Oncogenic hijacking of the stress response machinery in T cell acute lymphoblastic leukemia

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Cellular transformation is accompanied by extensive rewiring of many biological processes leading to augmented levels of distinct types of cellular stress, including proteotoxic stress. Cancer cells critically depend on stress-relief pathways for their survival. However, the mechanisms underlying the transcriptional initiation and maintenance of the oncogenic stress response remain elusive. Here, we show that the expression of heat shock transcription factor 1 (HSF1) and the downstream mediators of the heat shock response is transcriptionally upregulated in T cell acute lymphoblastic leukemia (T-ALL). Hsf1 ablation suppresses the growth of human T-ALL and eradicates leukemia in mouse models of T-ALL, while sparing normal hematopoiesis. HSF1 drives a compact transcriptional program and among the direct HSF1 targets, specific chaperones and co-chaperones mediate its critical role in T-ALL. Notably, we demonstrate that the central T-ALL oncogene NOTCH1 hijacks the cellular stress response machinery by inducing the expression of HSF1 and its downstream effectors. The NOTCH1 signaling status controls the levels of chaperone/co-chaperone complexes and predicts the response of T-ALL patient samples to HSP90 inhibition. Our data demonstrate an integral crosstalk between mediators of oncogene and non-oncogene addiction and reveal critical nodes of the heat shock response pathway that can be targeted therapeutically.

Multiple oncogenic insults converge on the transcriptional upregulation of anabolic pathways. Runaway cancer cell growth overwhelms the cellular proteome homeostasis and elicits the heat shock response to counter proteotoxic stress1–4. Stress alleviation is orchestrated by HSF1 and mediated by induced heat shock proteins (HSPs)5–8. The altered dependencies of cancer cells on stress response pathways have been proposed as an attractive therapeutic opportunity9,10.

Despite the importance of proteotoxic stress-relief mechanisms in cancer, the regulation of HSF1 by oncogenic signaling pathways remains elusive6,11. In experiments where HSF1 is activated by external stress, protein–protein interactions and extensive post-translational modifications have been shown to regulate HSF1 activity11. However, the molecular pathways responsible for the transcriptional initiation and maintenance of the heat shock response pathway in cancer are poorly understood6,11. Moreover, a comprehensive characterization of the direct effectors of HSF1 and the crosstalk of HSF1 with other transcription factors in disease conditions is missing6,11,12.

To gain insight into the cellular basis of heat shock response regulation in cancer, we focused on T-ALL as a disease model. Although the growth-promoting pathways driven by aberrantly activated oncogenes in T-ALL have been elucidated13–15, the regulation of supportive mechanisms (non-oncogenic per se) in acute leukemia cells has not been addressed. Current intensive chemotherapy regimens to pediatric and adult T-ALL patients come at the cost of serious side effects while a significant percentage of patients experience relapse, reinforcing the need to understand the altered dependencies of leukemia cells and target novel pathways to which tumor cells are specifically addicted.

Results
Expression of HSF1 and the downstream heat shock response are induced in human T-ALL. A plethora of post-translational modifications are critical for the stability and activation of HSF111,16–23. However, the transcriptional regulation of HSF1 expression in cancer remains unknown6,11. Gene expression profiling of pediatric T-ALL14 samples revealed significant upregulation of HSF1 expression compared to thymocyte subsets purified from healthy individuals (Fig. 1a). In addition, total HSF1 protein levels and HSF1 phosphorylated at Ser-326, a modification critical for HSF1 activation23, were significantly higher in primary T-ALL patient samples and T-ALL cell lines (the CUTLL1 line is shown as a representative example24) compared to normal T cells (Fig. 1b). We
HSF1 and gene members of the stress response pathway are highly expressed in human T-ALL. a, Box plot showing the expression of HSF1 among samples of T-ALL (n = 57 samples) and physiological thymocyte subsets (n = 21 samples; 7 T cell subsets derived from 3 donors) (quantile normalization across samples, see Methods). The boxes represent the first and third quartiles, and the line represents the median. The whiskers represent the upper and lower limits of the range (P = 1.7 × 10−16; two-sided Wilcoxon test). ***, significant. b, Protein levels of HSF1 (top) and pSer-326 HSF1 (center) in control T cells (CD4+ T cells) from the peripheral blood of healthy donors, primary bone marrow biopsies from T-ALL patients and the human T-ALL cell line CUTLL1. Representative blots from two independent studies (biological replicates) are shown. c, Volcano plot for changes in gene expression in T-ALL patients (n = 57 samples) versus physiological thymocyte subsets (n = 21 samples; 7 T cell subsets derived from 3 donors; P < 0.001; two-sided unpaired t-test). Red indicates genes expressed at higher levels and blue at lower levels in T-ALL patients versus physiological T cell subsets. Classic HSF1 targets are depicted. d, Annexin V staining following Renilla shRNA (shRenilla) and shHSF1 treatment (24 h) of CUTLL1 cells. The experiment was repeated three times (biological replicates) and a representative example is shown. e, Effects of HSF1 or Renilla knockout on human T-ALL (CUTLL1) survival. The mean ± s.d. from three representative studies is shown.

HSF1 is essential for disease progression in animal models of T-ALL. The significant upregulation of expression of HSF1 and classic HSF1 targets in T-ALL patient specimens suggested a potential involvement of this stress response program in the pathogenesis of acute leukemia. To test this hypothesis, we initially knocked down HSF1 in human T-ALL cell lines using previously validated short hairpin RNAs (shRNAs) (11–13). HSF1 depletion led to increased rates of apoptosis (Fig. 1d), defective proteostasis as exemplified by upregulation of endoplasmic reticulum stress markers (Supplementary Fig. 1c) and strongly affected growth of leukemic cells (Fig. 1e and Supplementary Fig. 1d,e). These experiments suggested that T-ALL cells are addicted to HSF1 function.

To establish conclusively whether HSF1 is required for T-ALL progression in vivo, we generated and studied a NOTCH1-induced T-ALL animal model (Supplementary Fig. 2a). We utilized this model since NOTCH1 pathway is hyper-active in the vast majority of T-ALL cases (14). We used an inducible Cre recombinase model to delete Hsf1 after disease establishment. Bone marrow progenitor cells derived from Hsf1/f−/−Mx1-Cre+ mice or littermate control mice (Hsf1−/−Mx1-Cre−) were retrovirally transduced with an ‘active’ NOTCH1 mutant (NOTCH1-ΔE-IRE-ES-GFP) and transplanted into lethally irradiated wild-type recipients. Following disease establishment, we genetically ablated Hsf1 (Supplementary Fig. 2b) and examined leukemia burden and disease progression. Hsf1 deletion led to a striking reduction of leukemic cell numbers and infiltration in all tissues surveyed (Fig. 2a–c and Supplementary Fig. 2c). Given the striking reduction in leukemia burden following Hsf1 deletion, we assessed animal survival. We found that, compared to littermate control mice, survival of Hsf1-depleted animals was dramatically extended (Fig. 2d). Notably, there was no fatality recorded in the Hsf1−/−Mx1-Cre− animal group for a period longer than a year, suggesting an absolute addiction of T-ALL cells to HSF1 function.

The in vivo NOTCH1-ΔE T-ALL model survival data suggested that Hsf1 loss could directly impact leukemia-initiating cell (LIC) function, as no relapse incidence was recorded. We have previously characterized LICs in this model of T-ALL and shown that Myc protein abundance is a bona fide LIC biomarker. This LIC subset corresponds to a small fraction of the overall T-ALL population and is characterized by a specific cell surface marker. The next examined whether elevated expression of HSF1 may induce transcriptionally the heat shock response pathway. To address this possibility, we surveyed the expression of classic gene members of the heat shock response pathway (15) in T-ALL primary patient samples. We found that well-characterized HSF1 targets such as HSPA8 (16), HSPA9 (17) and HSPH1 (18, 19) exhibit significantly higher expression in T-ALL samples (Fig. 1c and Supplementary Fig. 1a). In addition, using a second independent patient data set, we observed significantly higher expression of HSF1 and classic HSF1 targets in T-ALL samples compared to normal T cells (Supplementary Fig. 1b).
expression signature\textsuperscript{29}. To test the role of Hsf1–orchestrated stress response in T-ALL LICs, we studied the Myc\textsuperscript{+} LIC transcriptome and compared it to Myc\textsuperscript{−} cells that have no LIC capacity. We found that Myc\textsuperscript{+} LICs are characterized by high expression of a number of Hsf1 target genes (Fig. 2e). Together with our in vivo T-ALL remission data, these findings suggest that T-ALL cells are addicted to Hsf1 function and LICs are characterized by an enhanced Hsf1–driven stress response.

