empty vector (control) or *CASP1* containing lentiviral particles were treated with or without LPS/ATP, with or without 0.3 mM prednisolone, for 24 hours. RNA was extracted and hybridized to Affymetrix PrimeView oligonucleotide microarrays and RMA processed.

To be included as a gene that was transactivated by glucocorticoids, the median level of mRNA expression from 4 replicate experiments had to increase in control cells by at least 3-fold. To be included as a gene that was transrepressed by glucocorticoids, the median level of mRNA expression had to decrease by at least 50%.

Chromatin immunoprecipitation sequencing

NALM-6 transduced with either empty vector (control) or *CASP1* containing lentiviral particles were treated with or without LPS/ATP, and with or without 0.3 mM prednisolone for 24 hours. Chromatin immunoprecipation sequencing (ChIP-seq) was performed with an antibody to the glucocorticoid receptor (Santa Cruz sc-1003), as previously described[51]. Briefly, cells were cross-linked with formaldehyde (1% final concentration) for 10 minutes at room temperature and then incubated with glycine (0.125 M final concentration) for 5 minutes to stop the cross-linking reaction. Cells were pelleted and washed with 1X PBS containing protease inhibitors (Roche Protease Inhibitor Cocktail Tablets, 11836153001) and stored at –80C prior to further

experimentation. Crosslinked cell pellets were resuspended in cold lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl and 0.5% NP-40 solution) containing protease inhibitors (Roche Protease Inhibitor Cocktail Tablets, 11836153001), and pelleted to isolate nuclei. The pellet was resuspended in cold RIPA lysis buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS solution). Sonication was performed using a Bioruptor sonication system (Diagenode). Sonicated chromatin was added to Dynabeads (Invitrogen) coated with anti-glucocorticoid receptor antibodies (sc-1003) and incubated overnight at 4°C. The solution was washed with a LiCl wash solution (100 mM Tris pH 7.5, 500 mM LiCl, 1% NP-40 and 1% sodium deoxycholate) at $4^{o}C$, and a final wash using 1X TE buffer. Dynabeads were resuspended in an elution buffer (1% SDS and 0.1 M $NaHCO_3$) and placed at 65°C to reverse crosslinks. DNA was purified and a standard library preparation was performed prior to sequencing on an Illumina HiSeq 2000 sequencer. Glucocorticoid receptor binding sites were identified using the MACS peak caller[52].

ChIP-seq analyses

Reproducible glucocorticoid receptor ChIP-seq binding events were identified by a comparison of glucocorticoid receptor genome binding coordinates across replicate ChIP-seq experiments. Normalized read counts were assessed by compiling all reproducible binding events from control and CASP1 overexpressing cells (unique and common across both cells) and determining the number of reads, normalized to the total number of aligned reads per ChIP experiment, mapping to a 100-bp fragment of DNA centered on ChIP-seq binding site summits for all ChIP-seq replicate experiments. The average normalized read counts for each cell was determined and used to determine the ratio of read counts between control and CASP1 overexpressing cells. The

MEME program was used to identify enriched motifs at glucocorticoid receptor binding sites[53]. Normalized read count ratios are defined as the control divided by CASP1 overexpressing cell next-generation sequencing reads and a reproducible peak was defined as being identified by both replicate ChIP-seq experiments in each cell.

Expression of CASP1 and NLRP3 in leukemia cells at the time of disease relapse versus at diagnosis

Gene expression was assessed at diagnosis and relapse as previously described[17]. This dataset was MAS5[41] processed using the affy[42] Bioconductor[43] R-project package and then quantile normalized. A paired t-test was used for comparison of the matching diagnosis and relapse data. In a separate cohort of patients enrolled at St. Jude Children's Research Hospital, dexamethasone LC_{50} was measured at time of diagnosis or relapse.

Expression analysis of glucocorticoid sensitive and resistant xenograft ALLs

Affmetrix U133Plus2 expression data of primary ALL cells expanded as xenografts in mice were downloaded from the Pediatric Preclinical Testing Program, and MAS5 processed using the affy[42] Bioconductor[43] R-project package. Expression data was $\log_2$ transformed, and these ALL xenografts were categorized as either dexamethasone sensitive or dexamethasome resistant on the basis of their *in vivo* response to glucocorticoids[26,27]. Those xenografts determined to exhibit partial (intermediate) or complete resistance *in vivo* were categorized as resistant, and those that were sensitive *in vivo* were categorized as sensitive[26,27] .

Expression analysis of glucocorticoid-sensitive and resistant NCI60 ALL cell lines

Expression data of the NCI60 ALL cell lines (Chiron Pharmaceuticals) were constant translated (add 0.1) to eliminate zero values prior to $\log_2$ transformation and combined with expression data from xenografts (see Supplementary Fig. 7) measured from RNA isolated at diagnosis. Additionally, expression data from NALM-6 cells were obtained and combined with this dataset. Data were median centered and scaled by dividing by the intrasample standard deviation, followed by quantile normalization. NCI60 drug sensitivity measurements showed that all cells lines in the leukemia panel, except CCRF−CEM, were prednisolone resistant (accession number: NSC 19987, September 2014 release).

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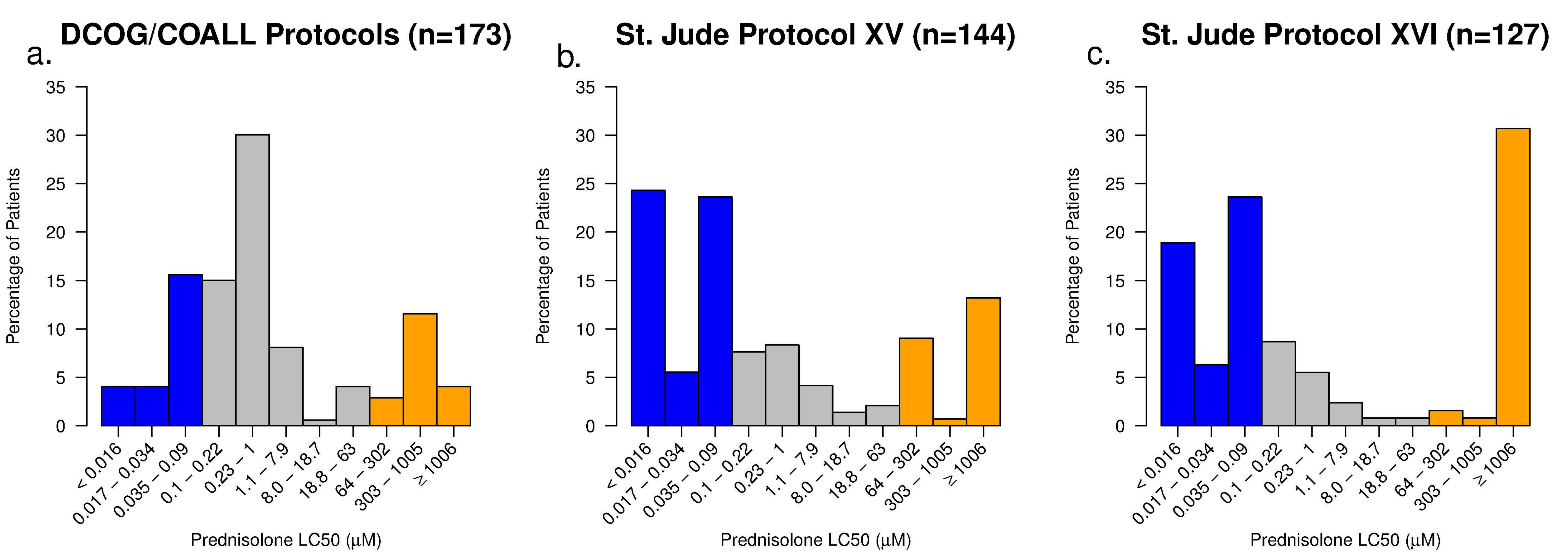
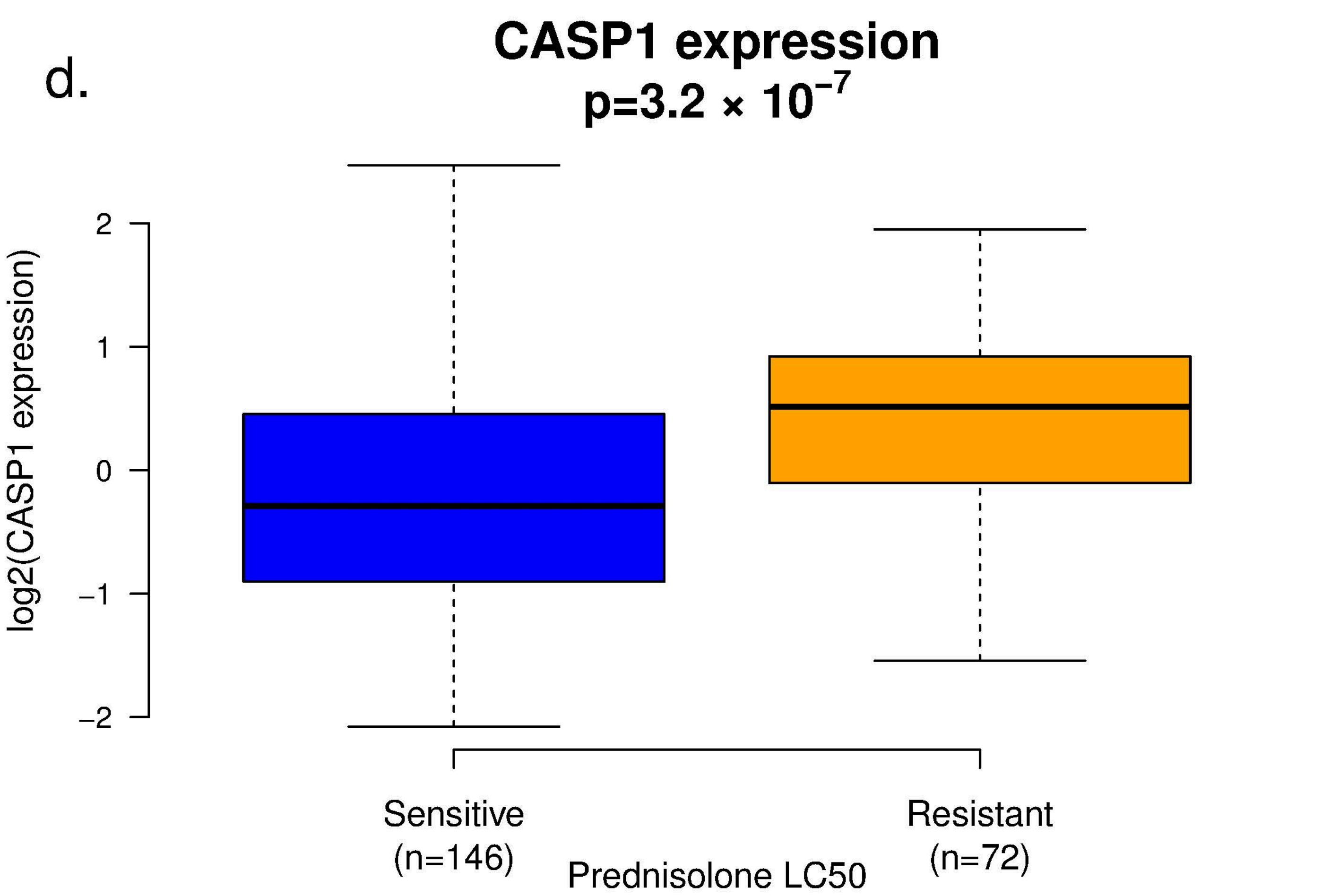
Competing Interests

