Variability and Robustness in T Cell Activation from Regulated Heterogeneity in Protein Levels

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In T cells, the stochasticity of protein expression could contribute to the useful diversification of biological functions within a clonal population or interfere with accurate antigen discrimination. Combining computer modeling and single-cell measurements, we examined how endogenous variation in the expression levels of signaling proteins might affect antigen responsiveness during T cell activation. We found that the CD8 co-receptor fine-tunes activation thresholds, whereas the soluble hematopoietic phosphatase 1 (SHP-1) digitally regulates cell responsiveness. Stochastic variation in the expression of these proteins generates substantial diversity of activation within a clonal population of T cells, but co-regulation of CD8 and SHP-1 levels ultimately limits this very diversity. These findings reveal how eukaryotic cells can draw on regulated variation in gene expression to achieve phenotypic variability in a controlled manner.

In biological systems, the stochasticity of protein expression can contribute to useful phenotypic variability within a clonal population (*1*), yet it also may compromise the reliability of a cellular response. How cells achieve robust physiological performance while experiencing substantial variation in the expression of molecules that control their functional activities has yet to be fully explained (*2*). Theoretical and experimental studies at the single-cell level have described the nature and origin of fluctuations in gene and protein expression (*3*–*7*) and have identified key molecular components controlling phenotypic variability. Most of these have been conducted on single-celled organisms, focusing on bacterial chemotaxis and competence (*8*–*10*).

Understanding the consequences of variation in protein expression is especially relevant for T lymphocyte activation in the immune system. Confrontation of these cells with foreign and self-ligands that bind their receptors initiates a signaling-based decision between productive activation and tolerance (*11*). Reliability of this process is critical for the proper functioning of the immune system and avoidance of autoimmunity (*12*). Nevertheless, ligand discrimination must also be flexible during T cell development (*13*) and diverse to allow differentiation between short-lived effector cells and long-lived memory cells during antigen-induced signaling responses (*14*, *15*). We examined how differences in protein expression within a clonal population might affect these dual features of robust and yet adaptable T cell responses to antigenic ligands.

We first theoretically tested how the natural variability in signaling-components expression within T cells might affect their response. To this end, a previously established computer model of T cell activation (*16*–*19*) was used, in which kinetic proofreading, enforced by two competing signaling feedback loops, accounts for sharp ligand discrimination (Fig. 1A). Signal transduction by the T cell receptor (TCR) of cytotoxic T cells is initiated when the co-receptor CD8 co-associates with TCRs engaged with their ligands [peptide-bound major histocompatability complex class I (pMHC) molecules]. Negative feedback, associated with the soluble hematopoietic phosphatase 1 (SHP-1), limits spurious stimulation by ligands that have weak affinity to the TCR (*20*). Positive feedback, involving the activation of the extracellular signal–regulated kinase 1 (ERK-1), maintains strong signaling if the cell is confronted with more strongly binding ligands (*20*). Because ERK-1 activation is digital ("all or none") in T cells (*19*), we defined the half-maximal effective concentration EC_{50} as the minimal number of ligands able to trigger phosphorylation of ERK-1 into ERK-pTpY (ppERK) in 50% of the cells. Our model predicts that EC_{50} is sharply dependent on the lifetime of the TCR-pMHC complex (Fig. 1B).

This model was then probed to examine how variability in the expression of signaling components might affect the T cell's response to antigen