To examine the effect of Hsf1 silencing in an additional T-ALL model (not driven by NOTCH1), we used T-ALL cells derived from a Tal1 mouse model. In this model, Tal1 is the initiating oncogenic trigger\textsuperscript{30}. Hsf1 knockdown strongly affected the survival of Tal1-overexpressing leukemia cells in vitro (Supplementary Fig. 1d,e). To test a requirement for Hsf1 in Tal1-driven leukemia progression in vivo, we infected primary mouse Tal1-driven T-ALL leukemia cells\textsuperscript{31} with lentiviruses encoding shRNAs to Hsf1 (or Renilla luciferase, as a control) and transplanted the infected cells into syngeneic recipients. Silencing of Hsf1 resulted in a striking reduction of peripheral T-ALL cells (characterized by co-expression of CD4 and CD8; Supplementary Fig. 2f) and significant increased overall animal survival (Supplementary Fig. 2g).

Hsf1 ablation does not affect normal hematopoiesis in vivo.

It was previously shown that Hsf1 expression is dispensable for survival of mice under normal growth conditions\textsuperscript{32}. To investigate possible effects of Hsf1 loss on cellular fitness specifically in the hematopoietic system, we undertook competitive bone marrow transplantation assays (using a 1:1 mixture of Hsf1-expressing and non-expressing bone marrow stem and progenitor cells). We found that Hsf1-depleted hematopoietic stem and progenitor cells (HSPCs) reconstituted recipient mice bone marrow efficiently (Fig. 2f,g) and were able to generate all blood lineages (data not shown), including T cells in the same proportions as their wild-type counterparts (Supplementary Fig. 3a,b). Moreover, Hsf1 deletion specifically in the hematopoietic system of adult mice did not affect steady-state hematopoiesis or the lymphoid compartment (Supplementary Fig. 3c). These experiments demonstrate that Hsf1 is specifically required for the viability of ‘stressed’ leukemic cells but is dispensable for hematopoietic stem cell function and normal T cell development, suggesting a potential therapeutic window for future HSF1 targeting for leukemia treatment.
Articles

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Genomic mapping of the HSF1-regulated stress response in acute leukemia. Studies in our laboratory and others have previously shown that HSF1 demonstrates cancer-type and mutation-specific genome occupancy patterns.\(^2\)\(^,\)\(^3\) To connect direct HSF1 gene targets to HSF1 function in leukemia, we sought to characterize the human T-ALL HSF1 transcriptional program. Chromatin immunoprecipitation followed by sequencing (ChiP-seq) studies in human T-ALL cells (CUTLL1) mapped the HSF1 chromatin occupancy signature. We found that the majority of HSF1 binding peaks (~75%) are located at transcription start sites (TSSs) of putative target genes (Supplementary Fig. 4a), suggesting a HSF1 preference for promoter regions. Bioinformatics analysis of these promoter regions revealed a strong enrichment for consensus HSF1 binding elements\(^3\)\(^,\)\(^4\) (Supplementary Fig. 4b; \(P < 10^{-20}\)). In addition, gene ontology analysis showed enrichment for protein folding functions (Supplementary Fig. 4c; \(P < 10^{-4}\)). On the basis of these ChiP-seq results, we defined a TSS-centric HSF1 gene target signature. We found that, in contrast to broad HSF1 genome occupancy in solid tumors\(^2\)\(^,\)\(^3\)\(^,\)\(^4\) and tumor stroma\(^4\), HSF1 occupies the promoters of a small number (fewer than 70) of genes in T-ALL. Examination of expression of the genes bound by HSF1 on their promoters demonstrated contrasting patterns between T-ALL patient samples and control thymocyte subsets (Fig. 3a). To further identify direct HSF1 transcriptional targets, we combined our ChiP-seq analysis with RNA sequencing (RNA-seq) following brief silencing of HSF1 in human T-ALL cells (Supplementary Fig. 4d). This analysis allowed us to define a group of genes that comprise the ‘direct HSF1 gene signature’ in this cancer type (Supplementary Table 1). We then asked whether these genes are differentially expressed in primary human T-ALL. We found that positively regulated HSF1 targets are upregulated in T-ALL samples (when compared to physiological thymocyte subsets) and negatively regulated targets are downregulated (Supplementary Fig. 4e). Thus, this combinatorial analysis uncovered direct HSF1 targets in acute leukemia and showed that the T-ALL HSF1 gene signature is distinct from the signatures previously defined in solid tumors and tumor stroma.

Functional mapping of the HSF1-regulated stress response in acute leukemia. An emerging question based on this definition of HSF1 gene signature is which of these targets mediate the HSF1 phenotype and are essential for T-ALL cell growth. A plausible scenario, pertinent to the strong requirement of HSF1 for T-ALL survival, involves the coordinated function of several direct HSF1 targets. To test this hypothesis, we surveyed all of the HSF1 positively regulated targets for their ability to mimic the effect of HSF1 knockdown on leukemia cell growth, using a targeted RNAi screen. We found that silencing of a number of single HSF1 direct targets (10 out of 23) was sufficient to suppress the growth of human T-ALL cells (Fig. 3b).
and Supplementary Fig. 4f). Interestingly, all of these ‘essential’ targets belong to the HSP90/HSP70 chaperone and co-chaperone interaction network. Overexpression of HSP90AB1, a key molecular chaperone that nucleates this functional network, was not sufficient to rescue the effect of HSF1 knockdown on T-ALL survival (Supplementary Fig. 4g), suggesting non-overlapping functions of the various HSF1 targets. HSF1 ChIP-seq analysis in a second human T-ALL cell line further supported these findings as it showed that common gene targets between the two cell lines include the ‘essential’ HSF1 targets identified in our screen (Fig. 3d). Together, our findings functionally characterize the HSF1 targets in human leukemia and indicate the presence of a well-defined functional HSF1 transcriptional program in acute T cell leukemia.

**NOTCH1 hijacks the stress response machinery.** The heat shock response pathway was described more than 50 years ago and has been implicated in the pathogenesis of diseases as diverse as neurodegeneration and cancer. However, the transcriptional trigger of the pathway remains elusive. On the basis of our findings showing upregulation of HSF1 messenger RNA expression in T-ALL, we sought to elucidate the mechanism underlying HSF1 transcriptional upregulation in this type of leukemia. We hypothesized that oncogenes may include anabolism-supportive pathways in their altered transcriptional program. Aberrant constitutively active NOTCH1 signaling is a defining feature of the vast majority (>90%) of human T-ALL cases. NOTCH1 ChIP-seq analysis in human T-ALL cells revealed binding of NOTCH1 on both the HSF1 TSS and gene body (Fig. 4a). The HSF1 promoter was also occupied by RNA polymerase II (Pol II) and marked by the presence of H3K4me3 (Fig. 4a), in agreement with the active status of the locus. NOTCH1 binding on the HSF1 promoter was further confirmed by analysis using ChIP coupled with quantitative PCR (Supplementary Fig. 5a). Moreover, NOTCH1 binding on the HSF1 promoter was detected in an additional T-ALL line (JURKAT; Supplementary Fig. 5b). By contrast, we did not detect binding of NOTCH1 on the HSF1 promoter in normal HSPCs, even following heat shock treatment, suggesting that oncogenic stress differs both in type and intensity from this of the classic heat shock (Supplementary Fig. 5c). Thus, our data directly link heat shock response to an oncogenic transcription factor and suggest oncogenic hijacking of the stress response.