Authors William E. Evans, Steven W. Paugh and Erik J. Bonten are named as co-inventors on a pending patent application that relates to the subject matter of the article, which was filed by St. Jude Children's Research Hospital.

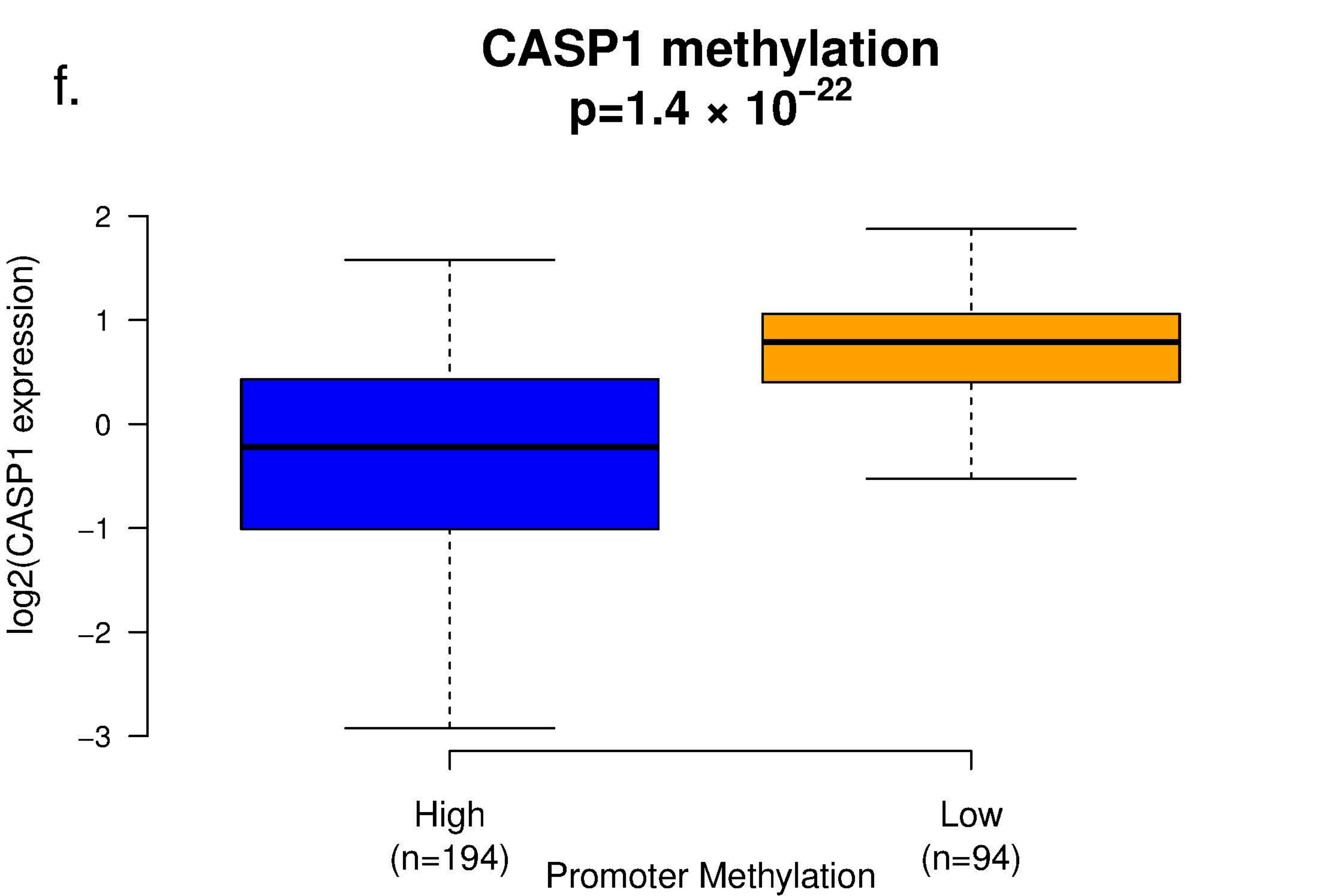


a. DCOG/COALL Protocols (n=173)
b. St. Jude Protocol XV (n=144)
c. St. Jude Protocol XVI (n=127)
Percentage of Patients
35
30
25
20
15
10
5
0
< 0.016
0.017 – 0.034
0.035 – 0.09
0.1 – 0.22
0.23 – 1
1.1 – 7.9
8.0 – 18.7
18.8 – 63
64 – 302
303 – 1005
≥1006
Prednisolone LC50 (μM)



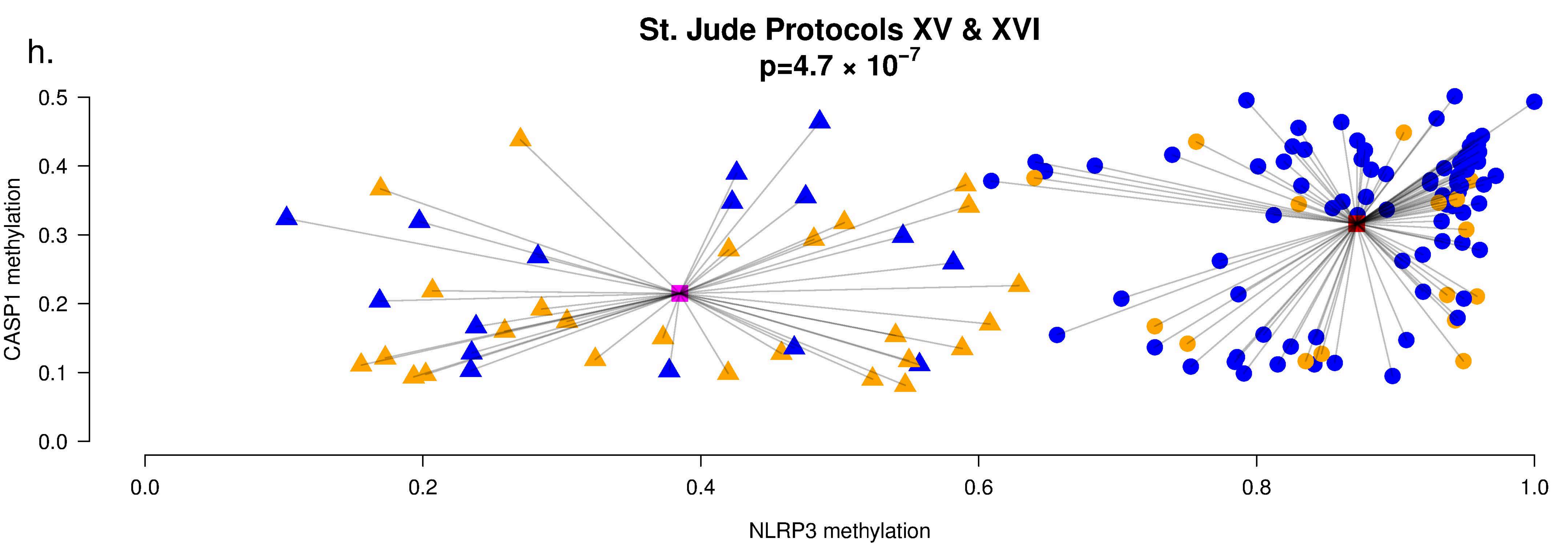
d.
CASP1 expression
p=3.2 × 10^−7
log2(CASP1 expression)
2
1
0
−1
−2
Sensitive (n=146)
Resistant (n=72)
Prednisolone LC50

e.
NLRP3 expression
p=3.5 × 10^−7
log2(NLRP3 expression)
2
1
0
−1
−2
−3
Sensitive (n=146)
Resistant (n=72)
Prednisolone LC50