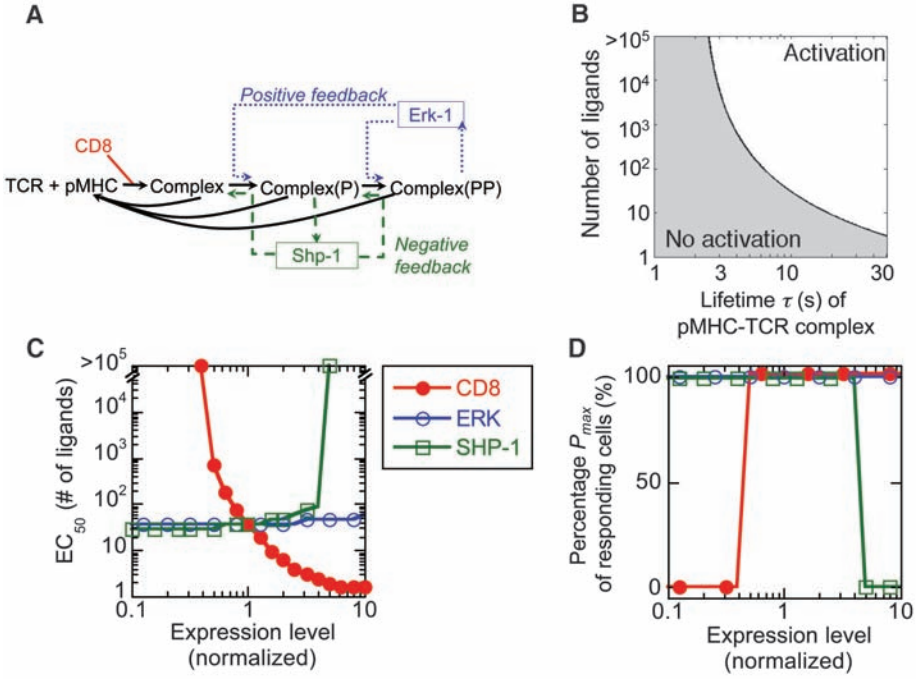

Fig. 1. Computational model of T cell activation links heterogeneity in protein expression with variability in cell response. (**A**) Model of proximal T cell signaling involved in ligand discrimination. (**B**) Predictions of digital ERK activation by different numbers of pMHC ligands, with varied lifetimes τ of interaction with TCR. We define activation as 100% of ERK becoming doubly phosphorylated and inactivation as no doubly phosphorylated ERK. The dependence of EC_{50} on τ is delineated by the black curve. (**C** and **D**) Predictions for the effect of varied expression of proteins on EC_{50} and on the percentage P_{max} of cells responding with $EC_{50} < 10^5$ (for an agonist ligand with $\tau = 10$ s; see fig. S8 for other ligands). Expression levels of proteins are normalized to the average wild-type level.

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(*21*), revealing that signaling components would fall into three categories: noncritical components, defined as signaling proteins whose variation in expression within the observed range does not measurably affect ligand response; analog regulators, which are signaling proteins whose level of expression fine-tunes EC_{50}; and digital regulators, whose level of expression switches a cell between responsive and unresponsive states (among responsive cells, the level of digital regulator does not affect the EC_{50}).

Despite its key role in regulating ligand discrimination, our model predicted ERK-1 to be a noncritical regulator because its average expression is in excess, so that variation within the physiological range would not affect the T cell's response (Fig. 1C). The CD8 co-receptor was predicted by the model to be an analog regulator (Fig. 1C): Higher levels of expression of CD8 considerably decrease the minimum number of ligands required for effective activation, without affecting the percentage of cells that can respond to the maximum dose of ligands (P_{max}). In contrast, the model predicted that SHP-1 would be a digital regulator. Thus, below a critical level (four times above the average level of SHP-1), the TCR signaling network responds consistently to strong agonists, with little variation in activation threshold EC_{50} (Fig. 1C). However, in cases where SHP-1 exceeds the critical level, the EC_{50} for the signaling network becomes infinite, and P_{max} becomes null (Fig. 1D). This means that, as SHP-1 levels increase, an increasing fraction of T cells becomes unresponsive to any physiologically relevant amount of ligands, whereas the other cells remain as sensitive as cells expressing low levels of SHP-1.