Following exposure to heat stress, HSF1 drives the expression of heat-inducible genes by increasing RNA Pol II release from promoter-proximal pause. However, the transcriptional regulation of HSF1 targets in cancer is unknown. Intriguingly, we observed a broad co-occupancy of HSF1 and NOTCH1 on the promoters of a large number of HSF1 targets (Fig. 4b; 48 out of 57 HSF1 targets, \( P = 2.2 \times 10^{-16} \), chi-squared test). Bioinformatics analysis revealed distinct binding motifs for these transcription factors on the promoters of stress response genes (Supplementary Fig. 5d). Occupancy of the promoters of HSF1 and heat shock proteins coding genes by NOTCH1 indicates a potential involvement of this oncogene in transcriptional control of the heat shock response pathway. To test this hypothesis, we initially monitored the expression of HSF1 following inhibition of NOTCH1 pathway. Treatment of T-ALL cells with \( \gamma \)-secretase inhibitor (\( \gamma SI \)) to block NOTCH1 cleavage and pathway activation, resulted in significant downregulation of HSF1 expression at both the mRNA and protein levels (Fig. 4c and Supplementary Fig. 6a). We observed similar effects following treatment of multiple T-ALL cell lines with \( \gamma SI \) (Supplementary Fig. 6b). Notably, NOTCH1 signaling inhibition had no effect on HSF1 expression in the leukemic cell line LOUCY that is characterized by the absence of NOTCH1 expression (Supplementary Fig. 6b). In addition, \( \gamma SI \) treatment reduced HSF1 expression in primary T-ALL patient samples (Supplementary Fig. 6c). To further examine the correlation between HSF1 expression and NOTCH1 pathway activity, we analyzed a T-ALL patient data set and categorized patients into 20% highest and 20% lowest NOTCH1 expressing. Consistent with our model suggesting regulation of the heat shock response by NOTCH1, we found that high-NOTCH1–expressing patient samples also expressed the highest levels of HSF1 and heat shock response genes (Supplementary Fig. 6d). By contrast, samples expressing low NOTCH1 levels were also characterized by low expression of HSF1 and heat shock response genes (Supplementary Fig. 6d). Interestingly, subcellular fractionation analysis of primary T-ALL patient samples (maintained on the bone marrow-derived stromal cell line OP9 that expresses the Notch ligand Delta-like 1) revealed variability in the levels of nuclear NOTCH1. In line with our model, patient samples characterized by high nuclear NOTCH1 protein levels also expressed higher levels of HSF1 and stress proteins, compared to samples expressing low or no nuclear NOTCH1 (Fig. 4d). In addition, patient samples characterized by high expression of stress response mediators showed increased sensitivity to HSF1 knockdown (Supplementary Fig. 6e).

We then assessed the expression of HSF1 targets whose promoters were also occupied by NOTCH1, following NOTCH1 pathway inhibition. We found that, similar to HSF1 knockdown, NOTCH1 inhibition resulted in reduction of expression of HSF1 targets (Fig. 4e). To further profile nascent RNAs during NOTCH1 signaling recovery after \( \gamma SI \) treatment, we performed global-run-on sequencing (GRO-seq). We observed increased transcriptional activity for HSF1 and HSF1 targets during recovery from NOTCH1 signaling inhibition (Supplementary Fig. 6f). What is the functional significance of NOTCH1–HSF1 co-occupancy on the promoters of stress response genes? To address this question, we knocked down HSF1 (using shRNAs) and also inhibited the NOTCH1 pathway activation (using \( \gamma SI \)). We observed cooperation in the reduction of expression of HSF1 target genes, including the chaperones and co-chaperones mediating the HSF1 effect on T-ALL survival (Fig. 4f). To investigate a potential direct interaction between NOTCH1 and HSF1, we characterized the nuclear NOTCH1 interactome in T-ALL and HEK293T cells, by mass spectrometry. Although we detected the majority of known interactors of NOTCH1, we did not identify interaction with HSF1 (Supplementary Table 2), in agreement with a previous report characterizing the NOTCH1 interactome in T-ALL. When we assessed the expression of HSF1 and heat shock response targets across T-ALL lines, we found that leukemic cells characterized by NOTCH1 expression had significantly higher levels of the stress signature, compared to leukemic cells not expressing NOTCH1 (Supplementary Fig. 7a). Cell cycle analysis revealed similar kinetics among these cell lines (Supplementary Fig. 7b), excluding the possibility of the stress response being a mere reflection of the cell cycle status. Finally, to investigate whether NOTCH1 pathway activation is sufficient to induce the heat shock response pathway in a non-transformed context, we expressed a form of NOTCH1 constitutively cleaved in a ligand-independent manner (\( \Delta E \) mutant) in HSPCs (Lineage\( ^{-}\)-c-Kit\( ^{-}\)-Sca-1\( ^{-}\) ) isolated from mouse bone marrow. We found that NOTCH1 pathway activation rapidly and significantly increased the expression of both Hsf1 and several heat shock genes coding direct Hsf1 targets (Fig. 4g). Simultaneous expression of Notch1-\( \Delta E \) and silencing of Hsf1 suppressed the effect of NOTCH1 on Hsf1 and stress response gene expression (Fig. 4g).

Finally, to test whether the NOTCH1–heat shock response axis is active in other blood malignancies, we tested distinct types of leukemia, including acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL). We chose CLL as, similar to T-ALL, it is associated with oncogenic NOTCH1 mutations. On the other hand, we have previously shown that the Notch pathway acts as a tumor suppressor in AML. Unlike T-ALL, we did not observe transcriptional upregulation of HSF1 in AML or CLL patient samples (Supplementary Fig. 7c). We investigated whether this is the result
Fig. 4 | Direct regulation of the heat shock response pathway by NOTCH1. a, Snapshots of NOTCH1 and RNA Pol II binding on the HSF1 promoter. Enrichment of the histone mark H3K4me3 and expression signal defined by RNA-seq analysis are also indicated. The scale represents reads per million. Snapshots of common peaks from two independent studies (biological replicates) are shown. b, Snapshots of HSF1, NOTCH1 and RNA Pol II binding on the promoters of representative HSF1 targets. Enrichment for the histone mark H3K4me3 and expression signal defined by RNA-seq analysis are also indicated. The scale represents reads per million. Snapshots of common peaks from two independent studies (biological replicates) are shown. The green region indicates the promoter of the corresponding genes. The stars denote the presence of binding motifs for NOTCH1 (RBPJ). c, HSF1 mRNA expression in human T-ALL (CUTLL1) cells following treatment with γS I or HSF1 knockdown. The mean ± s.d. from three representative studies is shown. The P values (two-sided unpaired t-test) are as follows: for DMSO versus γS I, 0.002; and for DMSO versus shHSF1, P < 0.0001. d, Protein levels of nuclear NOTCH1, HSF1, HSPA8 and HSP90AB1 from primary bone marrow biopsies of T-ALL patients. The numbered patient samples refer to samples further analyzed in Fig. 5 and Supplementary Fig. 10. Representative blots from two independent studies (biological replicates) are shown. e, A heatmap representation of changes in gene expression of HSF1 direct targets co-occupied at the promoter by NOTCH1, following γS I treatment, HSF1 knockdown, or a combination of γS I and HSF1 knockdown. Genes that showed cooperative reduction in expression following combined γS I and HSF1 knockdown are shown. f, A heatmap representation of changes in gene expression of HSF1 direct targets co-occupied at the promoter by NOTCH1, following Renilla knockdown, HSF1 knockdown or a combination of γS I and HSF1 knockdown. Genes that showed cooperative reduction in expression following combined γS I and HSF1 knockdown are shown. g, Hsf1 and HSF1 targets mRNA expression in murine bone marrow HSPCs following overexpression of Notch1-ΔE or overexpression of Notch1-ΔE and downregulation of HSF1 (HES1 was used as a positive control). mRNA expression was monitored 48 h after infection with retrovirus. The mean ± s.d. from three representative studies is shown. For pMIG-Empty versus pMIG-Notch1-ΔE, P values (two-sided unpaired t-test) are as follows: for HSF1, 0.0006; for HSP90AB1, 0.0007; for HSPA8, 0.0004; for HSPE1, 0.0055; for HSPH1, P < 0.0001; for DNAJA1, 0.0498; and for HES1, 0.0002.
NOTCH1 regulates the functional T-ALL chaperome. Previous studies have shown that, in the presence of oncogenic stress, heat shock proteins participate in a large functional and physical network nucleated by HSP90 and HSP70 chaperones, recently termed the ‘epichaperome’\(^{51,52}\). On the basis of our findings demonstrating transcriptional regulation of the heat shock response machinery by NOTCH1, we hypothesized that this oncogene might directly regulate the epichaperome in T-ALL. To test this hypothesis, we initially examined whether activation of NOTCH1 signaling in untransformed cells affects epichaperome formation. We found that active NOTCH1 (Fig. 5b and Supplementary Fig. 9a). Furthermore, we investigated whether activation of NOTCH1 signaling in untransformed cells affects epichaperome formation. We found that active NOTCH1 induced epichaperome formation in mouse HSPCs (Supplementary Fig. 9b).