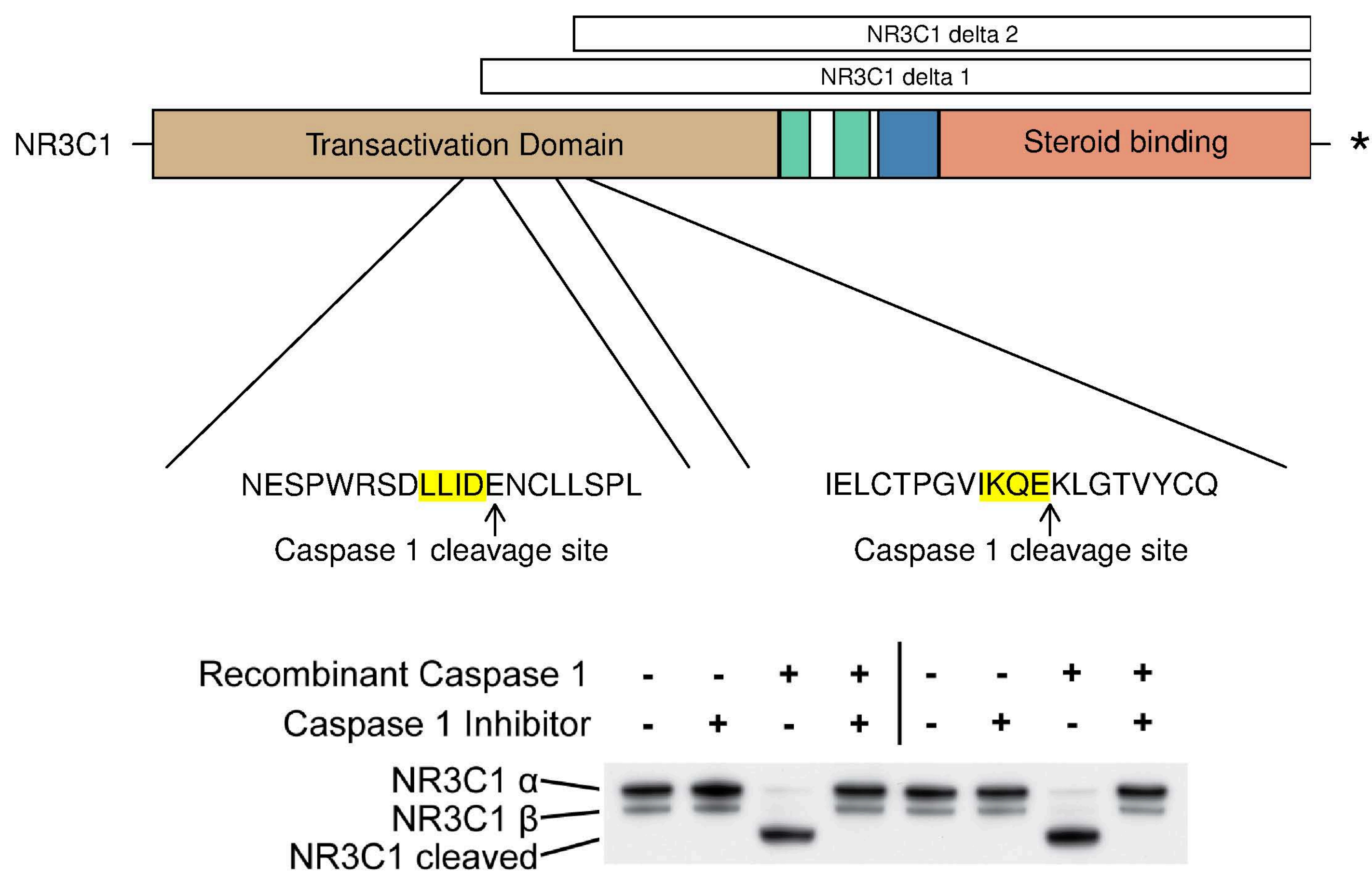
f.
CASP1 methylation
p=1.4 × 10^−22
log2(CASP1 expression)
2
1
0
−1
−2
−3
High (n=194)
Low (n=94)
Promoter Methylation

g.
NLRP3 methylation
p=6.7 × 10^−4
log2(NLRP3 expression)
2
1
0
−1
−2
−3
High (n=267)
Low (n=20)
Promoter Methylation

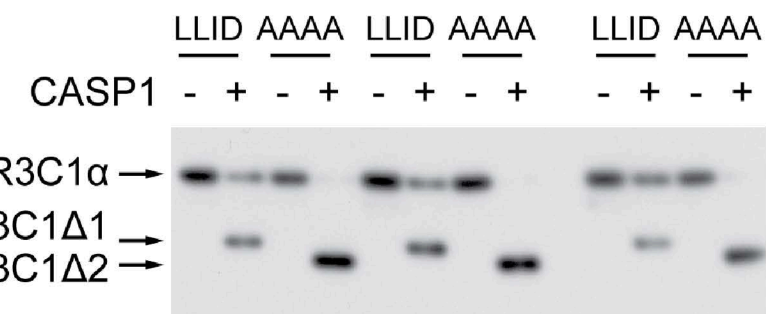


h.
St. Jude Protocols XV & XVI
p=4.7 × 10^−7
CASP1 methylation
0.5
0.4
0.3
0.2
0.1
0.0
0.2
0.4
0.6
0.8
1.0
NLRP3 methylation

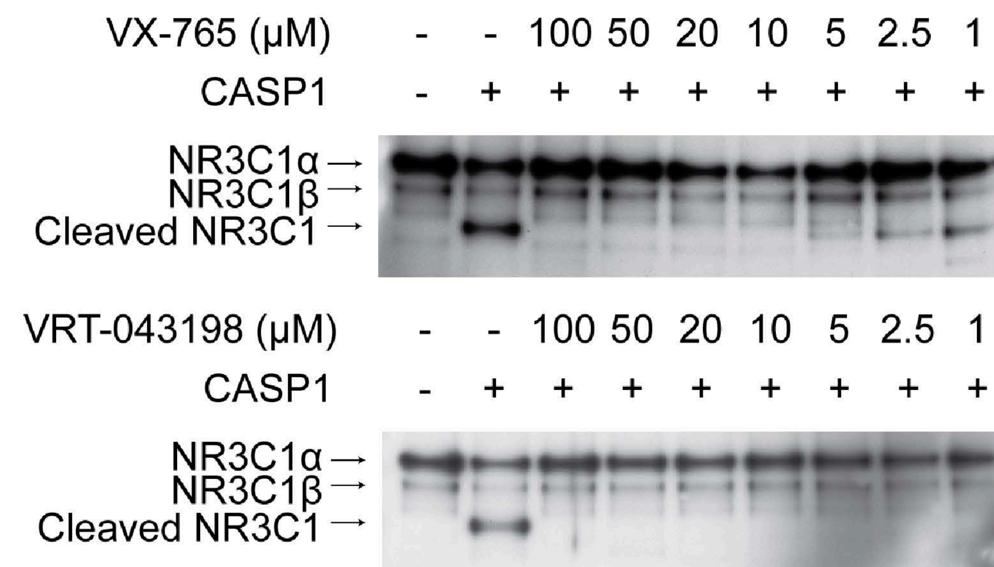
a.



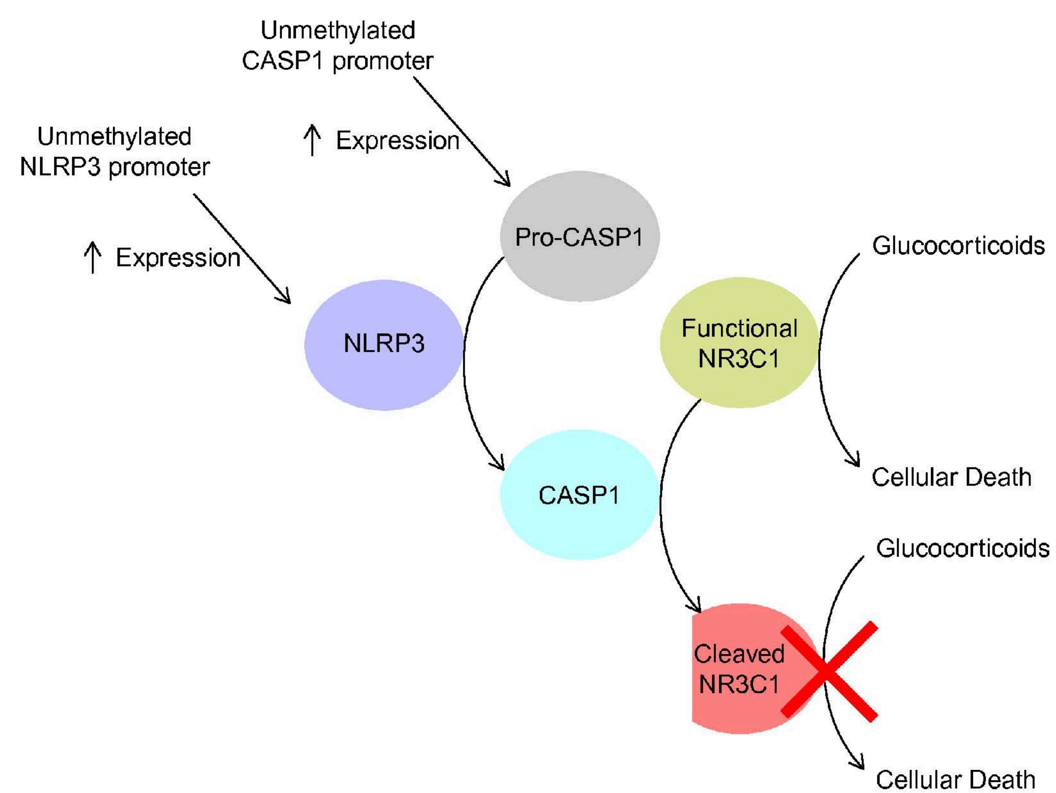
b.



