Insulin Stimulates Both Leptin Secretion and Production by Rat White Adipose Tissue

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ABSTRACT
Leptin, the peptide encoded by the obese gene, is secreted by adipose cells and plays a role in regulating food intake. Injection of leptin into the hypothalamus suppresses appetite. In addition, animals lacking leptin also exhibit decreased physical activity, hypothermia, and other metabolic defects. Body fat is the most important determinant of circulating leptin levels, but other factors also acutely regulate the production and secretion of leptin. For example, fasting decreases leptin, while refeeding restores the circulating leptin in both mice and humans. Because insulin levels also fall during fasting and are elevated by feeding, it has been hypothesized that insulin may regulate leptin levels. Some studies found that insulin increases the amount of leptin messenger RNA (mRNA) both in rats in vivo and in cultured adipocyte cell lines. However, in other experiments insulin appeared to increase leptin secretion by isolated adipose cells from both rats and humans. In particular, many studies found increased insulin secretion only after prolonged insulin treatment of isolated human or rat adipose cells or cultured 3T3-L1 adipocytes. Interestingly, insulin appeared to stimulate the transport of leptin from the endoplasmic reticulum rather than acting on a pool of regulated secretory vesicles.

LEPTIN IS a secreted peptide encoded by the obese gene and produced primarily by adipose cells (1). Leptin plays a vital role in controlling body weight, presumably by acting in the hypothalamus to suppress appetite. In addition, animals lacking leptin also exhibit decreased physical activity, hypothermia, and other metabolic defects. Body fat is the most important determinant of circulating leptin levels, but other factors also acutely regulate the production and secretion of leptin. For example, fasting decreases leptin, while refeeding restores the circulating leptin in both mice and humans (2, 3). Because insulin levels also fall during fasting and are elevated by feeding, it has been hypothesized that insulin may regulate leptin levels. Some studies found that insulin increases the amount of leptin messenger RNA (mRNA) both in rats in vivo and in cultured adipocyte cell lines (4–7), while in other experiments insulin appeared to increase leptin secretion by isolated adipose cells from both rats and humans (8, 9). However, these results are controversial, and contradictory results have also been reported (10, 11). In particular, many studies found increased leptin secretion only after prolonged insulin treatment of isolated human or rat adipose cells or cultured 3T3-L1 adipocytes (3, 5, 11–13). To address this controversy, we investigated the effect of insulin in a simple in vitro system.

In this study, insulin treatment acutely increased both secretion and production of leptin by rat adipose tissue fragments. Isolated adipose cells lost most of their leptin during preparation, but they also showed the stimulatory effects of insulin. Immediately after isolation, leptin was seen in the endoplasmic reticulum by confocal microscopy. After insulin treatment, there were two populations of cells. In many cells, leptin staining became fainter and was restricted to a narrow band near the plasma membrane. However, in other cells the leptin-staining pattern was unchanged. Leptin did not colocalize with GLUT4, the glucose transporter isofrom found primarily in insulin-responsive cells, in either basal or insulin-stimulated adipose cells. In this study, insulin increased both secretion and production of leptin by adipose tissue fragments. Interestingly, insulin appeared to stimulate the transport of leptin from the endoplasmic reticulum rather than acting on a pool of regulated secretory vesicles.

Materials and Methods

Chemicals
All chemicals were purchased from Sigma (St. Louis, MO) and were of reagent grade unless otherwise noted. The collagenase used to isolate adipose cells was obtained from Worthington Biochemical Corporation (Freehold, NJ), and the BSA used in the incubations was from Interagen Company (Purchase, NY).

Animals
Male rats (210–250 g; CD strain; Charles River Breeding Laboratory, Wilmington, MA) were housed with free access to laboratory chow and water. Animals were anesthetized using CO2/O2 (70%:30%) and killed by decapitation between 0009 h and 1100 h. All animals had food in their stomachs indicating they had eaten during the previous dark cycle. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Tissue and cell isolation
To obtain tissue samples, epididymal fat pads were dissected into proximal and distal segments during removal from the animal. These segments were further sectioned into three pieces in Krebs-Ringer-bicarbonate-HEPES buffer (KRHB) (10 mM NaHCO3, 200 mM adenosine, 30 mM HEPES, pH 7.4) containing 1% BSA (Fraction V) and 0.5 mg/ml bacitracin. Each experimental sample consisted of one proximal and one distal piece. This procedure ensured that each sample contained a mixture of large and small cells, as leptin levels appear to be correlated with the size of the adipose cells (data not shown and Ref. 16). Six samples were obtained from a single animal; lipid content was measured in one.