Previous studies have shown that the binding affinity of HSP90 for small-molecule inhibitors and the cancer cell sensitivity to HSP90 inhibition dramatically increase when this molecular chaperone participates in multi-chaperone complexes\(^{51,52,53}\). Therefore, we assessed the effect of epichaperome level reduction following γSI treatment on HSP90 inhibition outcome in T-ALL. We found that γSI pre-treatment suppressed the effect of PU-H71 on T-ALL survival and growth (Fig. 5c). However, γSI pre-treatment had no effect on PU-H71 efficacy in LOUCY T-ALL cells (Fig. 5c). Moreover, γSI pre-treatment of patient T-ALL samples significantly reduced cell death triggered by PU-H71 treatment (Supplementary Fig. 9c). Given the research efforts to identify optimal drug combinations in cancer, our findings suggest that drugs targeting the NOTCH1 signaling pathway may impede the efficiency of molecular chaperone inhibitors, providing a warning for future combinatorial treatments.

NOTCH1 activation predicts response to chaperome-targeted therapy. These studies suggest that high levels of expression of nuclear NOTCH1, and consequently high levels of epichaperome, provide an attractive biomarker for response to HSP90 inhibitors. Given the variability in the levels of nuclear NOTCH1 observed in T-ALL patient samples (Fig. 4d), we monitored the epichaperome levels in primary T-ALL patient samples. Intriguingly, patient samples expressing high levels of nuclear NOTCH1 (patients 6–10), expressed higher levels of epichaperome compared to those with low nuclear NOTCH1 expression (patients 1–5; Fig. 6a and Supplementary Fig. 10a,b).
Finally, we tested whether patient nuclear NOTCH1 levels can predict the response to HSP90 inhibition. We found that patient samples expressing high levels of nuclear NOTCH1 and high levels of epichaperome (patients 6–10) were significantly more sensitive to PU-H71 treatment compared to T-ALL with low nuclear NOTCH1 (patients 1–5; Fig. 6b). Our findings, in their totality, suggest that
the NOTCH1 signaling pathway regulates the epichromerome levels and impacts the outcome of HSP90 inhibition on leukemia survival.

**Discussion**

Our studies implicate HSF1 as a dominant orchestrator in acute leukemia and provide a number of key novel insights into the role and regulation of the heat shock response pathway, a universal response to which virtually all cancers are addicted. So far, and based primarily on studies where HSF1 is activated by heat, HSF1 has been shown to be regulated by numerous protein–protein interactions and extensive post-translational modifications. Our findings suggest that in T-ALL, the oncogene NOTCH1, a defining cancer trigger in this disease, hijacks the heat shock response pathway through transcriptional regulation of HSF1. We also provide evidence for the presence of a previously unsuspected cross-talk between mediators of oncogene and non-oncogene addiction. Specifically, we show interplay between an oncogene (NOTCH1) and HSF1 on the control of expression of critical effectors of the oncogenic stress-relief machinery. Our data mechanistically explain previous observations in the stress response field suggesting that transcription factors, other than HSF1, may regulate HSF1 target gene expression in disease. What is the molecular basis of this interplay? NOTCH1 promotes leukemia cell growth via direct transcriptional upregulation of anabolic pathways, including overactive transcription, ribosome biosynthesis and protein translation. We propose that, due to increased anabolism, the folding demands of leukemic cells are amplified and parallel activation of the heat shock response pathway might be vital for proteostasis maintenance. Characterization of the proteostasis machinery clientele in T-ALL will reveal crucial mediators of leukemogenesis. Interestingly, intracellular Notch1 itself has recently been proposed to be a novel client of HSP90.

Why might oncogene-mediated transcriptional hijacking of the stress response pathway be required in T-ALL and possibly in other cancer types? We propose that accumulation of a critical number of HSF1 molecules is necessary for the initiation of the HSF1 oncogene-specific transcriptional program. Work by our group and others has shown that this critical HSF1 protein abundance can be achieved through mechanisms such as mutation/silencing of E3 ligases or upregulation of oncogenic pathways that post-translationally modify residues that are critical for stability, and these mechanisms can be cancer-type specific. Our data suggest an additional—but not mutually exclusive—mechanism, that of oncogene-mediated transcriptional upregulation of HSF1 and HSF1-target expression. Further investigation will reveal whether NOTCH1 regulates the heat shock response pathway in other types of cancer or this cross-talk is mediated by tumor-specific oncogenes.

To date, there are no approved drugs targeting the NOTCH1 pathway, despite the development of a number of promising experimental compounds including γ-secretase. Our findings suggest that the heat shock response pathway may provide an alternative option for targeting cancers driven by NOTCH1, while sparing normal cells. Interestingly, our studies suggest that HSF1 targeting compromises the viability of cancer-initiating cells, an exciting finding given the potential role of such populations in disease relapse after treatment. In addition to the preclinical and early clinical promise of HSP90 and HSP70 inhibitors, our findings uncover a battery of chaperones and co-chaperones critical for T-ALL survival. We anticipate that these oncogenic stress-relief mediators could serve as novel intervention targets in an effort to battle leukemia and other types of cancer that are addicted to the heat shock response pathway for survival. Given that it is unlikely that every single chaperone and co-chaperone encoded by the genome will participate in the chaperome network and will be critical for a particular tumor type, an integrative approach similar to the one followed in our study will uncover tumor-specific altered dependencies on the chaperome machinery.

Currently, there is insufficient stratification of patients enrolled in trials targeting molecular chaperones, including HSP90. Our findings suggest that the status of NOTCH1 signaling may serve as a biomarker for the clinical outcome of therapeutic interventions targeting the stress response cellular machinery. Altered dependency of cancer cells on non-oncogenic stress-relief mechanisms has emerged as a novel anticancer therapy class. If its preclinical promise is to be realized, it is imperative that we decipher the regulation of this cancer anabolism-supportive pathway. Our study provides one of the first in-depth characterizations of the mechanism of regulation and action of the stress response in cancer.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at [https://doi.org/10.1038/s41591-018-0105-8](https://doi.org/10.1038/s41591-018-0105-8).

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**References**

1. Dai, C., Whitesell, L., Rogers, A. B. & Lindquist, S. Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell* **130**, 1005–1018 (2007).

2. Mendillo, M. L. et al. HSF1 drives a transcriptional program distinct from heat shock to support highly malignant human cancers. *Cell* **150**, 549–562 (2012).

3. Santagata, S. et al. Tight coordination of protein translation and HSF1 activation supports the anabolic malignant state. *Science* **341**, 545–555 (2013).

4. Dai, C. The heat-shock, or HSF1-mediated proteotoxic stress, response in cancer: from proteomic stability to oncogenesis. *Phil. Trans. R. Soc. B* **373**, 20160525 (2018).

5. Gomez-Pastor, R. et al. Abnormal degradation of the neuronal stress-protection transcription factor HSF1 in Huntington’s disease. *Nat. Commun.* **8**, 14405 (2017).

6. Li, J., Labbadia, J. & Morimoto, R. I. Rethinking HSF1 in stress, development, and organismal health. *Trends Cell Biol.* (2017).

7. Luo, J., Solomini, N. L. & Elledge, S. J. Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* **136**, 823–837 (2009).

8. Nagel, R., Senevra, E. A. & Berns, A. Drugging the addict: non-oncogene addiction as a target for cancer therapy. *EMBO Rep.* **17**, 1516–1531 (2016).

9. Gomez-Pastor, R., Burchiel, E. T. & Thiele, D. J. Regulation of heat shock transcription factors and their roles in physiology and disease. *Nat. Rev. Mol. Cell Biol.* **19**, 4–19 (2018).

10. Whitesell, L. & Lindquist, S. Inhibiting the transcription factor HSF1 as an anticancer strategy. *Expert Opin. Ther. Targets* **13**, 469–478 (2009).

11. Belver, L. & Ferrando, A. The genetics and mechanisms of T cell acute lymphoblastic leukaemia. *Nat. Rev. Cancer* **16**, 494–507 (2016).