Simple mechanisms can explain how CD8 and SHP-1 differently regulate TCR signaling. Because Lck, a kinase associated with the CD8 co-receptor, directly phosphorylates antigen-engaged TCRs, lower levels of CD8 may be compensated for by increased ligand availability, leading to analog regulation. In contrast, at a low level of SHP-1, ligands fail to activate enough of that phosphatase to prevent an early digital ERK response that, in turn, protects TCRs from dephosphorylation by SHP-1. This makes the precise level of SHP-1 irrelevant under these conditions. At higher levels of SHP-1, negative feedback associated with phosphatase activity rapidly affects enough TCRs to prevent ERK activation and suppress effective signaling: The cells become unresponsive to any level of ligand. In combination, these latter effects lead to digital regulation within the T cell population.

To test these predictions, we used a quantitative assessment of protein expression in individual cells, based on antibody staining and flow cytometry (figs. S1 to S4). We studied OT-1 TCR-transgenic Rag1 knockout $CD8^+$ T cell clones, which are cells that express only a single TCR specific for the ovalbumin peptide (residues 257 to 264) bound to the class I molecule K^b (K^b/OVA) pMHC (*13*). For these cells, the distributions of expression for CD8, ERK-1, and SHP-1 were log-normal, with coefficients of variations equal to 0.32, 0.56, and 0.62, respectively (Fig. 2A). Thus, 99% of cells express CD8 within a threefold range of one another, and ERK-1 and SHP-1 within a sixfold range. The large range in expression levels of these key signaling components, in conjunction with our computer predictions of response variability (Fig. 1, C and D), raised the possibility that a clonal population of T cells would not respond homogeneously to antigen stimulation.

To examine this, multiparameter flow cytometry was used to simultaneously analyze individual cells for both endogenous protein expression and the TCR signaling response to antigen. Ligand response was measured by intracellular antibody staining for ppERK (*22*) 5 min after stimulation by peptide-pulsed antigen-presenting cells (*4*–*7*, *21*). Consistent with our model's predictions, variations in the levels of CD8, ERK-1, and SHP-1 were found to have substantially different effects on T cell responsiveness (Fig. 2B and fig. S6). CD8 functioned as a positive analog regulator, such that subpopulations with varied CD8 levels displayed different EC_{50} but had nearly equal P_{max} (Fig. 2B). A sixfold difference in membrane CD8 expression was associated with a 100-fold change in EC_{50} (Fig. 2C and fig. S5). Our model predicted that very low CD8 levels would reduce P_{max}, although this would occur only beyond the normal physiological range of CD8 expression (Figs. 1C and 2A). SHP-1 acted as a negative digital regulator, again consistently with the predictions of the model: Subpopulations with varied levels of SHP-1 expression had different P_{max}, whereas the responsive fractions had the same EC_{50} (Fig. 2C). A twofold increase in SHP-1 levels was associated with a 2.5-fold decrease in P_{max} (Fig. 2D). In contrast to CD8 and SHP-1, and also in agreement with the simulations, variation in ERK-1 expression only marginally affected the T cell