Received March 12, 1997.
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sample, a second sample was used to determine the leptin content in freshly isolated tissue, and the remaining four samples were used to determine the change in leptin levels as a function of time. Two of these were incubated in the presence and two in the absence of insulin. Thus, insulin-treated tissue was always compared with untreated tissue from the same animal. After tissue samples were blotted and weighed, one sample was placed immediately into extraction buffer (1% Triton X-100, 0.3 M NaCl, 1 mM EDTA, 0.05 mM Tris, pH 7.4) with complete protease inhibitor cocktail (one tablet/50 ml, Boehringer Mannheim, Indianapolis, IN), while the remaining samples were incubated at 37 C in 2 ml KRBBH buffer with 5% BSA and bacitracin, with or without 700 nM insulin, IN), while the remaining samples were incubated at 37 C in 2 ml inhibitor cocktail (one tablet/50 ml, Boehringer Mannheim, Indianapolis, IN). We chose this insulin concentration, which is above the dose needed for maximal stimulation of leptin secretion (data not shown and Ref. 8) for two reasons: 1) to ensure high receptor occupancy quickly in tissue fragments where some time might be needed for diffusion of insulin; and 2) to ensure maximal stimulation throughout a 4-h time course. At the indicated times, the tissue was removed, blotted again, and homogenized in 2 ml extraction buffer with a Dounce homogenizer (12 strokes with loose pestle, followed by 12 strokes with tight pestle). Cell debris was removed by centrifugation at 16,000 x g for 60 min at 4 C.

For preparation of cells, epidydimal fat pads were removed, minced, and digested with Type I collagenase as described previously (17). Three separate samples were analyzed for each time point during each experiment; the cells were incubated at a concentration of 15% (wt/vol) in 1.5 ml KRBBH buffer with 5% BSA and bacitracin, separated by spinning through oil, and extracted as described for tissue.

Leptin antibody

Polyclonal antileptin antibodies were obtained by immunizing rabbits (CYT Immune Services; College Park, MD) with 250 µg glutathione S-transferase (GST)-murine leptin fusion protein produced in Escherichia coli. Affinity-purified antibody was produced by passing the serum over a GST-Sepharose column to remove anti-GST antibodies and then passing the unbound material over a GST-leptin column. Antileptin antibodies were eluted with isoglycine buffer (0.1 M isoglycine, 0.1 M NaCl, pH 3).

Immunoprecipitation and immunoblotting

The cells or tissue extracts and untreated media from each incubation were split into two samples that were immunoprecipitated and quantified separately. Identical samples varied by ±20%, as did different tissue samples from the same animal. For each sample, 25 µl Ultra Plus Protein A beads (Pierce Chemical Co., Rockford, IL) were washed three times in immunoprecipitation buffer (IB, 0.5% Triton X-100, 0.3 M NaCl, 25 mM NaPO4, pH 7.4, 0.02% azide) containing 1% BSA (IB-BSA) and incubated at 4 C in IB-BSA with 5 µl antileptin antisera. After 4–6 h, the beads were washed again and added to extracts or media samples and incubated overnight at 4 C. The beads were sedimented by centrifugation and washed once in IB-BSA, once in IB, and once in Tris-buffered saline, pH 7.4. The immunoprecipitates were solubilized and reduced by boiling in Laemmli sample buffer containing 3.6% (wt/vol) SDS with 20 mM dithiothreitol. Our antileptin antibody quantitatively immunoprecipitated rat leptin. The solubilized polypeptides were separated on 12.5% SDS-PAGE gels and transferred to nitrocellulose by standard methods (18). Leptin was detected by immunostaining with affinity-purified antimeurine leptin antibodies followed by goat antirabbit Fc-specific IgG coupled to horseradish peroxidase (Jackson Immunolabs, West Grove, PA). The resulting bands were visualized using enhanced chemiluminescence substrate (Pierce Chemical Co.). Band intensities were quantified on a Molecular Dynamics computing densitometer (Sunnyvale, CA). The relative amount of leptin was determined by comparing the integrated density of experimental bands to the integrated density of bands from immunoprecipitated recombinant leptin standards, obtained by Factor Xa digestion of GST-leptin fusion protein. After digestion with enzyme, the proteins were separated by HPLC, and the fractions containing leptin were combined. Our quantification consistently gave values 2- to 3-fold lower than the same samples analyzed in the Linco rat leptin RIA (St. Charles, MO). This discrepancy could be caused by a lower affinity of our antibody for rat samples compared with the mouse standards; however, this does not alter any of the conclusions based on comparisons of the relative amounts of leptin in different samples. The secretory rate was calculated as the quantity of leptin secreted into the medium per gram wet weight of tissue divided by the length of time the tissue was incubated.