12. Palomero, T. et al. NOTCH1 directly regulates e-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc. Natl. Acad. Sci. USA* **103**, 18261–18266 (2006).

13. Weng, A. P. et al. e-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukaemia/lymphoma. *Genes Dev.* **20**, 2096–2109 (2006).

14. Chou, S. D., Prince, T., Gong, J. & Calderwood, S. K. mTOR is essential for cancer trigger in this disease, hijacks the heat shock response pathway through transcriptional regulation of HSF1. We also provide evidence for the presence of a previously unsuspected cross-talk between mediators of oncogene and non-oncogene addiction. Specifically, we show interplay between an oncogene (NOTCH1) and HSF1 on the control of expression of critical effectors of the oncogenic stress-relief machinery.

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21. Kourtis, N. et al. FBXW7 modulates cellular stress response and metastatic potential through HSF1 post-translational modification. Nat. Cell. Biol. 17, 322–332 (2015).
22. Tang, Z. et al. MEK guards proteome stability and inhibits tumor-suppressive amyloidogenesis via HSF1. Cell 160, 729–744 (2015).
23. Westerheide, S. D., Anckar, J., Stevens, S. M., Ir, Sistonen, L. & Morimoto, R. I. Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. Science 323, 1063–1066 (2009).
24. Van Vlierbergh, P. et al. ETV6 mutations in early immature human T cell leukemias. J. Exp. Med. 208, 2571–2579 (2011).
25. Guettouche, T., Boellmann, F., Lane, W. S. & Voellmy, R. Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. BMC Biochem. 6, 4 (2005).
26. Palomero, T. et al. CUTLL1, a novel human T-cell lymphoma cell line with t(7;9) rearrangement, aberrant NOTCH1 activation and high sensitivity to gamma-secretase inhibitors. Leukemia 20, 1279–1287 (2006).
27. Brandvold, K. R. & Morimoto, R. I. The chemical biology of molecular chaperones—implications for modulation of proteostasis. J. Mol. Biol. 427, 2931–2947 (2015).
28. Astrin, I. C. et al. Oncogenic forms of NOTCH1 lacking either the primary binding site for RBP-Jkappa or nuclear localization sequences retain the ability to associate with RBP-Jkappa and activate transcription. J. Biol. Chem. 272, 11336–11343 (1997).
29. King, B. et al. The ubiquitin ligase FBXW7 modulates leukemia-initiating cell activity by regulating MYC stability. Cell 153, 1552–1566 (2013).
30. O’Neil, J. et al. Activating Notch1 mutations in mouse models of T-ALL. Blood 113, 1139–1142 (1988).
31. Roderick, J. E. et al. c-Myc inhibition prevents leukemia initiation in mice and impairs the growth of relapsed and induction failure pediatric T-ALL cells. Blood 123, 1040–1050 (2014).
32. Xiao, X. et al. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. EMBO J. 18, 5943–5952 (1999).
33. Xiao, H. & Lis, J. T. Germline transformation used to define key features of the activation mechanism. Cell 158, 564–578 (2014).
34. Taldone, T. et al. Synthesis of purine-scaffold fluorescent probes for heat shock protein 90 with use in flow cytometry and fluorescence microscopy. Bioorg. Med. Chem. Lett. 21, 5347–5352 (2011).
35. Li, C. & Sampson, S. B. HSF1: guardian of proteostasis in cancer. Nat. Med. 21, 1182–1189 (2015).
36. Wang, Z. et al. Stabilization of Notch1 by the Hsp90 chaperone is crucial for T-cell leukemogenesis. Clin. Cancer Res. 23, 3834–3846 (2017).
37. Rodina, A. et al. The epichaperome is an integrated chaperome network that facilitates tumour survival. Nature 538, 397–401 (2016).
38. Kourtis, N. et al. FBXW7 modulates proteome stability and inhibits tumor-suppressive amyloidogenesis via HSF1. Cell 160, 729–744 (2015).
39. Fabbri, G. et al. Common nonmutational NOTCH1 activation in chronic lymphocytic leukemia. Proc. Natl. Acad. Sci. USA 114, E2911–E2919 (2017).
40. Shrestha, L., Patel, H. J. & Chiosis, G. Chemical tools to investigate mechanisms associated with HSP90 and HSP70 in disease. Cell Chem. Biol. 23, 158–172 (2016).
41. Van Vlierbergh, P. et al. ETV6 mutations in early immature human T cell leukemias. J. Exp. Med. 208, 2571–2579 (2011).
42. Kourtis, N. et al. FBXW7 modulates cellular stress response and metastatic potential through HSF1 post-translational modification. Nat. Cell. Biol. 17, 322–332 (2015).
43. Smith, V. I. & Ligon, S. H. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137, 216–233 (2009).
44. Ben-Bassat, H., Shlomai, Z., Kohn, G. & Prokocimer, M. Establishment of a normal karyotype AML. Cancer Res. 49, 241–248 (1990).
45. Holmes, R. & Zuniga-Pflucker, J. C. The OP9-DLL1 system: generation of T-lymphocytes from embryonic or hematopoietic stem cells in vitro. Cold Spring Harb. Protoc. 2009, pdbprot5156 (2009).
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56.####################################################################################################
Bone marrow cell transplantation and quantification. For analysis of leukemia progression, c-kit+ HSPCs were enriched from bone marrow by magnetic separation (STEMCell Technologies) using an antibody cocktail (CD117 (c-kit) conjugated in the presence of 50 mg/ml FITC ligand, 50 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6). At 24 and 48 h after enrichment, c-kit+ cells were infected with concentrated retrovirus expressing NOTCH1::ΔE-ires-GFP or control vector. Transduction efficiency was determined by monitoring reporter fluorescence over a total period of four days. For induction of T-ALL, irradiated mice (2 rounds of 550 rads) received 5 × 10⁶ GFP+ cells, together with 5 × 10⁶ bone marrow mononuclear cells (wild type) for hemogenic support, via retro-orbital injection. The Mantel–Cox test was used for the analysis of survival data. No randomization or blinding method was used in these animal studies.

In vivo animal treatment. For inhibition of HSP90, mice were treated with intraperitoneal injections 75 mg/kg−1 PU–H71 (Selleckchem) or PBS, 3 times per week. Treatment was initiated 2 weeks after transplantation, following detection of ~10% leukemic (GFP+) cells in the peripheral blood.

Cell culture and primary cell samples. The human cell lines CUTILL1, P12, CEM and KOPTK1 were cultured in RPMI 1640 medium supplemented with 20% FBS, penicillin and streptomycin. The mouse T-ALL line Tal1 was cultured in RPMI 1640 medium supplemented with 10% FBS and penicillin and streptomycin. All cell lines were tested for the presence of mycoplasma and only mycoplasma-free lines were used for these studies. T-ALL patient samples were maintained on the bone marrow–derived stromal cell line OP9 expressing the NOTCH1 ligand Delta-like 1. Primary T-ALL patient samples and blood from healthy donors were collected at 4-week intervals after transplantation for 20 weeks, at which time mice were euthanized for assessment of chimerism in bone marrow and spleen.

In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis. In vitro drug treatment and cell growth and cell cycle analysis.

Epichaperome abundance measurement using PU–FITC flow cytometry assay. The PU–FITC assay was performed as previously described. HEL-60 and MV4-11 were used as negative and positive controls respectively for epichaperome abundance. The control derivative PU–FITC9 was used as a negative control. The mean fluorescence intensity of PU–FITC in viable cells (DAPI negative) was evaluated by flow cytometry.

Antibodies, reagents, kits and virus production. Antibodies against the following proteins were used: polyclonal rabbit HSF1 (Cell Signaling, 4356), monoclonal rabbit pSer-326 HSF1 (Abcam, 76076), monoclonal mouse HSP90A1 (GeneTex, 84342), monoclonal rabbit HSPA8 (Cell Signaling, 8444), monoclonal rabbit NOTCH1 (Cell Signaling, 4147), monoclonal mouse actin (Millipore, MA1B01) and monoclonal goat lamin A (Santa Cruz biotechnology, sc-6214). The siRNAs used against HSF1 and HSP70 were validated using the silencing strategy, have been described previously. Hsf1−/− mice were transplanted into syngeneic recipient mice as described previously and disease development was monitored. All animal experiments were performed in accordance with protocols approved by the New York University Institutional Animal Care and Use Committee (IACUC).