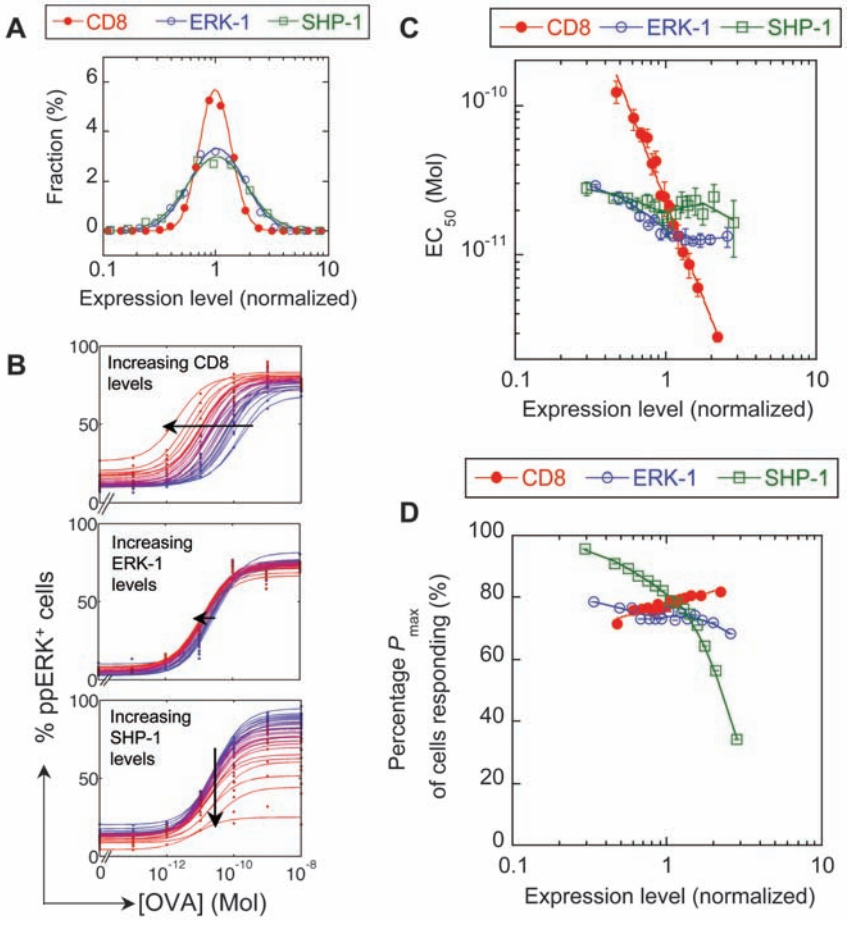

Fig. 2. Experimental single-cell analysis classifies CD8, ERK-1, and SHP-1 as analog, noncritical, and digital regulators of the T cell response, respectively. (**A**) Distribution of endogenous levels of these signaling proteins in OT-1 T lymphocytes. (**B**) Single-cell analysis of OT-1 T cell responses to varying concentrations of OVA peptide at different levels of expressions of CD8, ERK-1, and SHP-1 (blue, low levels; red, high levels). (**C**) EC_{50} and (**D**) P_{max} of different levels of signaling proteins, obtained by fitting the curves in (B). The solid lines are eye guides. Error bars are SEs for triplicate samples.

response in terms of either EC_{50} or P_{max} (Fig. 2, C and D).

These observations demonstrate that a clonal population of T cells can display substantial phenotypic variability based on stochastic variation in the expression of signaling proteins (here, CD8 and SHP-1). In contrast, experimental measurements of interferon-γ (*23*), calcium (*24*), or ppERK (*19*) responses suggest that the T cell antigen response can in some circumstances be highly uniform (robust) within a clonal population. We then identified two different mechanisms, associated with the different categories of signaling components, which enforce such robustness despite stochasticity in protein expression. Digital regulators such as SHP-1 maintain constant EC_{50} but at the expense of a fraction of cells made unresponsive (Fig. 2D), a parameter not often examined in published studies. Analog regulators modulate the cell's responsiveness in a sensitive manner, so their variability in expression levels must be constrained to limit variability in response (*6*, *7*), and this is seen for CD8 (Fig. 2, A and C).

We also considered a third, less obvious, constraint mechanism, based on recent evidence for covariation in gene expression among components in functional networks (*7*, *9*). The contradictory effects of positive and negative modulators might cancel out if their expression levels were co-regulated, reducing response variation in the population. To examine this hypothesis, we examined computer-modeled responses for the effects of varying levels of both CD8 and SHP-1 (Fig. 3A). These analyses revealed that, for any increase of SHP-1 level, there is a corresponding increased CD8 level that maintains a constant EC_{50} (Fig. 3A), which implies that co-regulation of SHP-1 and CD8 expression would limit the diversity of T cell activation.