Students’ t test was performed on Sigma Plot (Jandel Scientific Software, San Rafael, CA). Differences were accepted as significant at the P < 0.05 level.

Preparation and incubation of adipose cells for confocal microscopy

White adipose cells were isolated as described previously (17). At first we used a 1-h collagenase digestion and observed considerable variation in the brightness of leptin immunofluorescence. In fact, some freshly isolated cells had no detectable leptin staining. Shortening the digestion time to 35 min increased the number of cells with bright leptin immu-
nostaining. This shorter digestion was used in the experiments shown in Figs. 6–9. Isolated cells (2–4 × 10^6 cells/ml) were incubated without or with 700 nm insulin at 37 C for 15 min in KRBH with 1% BSA.

**Primary antibodies used in confocal microscopy**

Leptin immunostaining was detected with the same affinity-purified polyclonal antibodies used in the analysis of the immunoblots. For GLUT4, we employed a mouse monoclonal antibody F-27 directed to the C-terminal 14-amino acid sequence, kindly provided by Dr. P. N. Jongensen of Novo Nordisk (Bagsvaerd, Denmark). The specificity of this antibody was previously demonstrated by immunochemical and immunocytochemical techniques (19–22). The antigen calnexin-C rabbit polyclonal antibody SPA-860 from Stressgen Biotechnologies Corp. (Victoria, British Columbia, Canada) was previously used in immunofluorescence studies (23). Rhodamine-conjugated Lens culinaris agglutinin was purchased from Vector Laboratories Inc. (Burlingame, CA).

Fluorescein isothiocyanate (FITC)-and lissamine rhodamine sulfonyle chloride-conjugated IgG, monovalent F(ab) fragments, and divalent F(ab')_2 fragments from Jackson were the secondary antibodies. Specific antirabbit Ig antibodies were used with polyclonal primary antibodies, whereas antimouse Ig antibodies were used with monoclonal primary antibodies.

**Indirect immunofluorescence microscopy**

Single and double immunofluorescence experiments were performed using adipose cells in suspension following the protocol described in detail elsewhere (24). In some experiments, double-labeling immunofluorescence was carried out with two polyclonal antibodies (25, 26). In these cases, the cells were first labeled with the polyclonal anticalnexin antibody for 2 h at room temperature followed by an affinity-purified FITC-conjugated goat F(ab) antirabbit IgG (heavy and light chains) for 1 h at room temperature, following manufacturer’s instructions (Jackson ImmunoResearch) so that all the primary antibodies were sterically covered by the Fab fragments. Then, the cells were incubated with the second polyclonal, antileptin antibody for 2 h at room temperature, followed by a polyclonal affinity-purified lissamine rhodamine sulfonyle chloride-conjugated donkey F(ab')_2 antirabbit IgG (heavy and light chains) for 1 h at room temperature. Two control experiments confirmed the effectiveness of this approach. First, no reaction could be detected with a control, unconjugated polyclonal affinity-purified goat F(ab) antirabbit IgG (heavy and light chains), proving the efficacy of the monoclonal Fab fragment. Second, the pattern of the double labeled cells is identical to immunostaining with each of the respective polyclonal antibodies alone. In all experiments, the immunostained cells were viewed with a Nikon Optiphot 2 fluorescence microscope (Nikon Inc, Melville, NY) equipped with a Bio-Rad MRC1000 or 1024 confocal laser scanning imaging system from Bio-Rad Labs (Hercules, CA). This system uses a mixed argon/krypton laser (λ = 488 nm, blue line for FITC; λ = 568 nm, yellow line for rhodamine) and COSMOS/LaserSharp (Bio-Rad Labs, Hercules, CA) image analysis software. Specimens were viewed using planapochromat x10/1.4NA, x60/1.4NA, and x100/1.4NA objectives. For each experimental condition, 8–10 images per cell were recorded from at least 10–15 cells. Images were collected sequentially for the two fluorochromes in the double-labeling experiments and averaged with a Kalman filter at an optical zoom setting of 1 to 2.5. Colocalization was assessed throughout the cell by examination of several merged images. For presentation, images were further enhanced digitally using Adobe Photoshop 3.0 from Adobe Systems (Mountain View, CA) and printed with a Kodak PS 8650 digital printer (Eastman Kodak, New Haven, CT).