Chip and ChIP-seq library preparation. For HSF1 ChIP, 50×10⁴ cells were fixed with 1% formaldehyde for 10 min in room temperature and lysed on ice using 5 ml cell lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 and 0.25% Triton X-100). After centrifugation (5 min, 4000 rpm), the pellet was resuspended in 5 ml buffer II (10 mM HEPES-KOH, pH 7.4, 200 mM NaCl, 1 mM EDTA, pH 8.0 and 0.5 mM EGTA, 0.1% sodium deoxycholate and 0.5% N-lauroylsarcosine) and sonicated at a Bioruptor for 40 min. Triton X-100 was added to a final concentration of 1% and the chromatin proteinase K was pre-cleared using magnetic beads. The crosslinks were reversed, and the DNA was precipitated with ethanol and glycercol.

NOTCH1 ChIP was performed as described previously. The following primers were used to test NOTCH1 binding enrichment: HSF1 seq experiments and to avoid the effects of apoptosis due to transduction efficiency and RNA was collected 48 h after infection.

Quantitative real-time PCR. For mRNA quantification, total RNA was isolated from one million cells for each condition and replicate using the RNeasy Plus Mini Kit (Qiagen). RNA was quantified by absorbance at A260 and 1 µg of total RNA was used for cDNA synthesis using the Superscript III first-strand synthesis kit (Invitrogen). Real-time PCR reactions were carried out using SYBR Green Master Mix (Roche) and run with a Lightcycler 480 II (Roche). The following primer sequences were used for cDNA quantification: human HSF1 for 5′-GGAACCCTGCTGATTCGTT-3′, rev-5′-GTGCTCTGGGTCACCTTAG-3′, human HSP90A1 for 5′-AAAGGGAGAACGCTCACACC-3′, rev-5′-GGTCTCTGGGTCACCTTAG-3′; human HSP90A2 for 5′-ATGGACGAACTAATCTC TGAGAC-3′, rev-5′-GGTCTCTGGGTCACCTTAG-3′; mouse HSF1 for 5′-ATGGACGAACTAATCTC TGAGAC-3′, rev-5′-GGTCTCTGGGTCACCTTAG-3′; mouse HSP90A1 for 5′-ATGGACGAACTAATCTC TGAGAC-3′, rev-5′-GGTCTCTGGGTCACCTTAG-3′; and mouse HSP90A2 for 5′-ATGGACGAACTAATCTC TGAGAC-3′, rev-5′-GGTCTCTGGGTCACCTTAG-3′.
Protein was loaded on SDS–PAGE and analyzed with western blot and Coomassie staining. Proteins were digested with trypsin and tandem spectra were collected using an Orbitrap mass spectrometer.

RNA-seq library preparation. Whole RNA was extracted from 1–5 × 10^6 T-ALL cells or using the RNAeasy kit (QIAGEN) according to the manufacturer’s protocol. Libraries were constructed using the KAPA Stranded RNA-seq kit.

Protein–protein interaction network analysis. A protein interaction network was generated using the STRING database. The interaction network was generated with a required confidence score of 0.15 and ‘Active interaction sources’ based on experiments.

RNA-seq analysis. RNA-seq analysis was performed using lincRNA-screen. Specifically, sequencing reads were aligned to the reference genome hg19 using STAR aligner and default parameters. Differential expression analysis was performed using DESeq2. The comparisons tested were: y:5 treatment versus DMSO; HSF1 knocked down versus Renilla; HSF1 knocked down plus y:5 treatment versus HSF1 knockdown; and HSF1 knockdown plus y:5 treatment versus Renilla. Genes with a false discovery rate <0.1 were considered significantly differentially expressed. Other RNA-seq data sets in this study were also analyzed with the same platform and cutoffs.

Data sources and computational tools. Human assembly version hg19/GRCh37 and ENCODE release 69 were used for RNA-seq and ChIP-seq data and integration analyses. Bowtie (version 2.2.6) and STAR aligner (in the case of RNA-seq) were used for alignment of sequenced reads. HOMER (version 4.6) was used for ChIP-seq sequencing peak discovery. The R programming language (version 3.3.0), Bioconductor (version 3.4) and microarray–processing packages were used for microarray analysis. GenomicTools version 2.7.2 was used for performing genomic interval mathematical operations, genomic interval annotations and ChIP-seq profile construction.

Microarray analysis. Non-normalized expression data from T-ALL patients and normal T cells (GES33469 and GSE33470) were normalized using quantile normalization to eliminate any batch effects. Illumina identifiers were converted to gene names using Bioconductor (version 3.4) and Illumina HumanHT12v4 annotation data (R package version 1.26). Expression values were log-transformed and unpaired Student’s t-test with Benjamini–Hochberg correction and cutoff $p < 0.001$ was used to determine statistically significant differences between the T-ALL samples and their normal counterparts. Boxplots and volcano plots were generated with the untransformed normalized expression values (after quantile normalization), using ggplot2 in combination with ggrepel (in the case of volcano). R package version 0.6.5). Mouse microarray data (GSE46797) were processed with the affy Bioconductor package. Annotation of the probes was performed using Affymetrix Mouse Genome 430 2.0 Array annotation data. In the case of T-ALL cell line data (GSE48046), the analysis was performed as for mouse but using Affymetrix Human Genome U133 Plus 2.0 Array data for annotation. For heatmaps, the expression values were normalized per row (z-score). Heatmaps were generated using R (version 3.3.0).

ChIP-seq analysis. Sequenced reads were aligned using Bowtie (version 2.2.6) with default parameters, except for −m 1 so as to report only unique alignments). Human assembly version hg19. Bases with mapping quality MAPQ <30 were discarded. Peak discovery for transcription factors (NOTCH1 and HSF1) was performed with HOMER (version 4.6) using default parameters and the ‘factor’ peak style option, optimized for transcription factors. The motif analysis and peak annotation was also performed using HOMER, with the default settings and the previously identified peaks as input. Histone modification ChIP-seq samples were analyzed using MACS and the following settings: --broad --nomodel --extsize= 150 --pvalue= 1 × 10^-4. Sonicated input was used as a control for peak discovery. HSF1 peaks were characterized on the basis of their genome-wide distribution as: TSS (1 kb TSS-flanking regions of transcript isoforms), Gene Body (peaks falling within the gene body, excluding any overlaps with TSS-flanking regions), TSS upstream (regions 10 kb–100 kb from the TSS excluding those that overlapped with the aforementioned categories) and Intergenic (the rest of the peaks). Venn diagrams showing the common targets of certain transcription factors were plotted using the online tool: http://bioinformatics.psb.ugent.be/webtools/Venn/. Snapshots of genomic areas were created using the Human Epigenome Browser at Washington University.

Motif analysis. The raw sequence of the human genome (hg19) was downloaded using the UCSC browser and the sequence corresponding to ±1 kb from the TSS of genes of interest was extracted. Then, FIMO was used with default settings to search for motifs.

Statistics. For statistical analysis, we used GraphPad Prism software v.7.0c and variance was similar between the groups that were compared. Figure legends specify the statistical analysis used and define error bars. For RNA-seq, ChIP-seq and microarray analysis, the statistical analysis performed is described in the corresponding sections of the Methods. The statistical analysis of the intersection of NOTCH1 and HSF1 targets was performed using the SuperExactTest R package (version 0.9.9.4).

GO analysis. Gene ontology (GO) analysis was performed on the basis of ChIP-seq data and using DAVID v6.8. The top significantly enriched GO terms were selected for plotting.

GRO-seq analysis. GRO-seq analysis was performed as previously described.

Data availability. The data sets generated for this study can be accessed at GEO (GSE90717). Previously published data sets that were reanalyzed during this study include microarray data from patients with T-ALL and normal T cells (GSE33469 and GSE33470), AML (GSE30029), CLL (GSE66117), human T-ALL cell lines (GSE48046), mouse microarray data (GSE46797) and ChIP-seq data for CUITLL1 cells (GSE51800). Uncropped immunoblots are available in Supplementary Fig. 11.