To explore this prediction experimentally, we analyzed the ppERK dose-response for different CD8 and SHP-1 levels in individual OT-1 T cell blasts and found a good match with our computer simulations (Fig. 3B). Moreover, our analyses revealed that T cell blasts co-regulate their CD8 and SHP-1 levels (Fig. 3C). To quantify how this correlation might limit response functional diversity, we estimated the distribution of EC_{50} by integrating over the distribution of CD8 and SHP-1 levels (Fig. 3, B and C) (*21*). For the natural distribution of signaling proteins (Fig. 3D, left), we found that the resulting distribution of EC_{50} was log-normal with a coefficient of variation of 5.0 (fig. S9). For a hypothetical distribution of CD8 and SHP-1, with the same individual distributions but without co-regulation (Fig. 3D, right), the distribution of EC_{50} would also be log-normal but with a larger coefficient of variation of 18 (fig. S9). In other words, the actual fraction of hyperresponsive OT-1 T cell blasts whose EC_{50} is below 0.1 pM is 0.03%, but this fraction would be predicted to be 10-fold higher (0.3%) without co-regulation of CD8 and SHP-1 levels (Fig. 3D, arrows). Thus, co-regulation decreases the number of hyperresponsive cells and potentially limits the risk of self-responsiveness and autoimmune activation among T cells. Several nonexclusive mechanisms—including gene locus colocalization (*4*), control of transcription by shared factors, and the targeting of transcripts by the same microRNA—may account for this co-regulated expression and will require further analysis to elucidate.

This study predicted theoretically and confirmed experimentally that signaling molecules within a network can have qualitatively distinct effects on cell responsiveness. It also revealed that intraclonal differences in protein expression generate dispersion in responsiveness among individual cells, and demonstrated how co-regulation of expression among these proteins limits this phenotypic variability. For T lymphocytes, these insights help explain how ligand discrimination can be tuned through the regulation of signaling components (*13*, *24*) and diversified (*14*) so that pathogen reactivity is preserved without encouraging autoimmunity. More generally, these observations suggest how cells may leverage regulated stochasticity of intracellular processes to generate controlled variability in physiological performance.