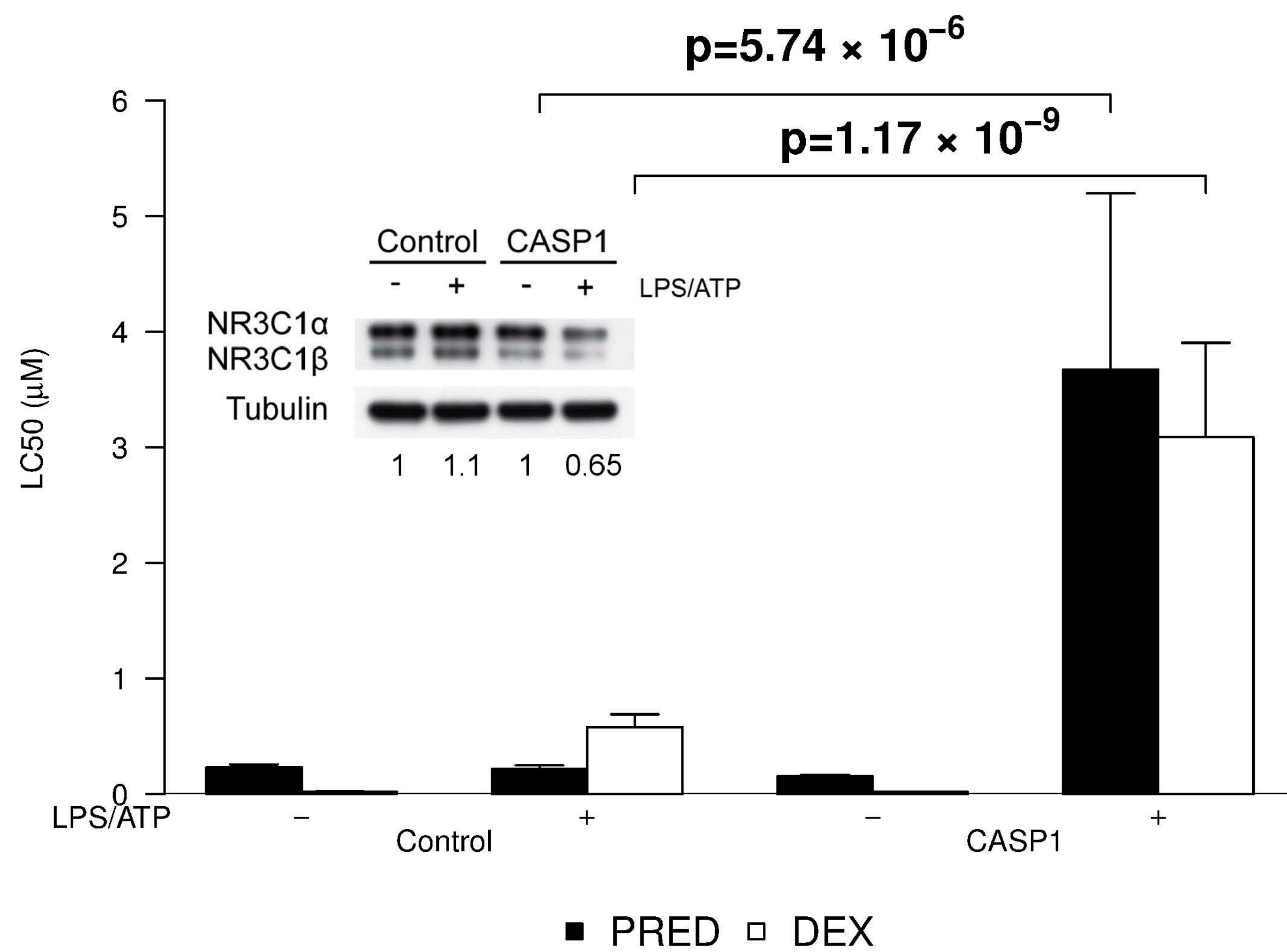
c.

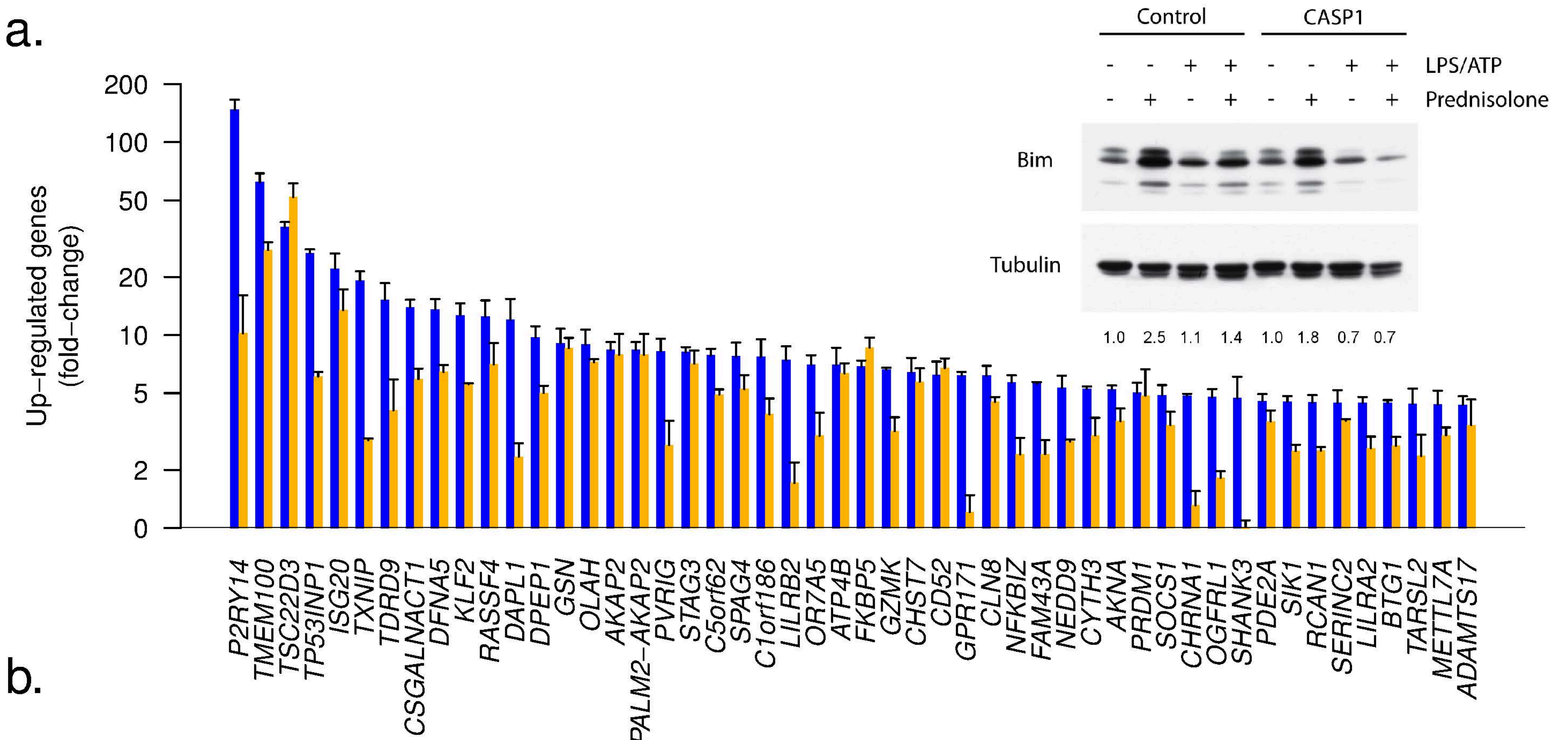


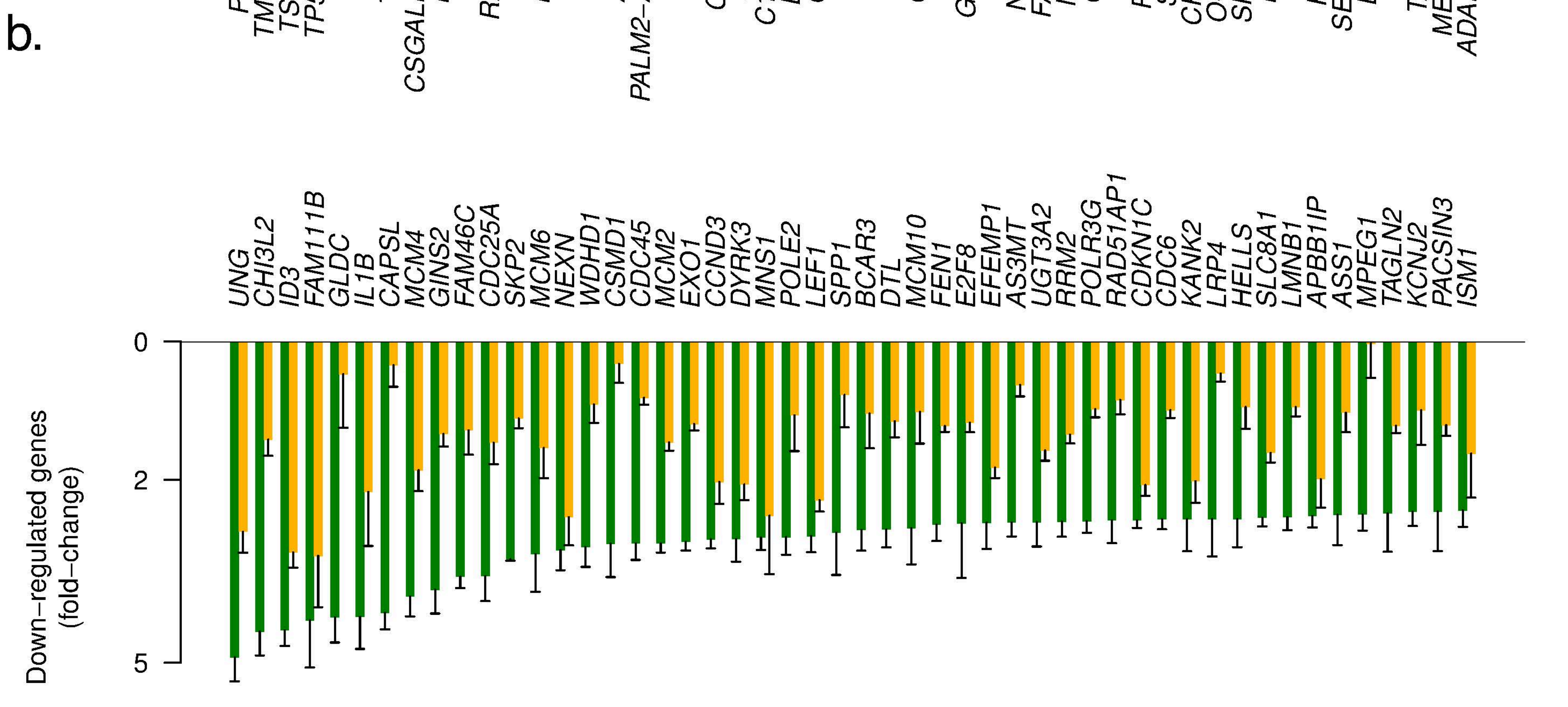
d.