**Results**

**Insulin stimulates leptin secretion and production**

Rat adipose tissue was incubated in vitro for 10–240 min in either the absence or presence of insulin (700 nm). While the amount of leptin secreted into the medium increased with increasing incubation time under both conditions, insulin led to an 80% increase in the amount of leptin secreted (Figs. 1A and 2A). This increase was significant at all times studied.
After 10 min of incubation, the amount of leptin remaining in insulin-treated tissue was less than the amount in untreated tissue. After a 4-h incubation without insulin, the amount of leptin in the tissue extracts decreased to about 50% of the starting amount. However, insulin-stimulated tissue retained 70–80% of the starting amount of leptin (Figs. 1B and 2B). Thus, at all times later than 10 min, insulin increased the total amount of leptin produced, calculated by adding the amount of leptin in tissue to the amount in the medium (Fig 2C). The increases in tissue-associated leptin and total leptin were significant at all times greater than 10 min ($P < 0.05$).

These studies also demonstrated a large variation in the amount of leptin in samples from different animals (Fig. 3). Similar variability in leptin levels was seen in isolated rat adipose cells. However, fresh tissue contained 4-fold more leptin per gram wet weight than an equivalent weight of freshly isolated rat adipose cells.

Nonetheless, the effect of insulin on isolated rat adipose cells was similar to that seen in tissue fragments (Fig. 4). However, in isolated cells, insulin stimulation of leptin secretion was maintained for only 2 h (Fig. 4A). In contrast, insulin increased leptin secretion from tissue fragments throughout the 4-h time course. Insulin-treated cells also contained more leptin than untreated cells (Fig. 4B), although the actual amount of leptin in all adipose cells decreased during the in vitro incubation, as shown for tissue in Fig. 2. Thus insulin-treated cells produced more leptin during the first 2 h of the in vitro incubation (Fig. 4C).

**Subcellular localization of leptin**

We then investigated how insulin affects the subcellular localization of leptin in isolated rat adipose cells using confocal microscopy (Figs. 5–8). In cells with detectable staining, leptin immunostaining was seen in a honeycomb pattern, typical of proteins in the ER of adipose cells (Fig. 5, A–C). This compartment is very distinctive in adipose cells. Previous electron microscopy studies showed highly organized interconnected ER membranes closely associated with the central large lipid droplet and the small cytoplasmic lipid droplets;
in fact, ER cisternae often encase the lipid droplets (27–29). Because of this organization, at the light level ER proteins display an immunofluorescence pattern that outlines the lipid droplets. After 15 min of insulin treatment, two patterns of leptin staining were found (Fig. 5, D-F). In many cells, the staining was faint and restricted to a narrow rim near the plasma membrane (Fig. 5D). In other cells, the leptin staining was bright, and the outlines of the lipid droplets were still visible, indicating an ER localization (Fig. 5, E and F). After 45 min of insulin treatment, more cells showed bright staining, particularly in the presence of insulin, perhaps due to the stimulation of *de novo* synthesis (data not shown).

The ER localization of leptin was confirmed by colocalization with calnexin, an integral membrane protein found in ER membranes (Fig 6, A-C). In cells not treated with insulin, leptin staining was almost identical to calnexin staining. This colocalization was seen in all focal planes throughout the adipose cells. In cells where only dim leptin staining remained after insulin treatment, most of the calnexin was present in regions that no longer stained for leptin (Fig. 6, D-F). Leptin was still visualized in some parts of the ER, possibly places in which leptin was being synthesized. Leptin was never seen in a punctate staining pattern indicative of storage in secretory vesicles, and leptin immunostaining did not resemble the pattern of TGN 38 in the Golgi complex (data not shown).

Variability in the amount of leptin was further documented by immunofluorescence in both basal and insulin-treated cells. When whole cells were fixed and stained immediately after isolation, fluorescent signal was present in all cells, although the intensity of the staining was variable (Fig. 7A). Generally, large cells displayed brighter staining than small cells, which is consistent with biochemical evidence that the former express higher levels of leptin than the latter (data not shown and Ref. 16). When cells were incubated for 15 min without insulin, the fluorescence intensity decreased, consistent with constitutive secretion of leptin (Fig. 7B).
Nonetheless, all the cells still exhibited detectable leptin staining. In contrast, cells that were incubated in the presence of insulin for 15 min showed a greater decrease in overall fluorescence, and about 20% of the cells (74/380) no longer had any detectable leptin staining (illustrated in the left panels of Fig. 8). We then investigated whether this feature is spe-

Fig. 6. Dual immunofluorescence of leptin and calnexin in untreated (A–C) and insulin-treated (D–F) rat adipose cells. In the absence of insulin, leptin (A) and calnexin (B) show very similar staining patterns. A high degree of colocalization is shown in yellow in the merged image (C). In the presence of insulin, leptin immunostaining is restricted to a narrow peripheral rim (D). Calnexin staining is not changed by insulin treatment (E). Merged image (F) shows little overlap (yellow). A few areas of overlap are still observed, particularly where the leptin staining has the distinctive “honeycomb” pattern of the ER. Bar, 20 μm.