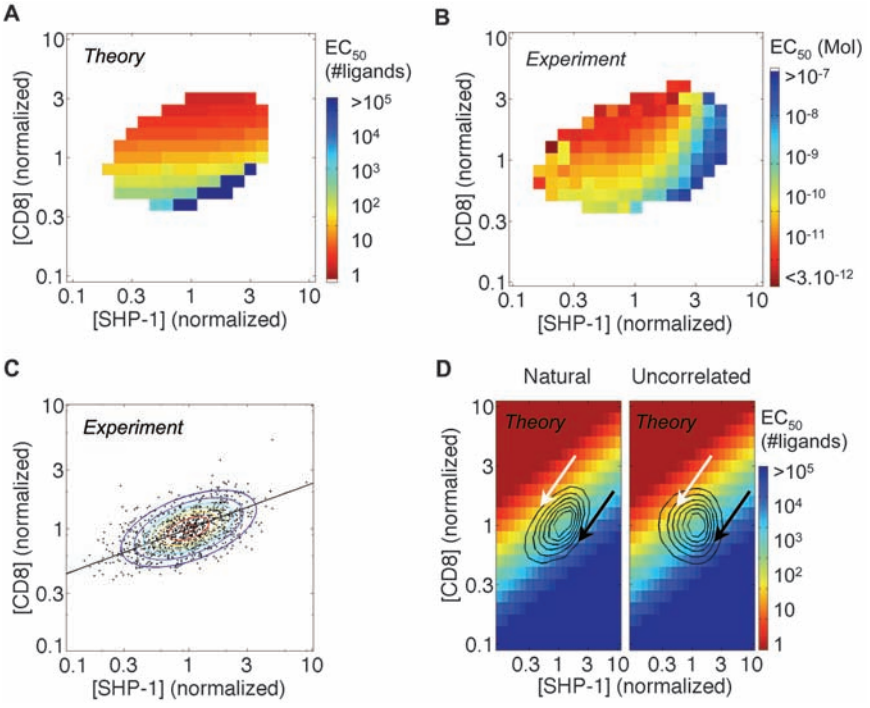

Fig. 3. Covariation of CD8 and SHP-1 levels limits the frequencies of hypo- and hyperresponsive cells. Computer model (**A**) and single-cell experimental measurements (**B**) for T cell activation demonstrate how the EC_{50} for ppERK responsiveness is determined by both CD8 and SHP-1 levels. (**C**) Single-cell analysis of OT-1 T cell blasts shows a correlation between the expression of SHP-1 and CD8. The line is the fit $\log([CD8]) = 0.4 \times \log([SHP\text{-}1])$. (**D**) Overlay of a fitted color map of EC_{50} with the contour plot of the distribution of the levels of CD8 and SHP-1 (left, the endogenous/co-regulated population; right, a theoretical population with uncorrelated levels of CD8 and SHP-1 keeping their individual measured distributions). CD8/SHP-1 co-regulation limits the number of hypo- and hyperresponsive cells (indicated by black and white arrows, respectively).

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25. We thank M. Van der Brink for the generous use of the flow cytometer and J. Allison, N. Altan-Bonnet, P. Cluzel, L. Denzin, M. Huse, E. O'Shea, N. Socchi, and D. Sant'Angelo for comments and discussion.

Supporting Online Material
www.sciencemag.org/cgi/content/full/321/5892/1081/DC1
Materials and Methods
Figs. S1 to S10
References

19 March 2008; accepted 3 July 2008
10.1126/science.1158013

Adenovirus Small e1a Alters Global Patterns of Histone Modification

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Adenovirus small early region 1a (e1a) protein drives cells into S phase by binding RB family proteins and the closely related histone acetyl transferases p300 and CBP. The interaction with RB proteins displaces them from DNA-bound E2F transcription factors, reversing their repression of cell cycle genes. However, it has been unclear how the e1a interaction with p300 and CBP promotes passage through the cell cycle. We show that this interaction causes a threefold reduction in total cellular histone H3 lysine 18 acetylation (H3K18ac). CBP and p300 are required for acetylation at this site because their knockdown causes specific hypoacetylation at H3K18. SV40 T antigen also induces H3K18 hypoacetylation. Because global hypoacetylation at this site is observed in prostate carcinomas with poor prognosis, this suggests that processes resulting in global H3K18 hypoacetylation may be linked to oncogenic transformation.

The adenovirus small e1a protein drives contact-inhibited primary cells through the cell cycle dependent on three conserved regions (CRs) in e1a (fig. S1) (*1*). e1a is not a DNA binding protein, but it binds several other proteins. e1a CR2 binds the retinoblastoma protein (RB) and its paralogs p107 (RBL1) and p130 (RBL2) with high affinity and, together with a lower affinity binding region within CR1, displaces RB proteins from E2F family transcription factors (heterodimers of E2F1 through E2F5 with DP1 or DP2) (*1*–*3*). Release of RB family proteins and their associated repressing chromatin modifying activities (*4*) results in de-repression of cell cycle genes (*1*). Although the N terminus of e1a is not as extensively conserved among primate adenoviruses as CR1 and CR2, it is nonetheless well conserved among these viruses (*5*) and is required to drive cells into the cell cycle (*1*). The N terminus and residues in CR1 that are required for e1a transforming activity bind to several cellular proteins involved in the regulation of chromatin structure, including p300 and CBP, PCAF, GCN5, and p400 (*1*). However, the interaction with p300/CBP is the most important for transformation (*5*) and inhibits p300/CBP-dependent co-activator activity in transient transfection assays (*6*). It is not understood how the e1a interaction with p300/CBP contributes to cell cycling.