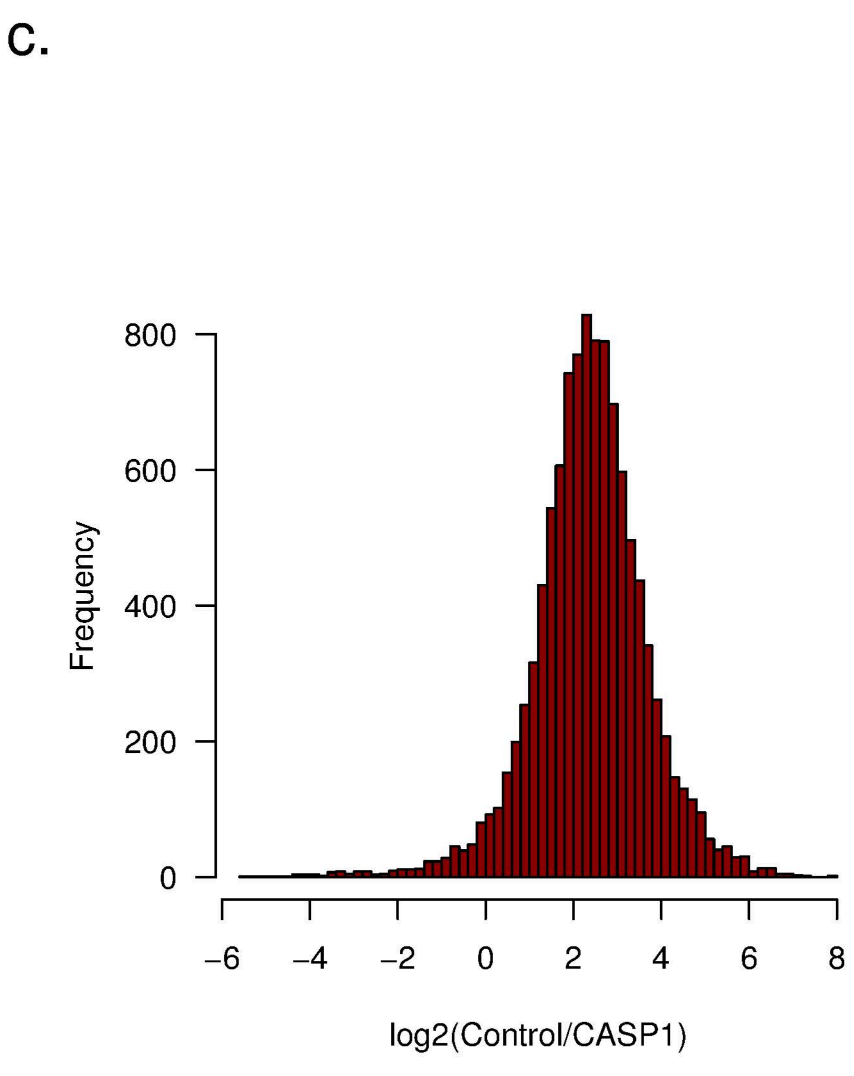


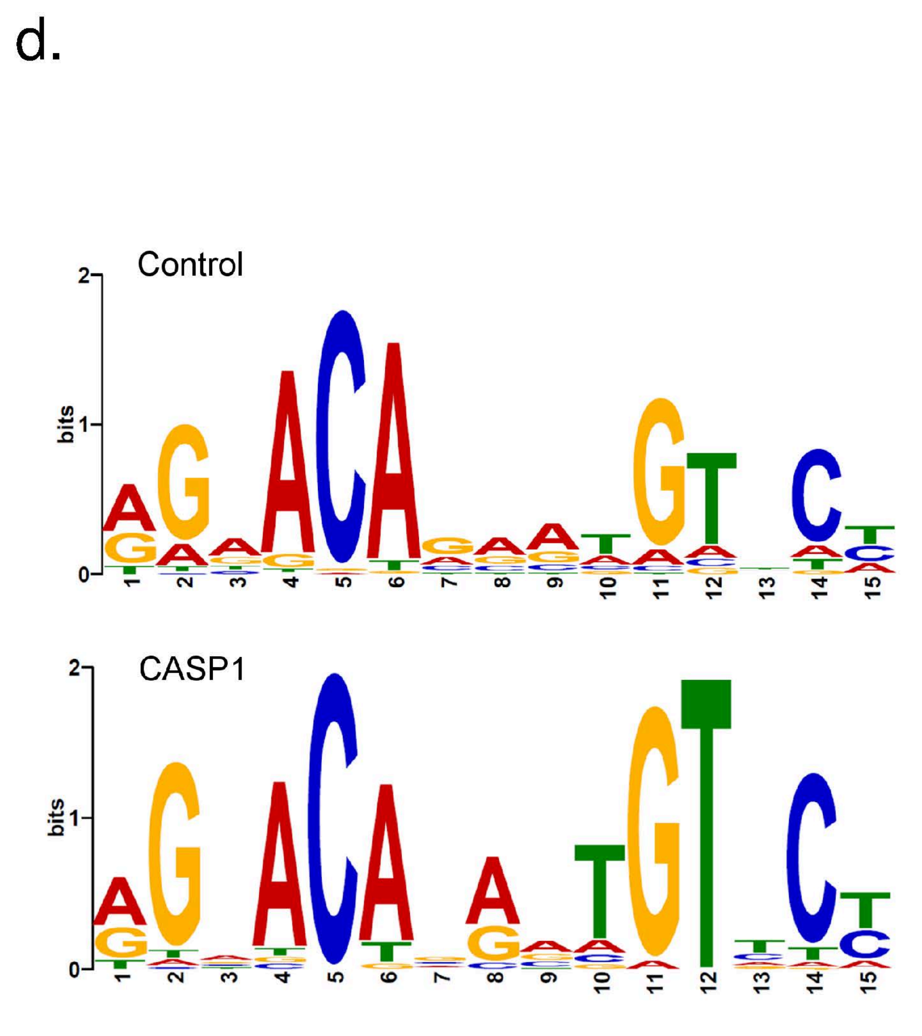
e.