References

1. Le Masson, F. et al. Identification of heat shock factor 1 molecular and cellular targets during embryonic and adult female matiosis. Mol. Cell. Biol. 31, 3410–3423 (2011).
2. Tatarek, J. et al. Notch1 inhibition targets the leukemia-initiating cells in a Tα1/Lmo2 mouse model of T-ALL. Blood 118, 1579–1590 (2011).
3. Taldone, T. et al. Heat shock protein 70 inhibitors. 2. 2,5-thiodipyrimidines, 5-(phenylthio)pyrimidines, 2-(pyridin-3-ylthio)pyrimidines, and 3-(phenylthio)pyridines as reversible binders to an allosteric site on heat shock protein 70. J. Med. Chem. 57, 1208–1224 (2014).
4. Nizich Kirstos, P. et al. Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia. Nat. Med. 18, 298–301 (2012).
5. Jensen, L. J. et al. STRING 8—global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res. 37, D412–D416 (2009).
6. Gong, Y. et al. IncRNA-screen: an interactive platform for computationally screening long non-coding RNAs in large genomes datasets. BMC Genomics 18, 434 (2017).
7. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–23 (2013).
8. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550 (2014).
9. Hou, R. C., Gilmour, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359 (2012).
10. Heinz, S. et al. Simple combinations of lineage-determining transcription factors prime cistrome regulatory elements required for macrophage and B cell identities. Mol. Cell 38, 576–589 (2010).
11. Huber, W. et al. Orchestrating high-throughput genomic analysis with Bioconductor. Nat. Methods 12, 115–121 (2015).
12. Tsirigos, A., Haiminen, N., Bilal, E. & Utro, F. GenomicTools: a computational platform for developing high-throughput analytics in genomics. Bioinformatics 28, 282–283 (2012).
13. Wickham, H. Ggplot2: Elegant Graphics for Data Analysis (Springer, New York, 2009).
14. Gautier, L., Cope, L., Bolstad, B. M. & Irizarry, R. A. affy—an analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20, 307–315 (2004).
15. Zhou, X. et al. The Human Epigenome Browser at Washington University. Nat. Methods 8, 989–990 (2011).
16. Grant, C. E., Bailey, T. L. & Noble, W. S. FIMO: scanning for occurrences of a motif given a PSSM. Bioinformatics 27, 1017–1018 (2011).
17. Wang, M., Zhao, Y. & Zhang, B. Efficient test and visualization of multi-set intersections. Sci. Rep. 5, 16923 (2015).
18. Huang da, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57 (2009).
19. Rahl, P. B. et al. c-Myc regulates transcriptional pause release. Cell 141, 432–445 (2010).
20. Wang, H. et al. NOTCH1-RBPJ complexes drive target gene expression through dynamic interactions with superenhancers. Proc. Natl. Acad. Sci. USA 111, 765–770 (2014).
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Software and code

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Data collection

Flow cytometry experiments were analyzed with FlowJo 9.3.2. For mass spectrometry experiments we used the SEQUEST algorithm and the Integrated Proteomics Pipeline (IP2, Integrated Proteomics Inc., CA) on Intel Xeon X5450 X/3.0 PROC processor clusters running under the Linux operating system. The validity of peptide/spectrum matches was assessed in DTASelect2.

Data analysis

RNA-seq analysis was performed using lncRNA-screen. Differential expression analysis was performed using DESeq2. Bowtie (version 2.2.6) and STAR aligner (in the case of RNA-seq) were used for alignment of sequenced reads. HOMER (version 4.6) was used for ChIP-seq sequencing peak discovery. The R programming language (version 3.3.0), Bioconductor (version 3.4) and microarray-processing packages were used for microarray analysis. GenomicTools version 2.7.2 was used for performing genomic interval mathematical operations, genomic interval annotations and ChIP-seq profile construction. Illumina identifiers were converted to gene names using Bioconductor (version 3.4) and Illumina HumanHT12v4 annotation data (R package version 1.26). Boxplots and Volcano plots were generated with the untransformed normalized expression values (after quantile normalization), using ggplot2 in combination with ggrepel (in the case of Volcano; R package version 0.6.5). Mouse microarray data were processed with the affy Bioconductor package. Annotation of the probes was performed using Affymetrix Mouse Genome 430 2.0 Array annotation data. In the case of T-ALL cell lines data the analysis was performed as for mouse but using Affymetrix Human Genome U133 Plus 2.0 Array data for annotation. For heatmaps, the expression values were normalized per row (z-score). Heatmaps were generated using R (version 3.3.0). For ChIP-seq analysis sequenced reads were aligned using Bowtie (version 2.2.6). Peak discovery for transcription factors (NOTCH1 and HSF1) was performed with HOMER (version 4.6). FIMO was used for motif analysis. For statistical analysis, we used GraphPad Prism software v.7.0c.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
For all Experiments at least 3 or more replicates/number of animals was used in order to obtain statistical significance.
For all animal studies, >3 animals were used for each experimental cohort per specified genotype/treatment.
For patient samples, at least five samples of high or low nuclear NOTCH1 levels were examined for epichaperome levels and response to HSP90 inhibitors.

Data exclusions
No data were excluded

Replication
All experiments were reproduced two or more times using the same experimental approach. All attempts to replicate were successful.

Randomization
Wild type recipients upon irradiation were randomly selected for transplantation with hematopoietic progenitors derived from HSF1wt or HSF1f/f animals. Also, wild type animals transplanted with hematopoietic progenitors (all having 2-4% GFP positive leukemic cells in the peripheral blood) were randomly selected for treatment with PU-H71 or PBS.

Blinding
Post-mortem tumor burden analysis and IHC in animals was quantified in a blinded fashion. The researcher was unaware of the genotype of the hematopoietic progenitors transplanted to the wild type recipients or the type of treatment (PBS or PU-H71).

For in vitro experiments, the researcher was unaware of the abundance of nuclear NOTCH1 during the epichaperome monitoring and the response to HSP90 inhibitors of patient samples.

Materials & experimental systems

Policy information about availability of materials

Involved in the study

Antibodies
Antibodies against the following proteins were used: polyclonal rabbit HSF1 (Cell Signaling, 4356, lot 2, dilution 1:1000), monoclonal rabbit pS326 HSF1 (Abcam, 76076, lot 1, dilution 1:1000), monoclonal mouse Actin (Millipore, MAB1501, clone C4, lot 2, dilution 1:10000) and goat polyclonal Lamin A (Santa Cruz biotechnology, sc-6214, lot 1, dilution 1:1000), mouse monoclonal HSP90AB1 (GeneTex, 8A342, 4C10, lot1, dilution 1:3000), monoclonal rabbit HSPA8 (Cell signaling, 8444, lot 2, 1:2000), monoclonal rabbit NOTCH1 (Cell Signaling, 4147, Val1744, lot1, dilution 1:1000).
Validation

All Cell Signaling antibodies were validated by the manufacturer using various approaches (https://www.cellsignal.com/contents/our-approach/cst-antibody-validation-principles/ourapproach-validation-principles). For HSP90 antibody please see validation: http://www.genetex.com/HSP90-beta-antibody-4C10-GTX84342.html

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

Cell lines were obtained from ATCC. The CUTLL1 cell line was a gift from Adolfo Ferrando’s lab at Columbia.

Authentication

Cell lines have been authenticated by PCR detection of originally described translocations, detection of intra-nuclear NOTCH1 and sensitivity to originaly described drugs

Mycoplasma contamination

All cell lines tested were mycoplasma negative

Commonly misidentified lines

No misidentified cell lines were used

Research animals

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Animals/animal-derived materials

Mx1-Cre (6-8 weeks old; females; Jackson Laboratory) mice were crossed to Hsf1fl/fl animals (6-8 weeks old; males; a gift from Elizabeth Christians). For NotchDE experiments, C57BL/6 mice (6-8 weeks old; females; Jackson Laboratory) were lethally irradiated and transplanted with infected progenitors isolated from Hsf1+/+;Mx1-Cre or Hsf1fl/fl;Mx1-Cre animals (6-8 weeks old). For the PU-H71 in vivo treatment, C57BL/6 mice (6-8 weeks old; no sex-bias; Jackson Laboratory) were lethally irradiated and transplanted with infected progenitors (NotchDE) from wt syngeneic animals (6-8 weeks old). For Tal1 in vivo experiments, freshly isolated tumor cells were infected with shRNAs against Renilla or Hsf1 and transplanted to FVB/NJ mice (6-8 weeks old; no sex-bias; Jackson Laboratory)

Human research participants

Policy information about studies involving human research participants

Population characteristics

Primary T-ALL patient samples were collected by Columbia Presbyterian Hospital with informed consent and approved and analyzed under the supervision of the Columbia University Medical Center Institutional Review Board.