In studies potentially related to this question, Seligson *et al*. (*7*) reported that the risk of tumor recurrence in patients with low-grade prostate cancer (tumors with well-formed glandular structures) is related to the global cellular levels of H3K18 acetylation and H3K4 methylation observed by the intensity of nuclear staining with antibodies specific for these histone modifications. Global H3K18 hypoacetylation plus H3K4 hypomethylation strongly correlate with increased risk of tumor recurrence (*7*). Because the e1a interaction with p300/CBP is required for transformation by e1a, and because all the p300 and CBP in a nuclear extract of adenoviros type 5 (Ad5)–transformed 293 cells co-elutes with e1a on a gel filtration column in a complex of ~600 kD (*8*), we asked whether e1a induces a global decrease in histone acetylation and whether such an activity might contribute to e1a's transforming activity.

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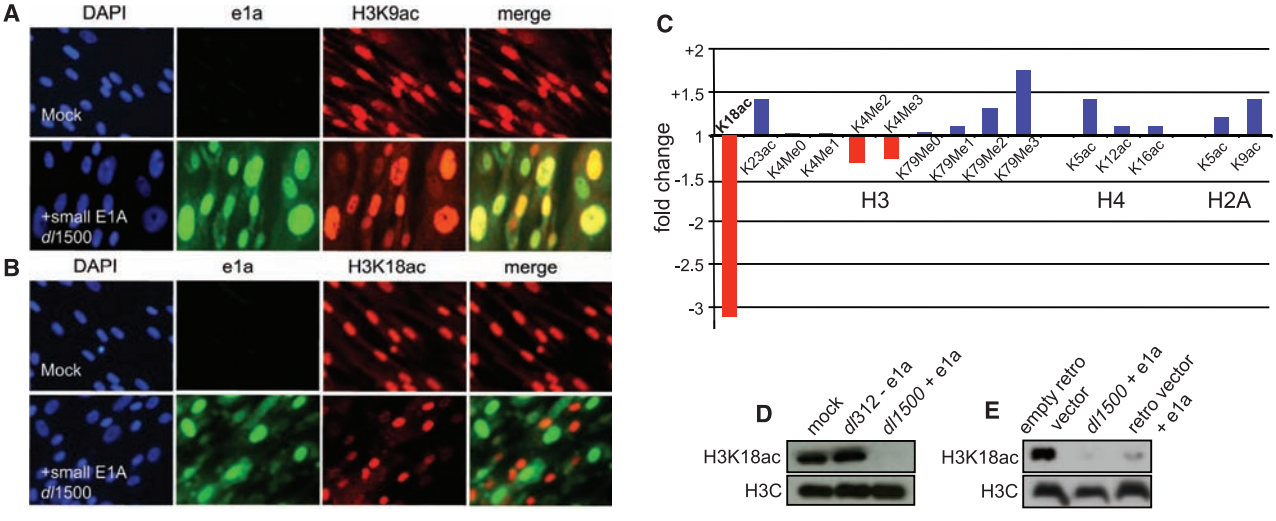

Fig. 1. e1a induces hypoacetylation of H3K18. Contact inhibited human primary IMR90 fibroblasts were mock-infected or infected with Ad5 *dl*1500 (e1a only). 24 hours p.i., cells were fixed and immunostained with antibodies to e1a (green), either (**A**) H3K9ac (red) or (**B**) K3K18ac (red), and 4′,6′-diamidino-2-phenylindole (DAPI) to stain nuclei. Merges of green and red signals are also shown. (**C**) Fold change in histone modifications after *dl*1500-infection of IMR90 cells determined by mass spectrometry (fig. S2). Western blots of histones from (**D**) IMR90 cells infected with *dl*312 (no e1a) or *dl*1500 (e1a only) and (**E**) HeLa cells transduced with the indicated retrovirus vectors or *dl*1500-infected.

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Science **321** (5892), 1081-1084. [doi: 10.1126/science.1158013]

Editor's Summary