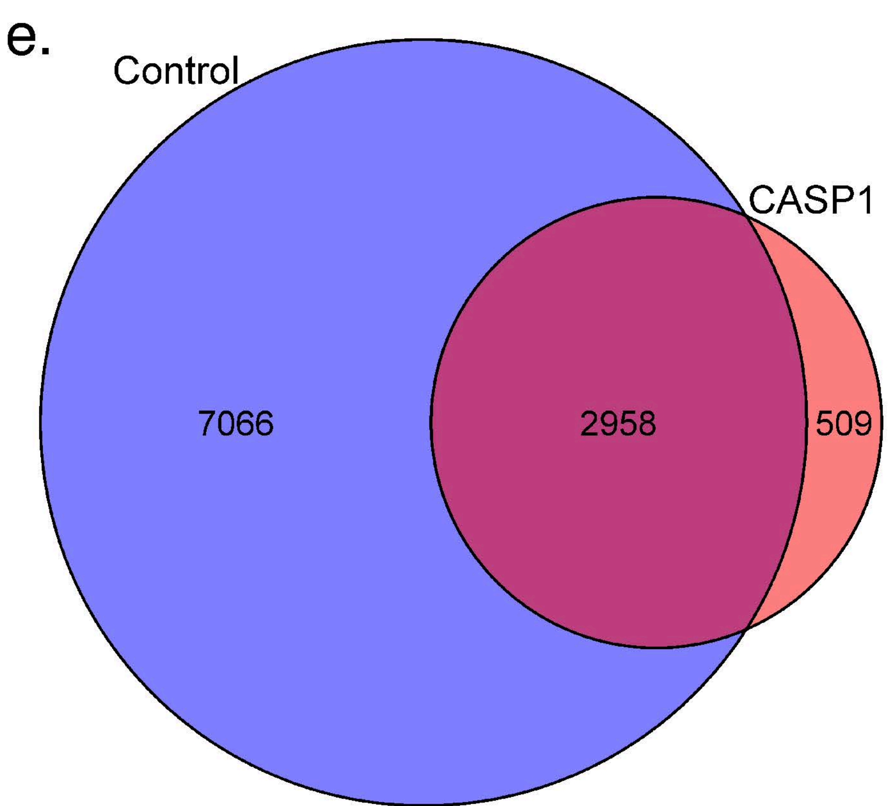


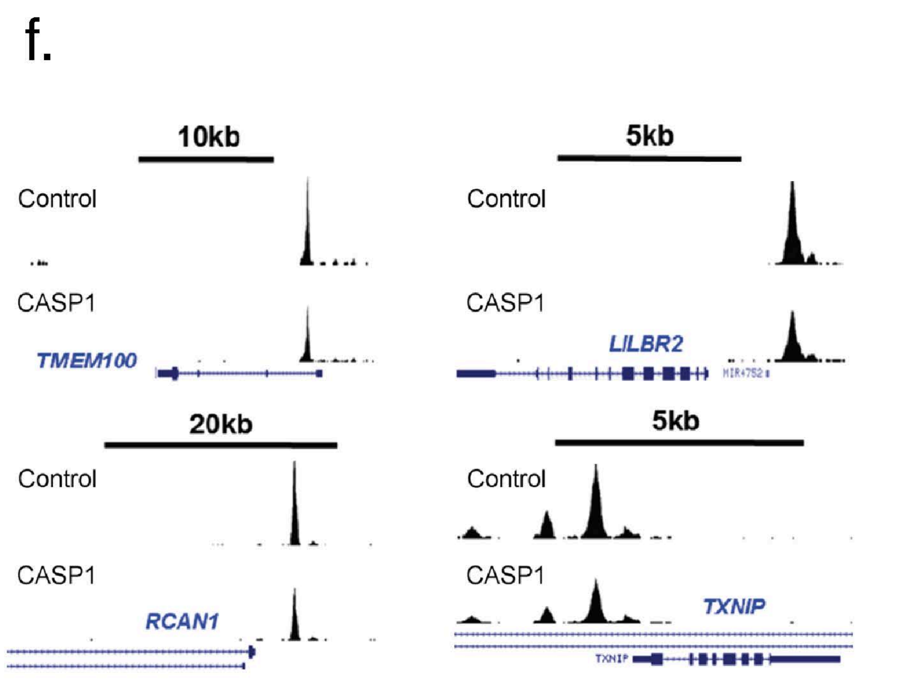

a.
Control
CASP1
LPS/ATP
Prednisolone
Bim
Tubulin
1.0 2.5 1.1 1.4 1.0 1.8 0.7 0.7
200
100
50
20
10
5
2
0
Up-regulated genes (fold-change)
P2RY14
TMEM100
TSC22D3
TP53INP1
ISG20
TXNIP
TDRD9
CSGALNACT1
DFNA5
KLF2
RASSF4
DAPL1
DPEP1
GSN
OLAH
AKAP2
PALM2-AKAP2
PVRIG
STAG3
C5orf62
SPAG4
C1orf186
LILRB2
OR7A5
ATP4B
FKBP5
GZMK
CHST7
CD52
GPR171
CLN8
NFKBIZ
FAM43A
NEDD9
CYTH3
AKNA
PRDM1
SOCS1
CHRNA1
OGFRL1
SHANK3
PDE2A
SIK1
RCAN1
SERINC2
LILRA2
BTG1
TARSL2
METTL7A
ADAMTS17

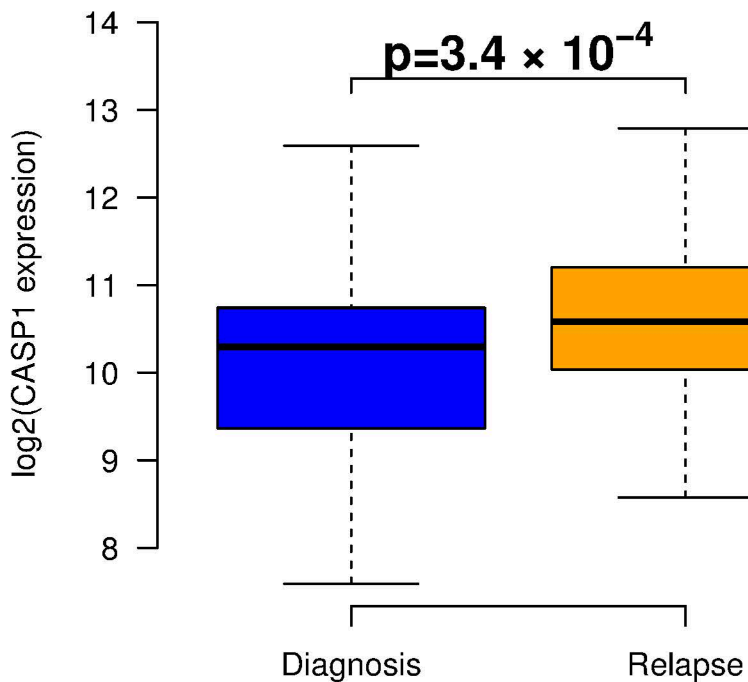

b.
UNG
CHI3L2
ID3
FAM111B
GLDC
IL1B
CAPSL
MCM4
GINS2
FAM46C
CDC25A
SKP2
MCM6
NEXN
WDHD1
CSMD1
CDC45
MCM2
EXO1
CCND3
DYRK3
MNS1
POLE2
LEF1
SPP1
BCAR3
DTL
MCM10
FEN1
E2F8
EFEMP1
AS3MT
UGT3A2
RRM2
POLR3G
RAD51AP1
CDKN1C
CDC6
KANK2
LRP4
HELLS
SLC8A1
LMNB1
APBB1IP
ASS1
MPEG1
TAGLN2
KCNJ2
PACSIN3
ISM1
0
2
5
Down-regulated genes (fold-change)


c.
800
600
400
200
0
Frequency
−6 −4 −2 0 2 4 6 8
log2(Control/CASP1)

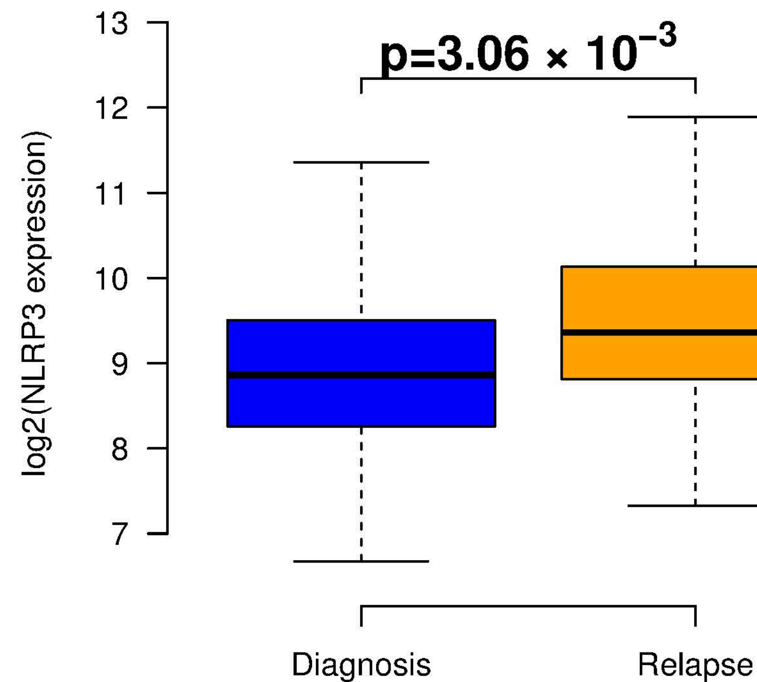

d.
Control
CASP1
bits
2
1
0


e.
Control
CASP1
7066
2958
509

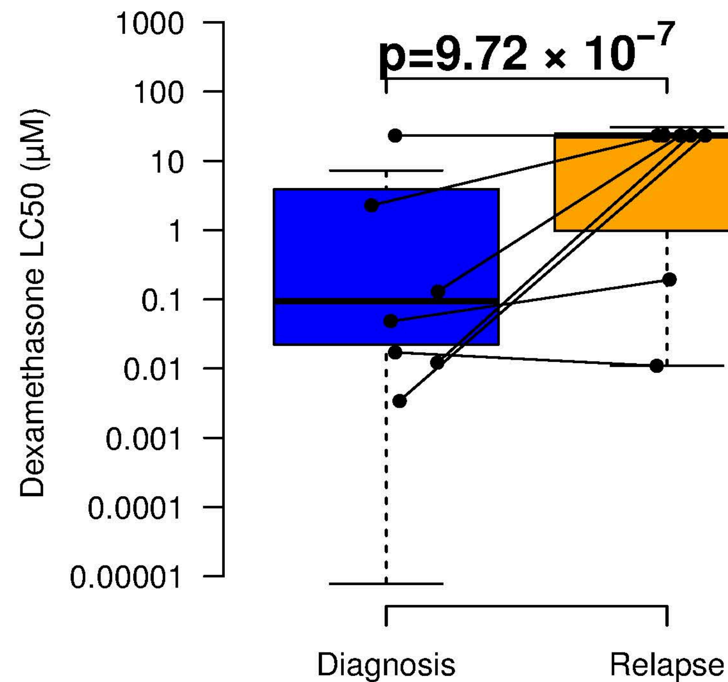

f.
10kb
5kb
20kb
5kb
Control
CASP1
TMEM100
LILRB2
RCAN1
TXNIP



a.
CASP1
p=3.4 × 10^−4
log2(CASP1 expression)
14
13
12
11
10
9
8
Diagnosis
Relapse