Method-specific reporting

ChIP-seq

Data deposition

☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

May remain private before publication.

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE90717

Files in database submission

GSM2411124 RNA-Seq analysis in CUTLL1 cells treated with DMSO (rep1)
GSM2411125 RNA-Seq analysis in CUTLL1 cells treated with DMSO (rep2)
GSM2411126 RNA-Seq analysis in CUTLL1 cells treated with shHSF1 (rep1)
GSM2411127 RNA-Seq analysis in CUTLL1 cells treated with shHSF1 (rep2)
GSM2411128 RNA-Seq analysis in CUTLL1 cells treated with GSI (rep1)
GSM2411129 RNA-Seq analysis in CUTLL1 cells treated with GSI (rep2)
GSM2411130 RNA-Seq analysis in CUTLL1 cells treated with GSI and shHSF1 (rep1)
GSM2411131 RNA-Seq analysis in CUTLL1 cells treated with GSI and shHSF1 (rep2)
GSM2411132 ChiP-Seq analysis of HSF1 in CUTLL1 cells (rep1)
GSM2411133 ChiP-Seq analysis of HSF1 in CUTLL1 cells (rep2)
GSM2411134 Sequencing analysis of Input (for HSF1 ChiP) in CUTLL1 cells
GSM2411135 ChiP-Seq analysis of HSF1 in P12 cells (rep1)
GSM2411136 ChiP-Seq analysis of HSF1 in P12 cells (rep2)
GSM2411137 Sequencing analysis of Input in P12 cells
GSM2411138 ChiP-Seq analysis of MYC in CUTLL1 cells (rep1)
GSM2411139 ChIP-Seq analysis of MYC in CUTLL1 cells (rep2)
GSM2411140 Sequencing analysis of Input (for MYC ChIP) in CUTLL1 cells

No longer applicable

Methodology

Replicates

Two replicates for each of the ChIPSeq and RNASeq experiments. Only common targets were selected for further analysis.

Sequencing depth

For RNA-seq, here is the number of reads for each one of the samples:

| Sample                                      | Reads   |
|---------------------------------------------|---------|
| REN_dmso REN_dmso.rep1                      | 41700000|
| REN_dmso REN_dmso.rep2                      | 47600000|
| REN_dmso REN_dmso.rep3                      | 45300000|
| REN_gsi REN_gsi.rep1                        | 43100000|
| REN_gsi REN_gsi.rep2                        | 41100000|
| REN_gsi REN_gsi.rep3                        | 38400000|
| sh7483_dmso sh7483_dmso.rep1                | 42100000|
| sh7483_dmso sh7483_dmso.rep2                | 42700000|
| sh7483_dmso sh7483_dmso.rep3                | 40700000|

For ChIP-seq:

| Sample                                      | Total reads | Aligned reads | De-duplicated alignments |
|---------------------------------------------|-------------|---------------|--------------------------|
| P12-untreated-HSF1-rep1                      | 27526284    | 22718402      | 17678637                 |
| CUTLL12014-untreated-HSF1-rep1               | 26233168    | 22013081      | 21591054                 |
| CUTLL12013-untreated-HSF1-rep2               | 31022966    | 25516468      | 22615138                 |
| P12-untreated-Input-rep1                     | 57138897    | 46644178      | 38400000                 |
| P12-untreated-Input-rep1                     | 38027721    | 30233354      | 25669003                 |
| CUTLL12013-untreated-Input-rep1              | 25239588    | 20830291      | 17918239                 |
| P12-untreated-HSF1-rep2                      | 16433693    | 13673713      | 3646088                  |
| CUTLL12014-untreated-Input-rep2              | 27449449    | 23247930      | 17918239                 |
| CUTLL12013-untreated-Input-rep1              | 23247930    | 20830291      | 17918239                 |
| P12-untreated-HSF1-rep2                      | 16433693    | 13673713      | 3646088                  |
| CUTLL12014-untreated-HSF1-rep2               | 23961647    | 20052138      | 19645211                 |
| CUTLL12014-untreated-HSF1-rep2               | 37901004    | 31666093      | 27718374                 |
| P12-untreated-Input-rep1                     | 42352106    | 35873347      | 28460401                 |
| P12-untreated-Input-rep1                     | 19581496    | 16028753      | 14757933                 |
| CUTLL1-DMSO-MYC-rep1                         | 18563176    | 15259636      | 12785456                 |
| CUTLL1-DMSO-MYC-rep2                         | 15259636    | 12785456      | 10523456                 |
| CUTLL1-DMSO-Input-rep1                       | 42352106    | 35873347      | 28460401                 |
| CUTLL1-DMSO-Input-rep1                       | 38573347    | 31666093      | 27718374                 |
| CUTLL1-DMSO-MYC-rep2                         | 31666093    | 27718374      | 22615138                 |
| CUTLL1-DMSO-MYC-rep1                         | 27718374    | 22615138      | 17918239                 |
| CUTLL1-DMSO-Input-rep1                       | 42352106    | 35873347      | 28460401                 |
| CUTLL1-DMSO-Input-rep1                       | 38573347    | 31666093      | 27718374                 |
| CUTLL1-DMSO-MYC-rep2                         | 31666093    | 27718374      | 22615138                 |
| CUTLL1-DMSO-Input-rep1                       | 42352106    | 35873347      | 28460401                 |
| CUTLL1-DMSO-Input-rep1                       | 38573347    | 31666093      | 27718374                 |

Read length: 51nt. Single-end for ChIP-seq and paired for RNA-seq

Antibodies

Polyclonal rabbit HSF1 (Cell Signaling, 4356)

Peak calling parameters

ChIP-seq:
Read mapping was performed with Bowtie2 (v. 2.2.6) against genome build hg19 setting base calling quality cutoff MAPQ=30
Peak calling:
For HSF1, NOTCH1 and MYC: analysis was performed with HOMER (default settings) and with "factor" peak style option activated
For histone marks: Using MACS2 (2.0.10.20131216) and the following settings: --broad --nomodel --extsize=150 --pvalue=1e-4

RNA-seq was performed with lncRNA-screen and default settings.

Data quality

For both the RNA-seq and ChIP-seq analysis, QC took place. Quality metrics used included base calling quality (MAPQ>30).
Data quality

Specifically for ChIP-seq, the plotFingerprint function from Deeptools (http://deeptools.readthedocs.io) was used to visualize sufficient enrichment over background.

Software

Our pipeline used for ChIP-seq analysis is available here: https://github.com/NYU-BFX/hic-bench/tree/master/pipelines/chipseq-standard

HOMER is available here: http://homer.ucsd.edu/homer/ (Heinz S, Benner C, Spann N, Bertolino E et al. Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. Mol Cell 2010 May 28;38(4):576-589)

For RNA-Seq analysis we used: lncRNA-screen (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5458484/)

Flow Cytometry

Plots

Confirm that:

☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☑ All plots are contour plots with outliers or pseudocolor plots.
☑ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cells isolated through mechanical disruption of thymus, bone marrow and spleen were used from mice. T-ALL patient samples frozen after biopsy were also used.

Instrument

BD LSRFortessa

Software

FlowJo 9.3.2

Cell population abundance

No sorting was performed

Gating strategy

For all Annexin V experiments, debris was excluded by plotting SSC-A versus FSC-A and gating around the cell population. Doublets were excluded by plotting FSC-H versus FSC-A and excluding cells below the 45° axis of a 1:1 ratio. Live cells were then identified by plotting FSC-A versus DAPI and gating around cells negative for DAPI. Finally, Annexin V positive cells were distinguished from Annexin V negative cells by using a heat induced apoptosis positive control and a fluorescence minus one (FMO) negative control. This gate had a frequency of 0.0 in the FMO control.

For all PU-FITC/PU-FIT9 experiments, debris was excluded by plotting SSC-A versus FSC-A and gating around the cell population. Doublets were excluded by plotting first SSC-H versus SSC-A and then FSC-H versus FSC-A and excluding in both cells below the 45° axis of a 1:1 ratio. Live cells were then identified by plotting SSC-A versus DAPI and gating around cells negative for DAPI. Finally, mean fluorescence intensity was calculated by plotting live cells in a histogram in the FITC channel.

☑ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.