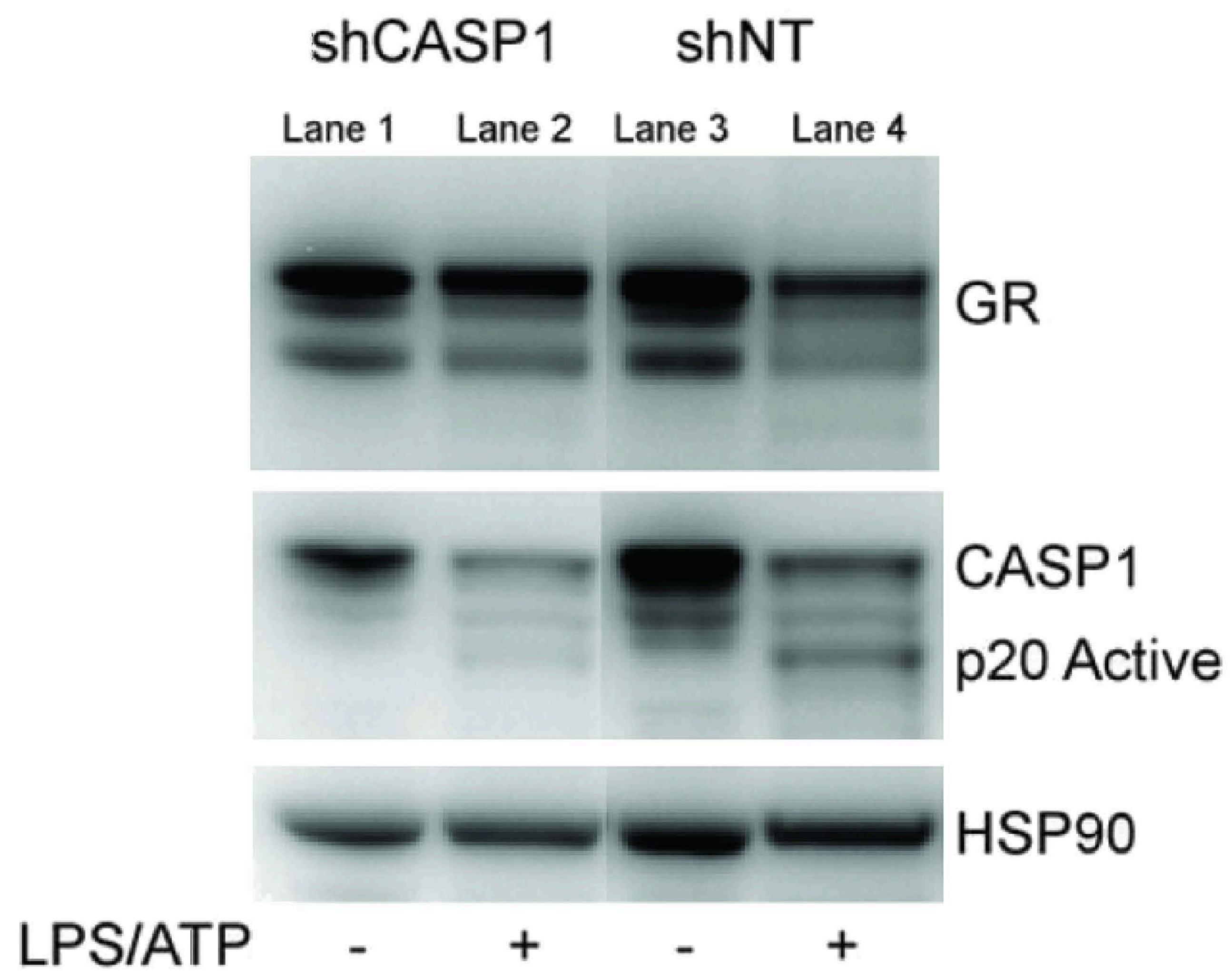


b.
NLRP3
p=3.06 × 10^−3
log2(NLRP3 expression)
13
12
11
10
9
8
7
Diagnosis
Relapse

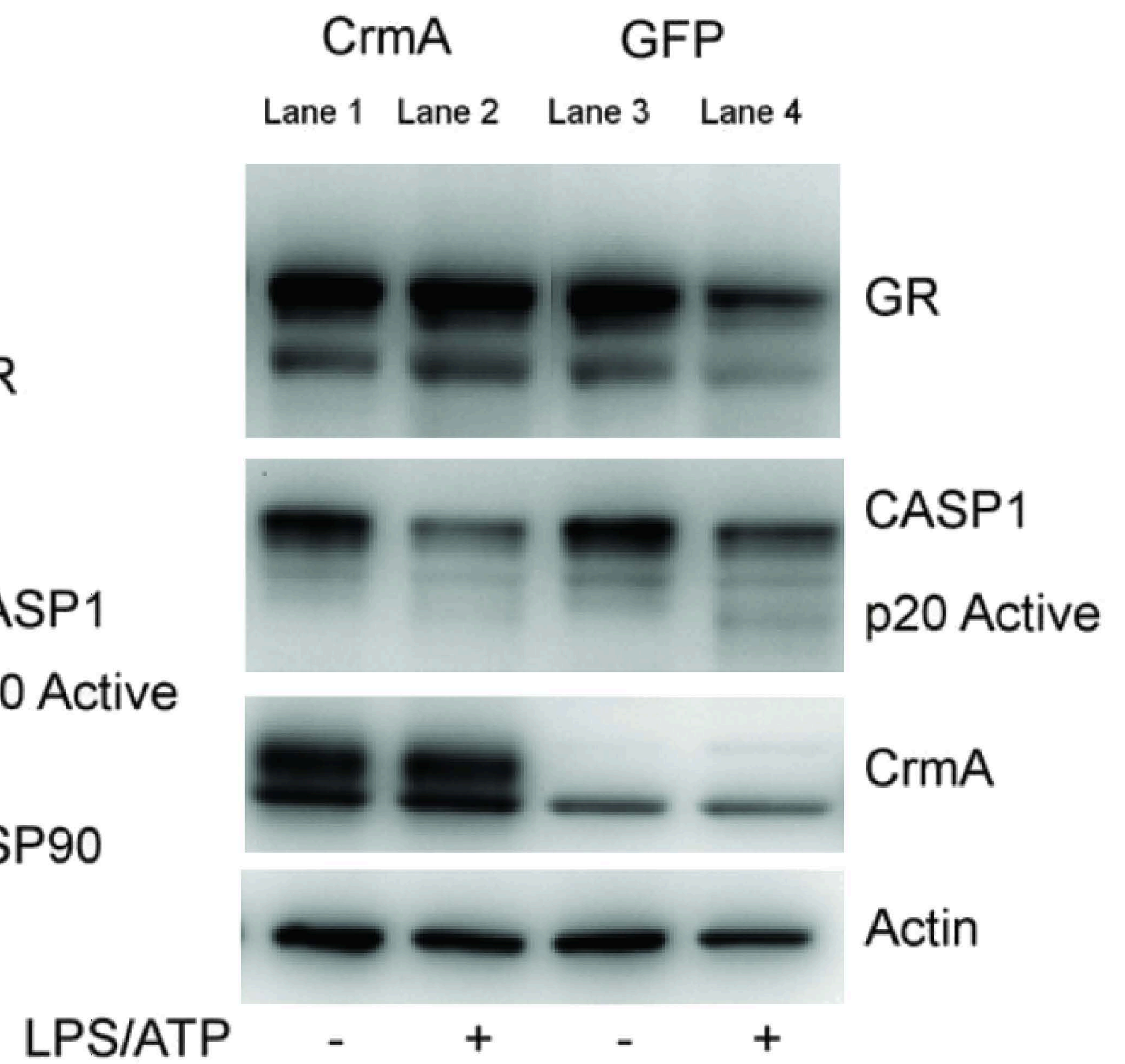


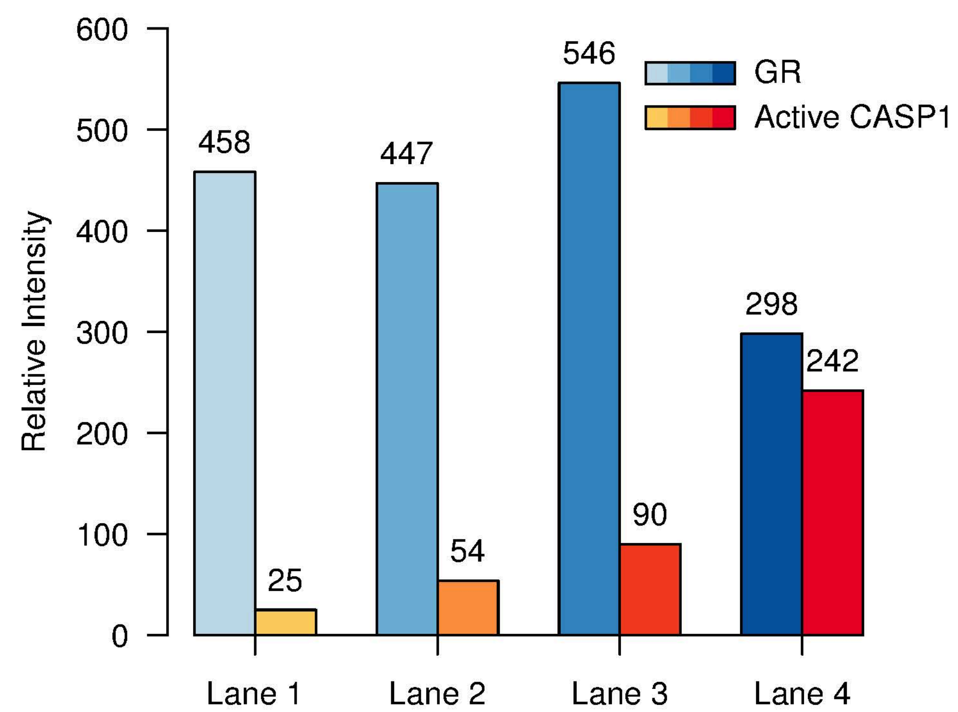
c.
Dexamethasone LC50
p=9.72 × 10^−7
Dexamethasone LC50 (µM)
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
Diagnosis
Relapse

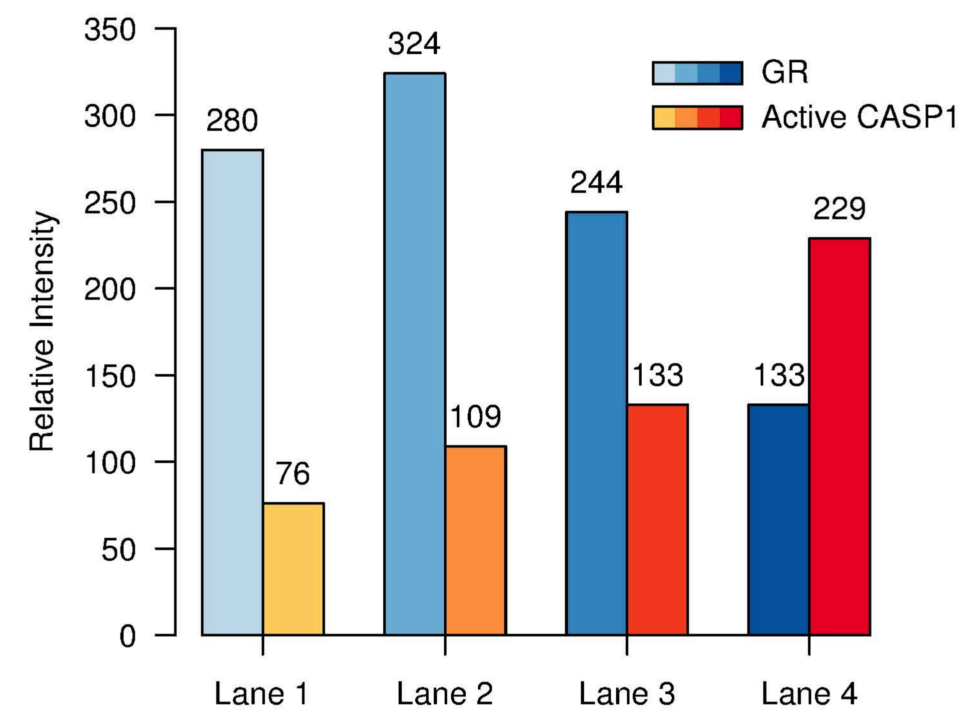
a.



b.

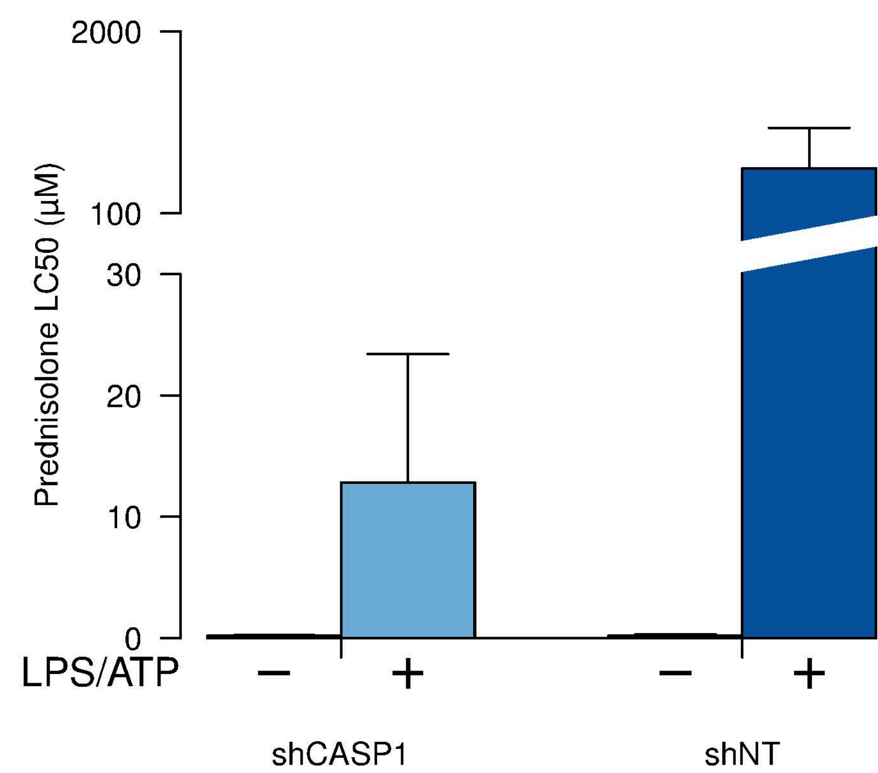


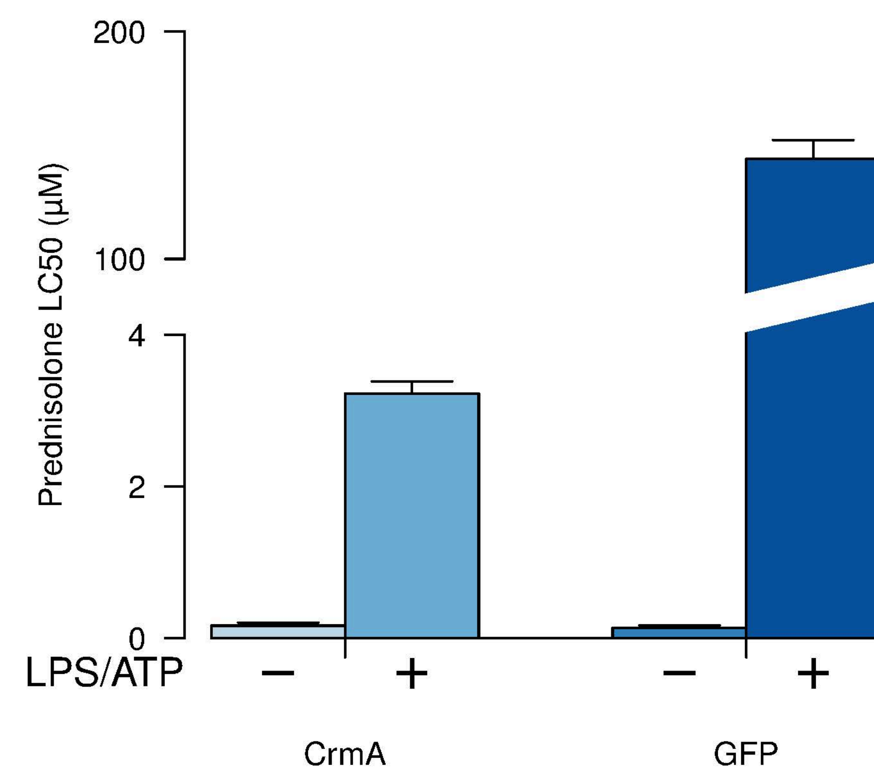


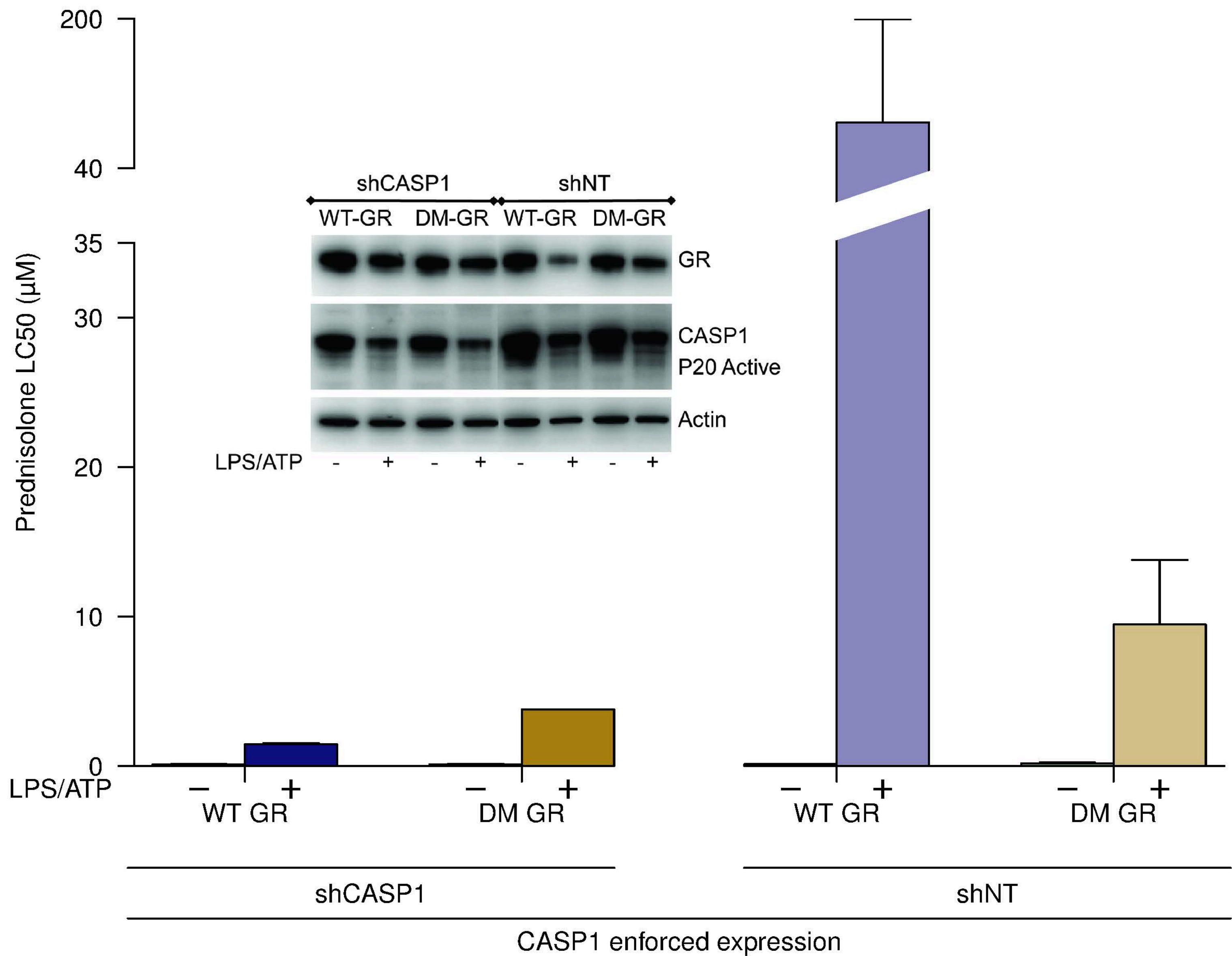


c.

d.







Prednisolone LC50 (μM)
200
40
35
30
20
10
0
shCASP1
shNT
WT-GR
DM-GR
WT-GR
DM-GR
GR
CASP1
P20 Active
Actin
LPS/ATP - + - + - + - +
LPS/ATP
− + − + − + − +
WT GR
DM GR
WT GR
DM GR
shCASP1
shNT
CASP1 enforced expression