Fig. 7. Time-dependent decrease of leptin immunofluorescence in basal adipose cells. When cells were fixed and stained immediately after isolation (A), leptin staining is seen in all the cells, although the fluorescence intensity is somewhat variable. Most of the large cells show very bright leptin staining (indicated by arrowheads). After 15 min of incubation (B), the leptin immunofluorescence decreased in intensity but is still present in all cells. The instrument settings of the confocal microscope were the same for both micrographs. Bar, 50 μm.
specific for leptin by examining the fate of the ER-resident protein calnexin. Figure 8 shows a montage of adipose cells treated with insulin for 15 min and double stained for leptin and calnexin. While the intensity of calnexin staining was relatively uniform (Fig. 8, D–F), the intensity of leptin staining was quite variable (Fig. 8, A–C). In cells with bright leptin staining, leptin staining matched calnexin staining. However, in many cells the leptin staining was faint or undetectable. Apparently these cells secreted their leptin in response to insulin.

The immunostaining of leptin is different from the punctate immunostaining of the GLUT4 glucose transporter (Fig. 9). In basal cells (incubated in the absence of insulin), leptin and GLUT4 did not colocalize (Fig. 9, A and B). Both proteins moved in response to insulin, but little overlap was observed in the two distributions (Fig. 9, C and D). The focal plane for these images was set at the cell surface where a secreted protein such as leptin would not be expected to be found. Presumably, the leptin staining came from protein on the way to the cell surface, whereas...
the GLUT4 staining originates mainly at the plasma membrane.

**Discussion**

Previous studies have shown that refeeding after fasting increases circulating levels of leptin. Our observations are consistent with the hypothesis that this effect may be mediated, at least in part, by the ability of insulin to stimulate leptin secretion by adipose tissue. We have shown that insulin increased leptin production in both rat adipose tissue and isolated rat adipose cells. It is possible that increased leptin synthesis would lead to increased efflux of leptin without a direct effect upon leptin secretion per se. However, two observations suggest that insulin’s action may also be exerted directly at the level of leptin secretion. First, after 10 min of insulin treatment, leptin secretion was increased while the amount of leptin in the tissue decreased. If the increase in leptin efflux had been due only to increased synthesis in the absence of an increase in the rate constant for insulin secretion, this would have required an increase in the cellular content of leptin. Second, insulin changed the subcellular localization of leptin and decreased leptin immunostaining, consistent with secretion from the cell.

In 3T3-L1 and 3T3-F422A adipocytes, it has been consistently reported that insulin increases the amount of leptin mRNA and the rate of leptin secretion (6, 7). However, it is not clear whether insulin exerts a specific effect to regulate synthesis and secretion of leptin or merely promotes adipocyte differentiation. In contrast, studies on insulin treatment of isolated human adipose cells have generally not shown acute stimulation of leptin secretion (12, 30). Studies in isolated rat adipose cells are equivocal: two studies reported effects at both the mRNA and protein levels while a third study showed no increase in leptin mRNA in response to incubation with insulin (4, 8, 10). Our results document stimulation of both production and secretion of leptin in tissue fragments. Moreover, these effects are easier to quantify in tissue fragments because isolated adipose cells contain much less leptin, thus making it difficult to see an effect. Furthermore, after 2 h in culture, isolated adipose cells no longer respond to insulin with increased leptin secretion, indicating they can easily lose the ability to produce leptin.

We observed that insulin increased leptin secretion in vitro. Why then have no increases in circulating leptin been detected in in vivo insulin clamp studies (12, 13, 31–33)? Possible simple explanations include a species difference between rats and humans or the possibility that some adipose cells do not respond like those in epididymal fat tissue. It could be that a delay occurs between secretion by fat cells and appearance in the blood. Another possibility is that insulin affects the clearance of leptin even though leptin appears to be cleared by passive filtration (34). However, the most likely explana-

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**FIG. 9.** Dual immunofluorescence of leptin and GLUT4 in untreated (A and B) and insulin-treated (C and D) rat adipose cells. In a confocal grazing section of an untreated cell, leptin immunostaining (A) shows the characteristic reticular pattern that is quite different from the punctate pattern of GLUT4 (B). After insulin treatment, leptin immunostaining (C) is very weak in a similar grazing section and does not overlap with the strong punctate labeling of GLUT4 (D) staining in the same optical section. Bar, 20 μm.
tion is that the direct effects of insulin are blunted by other effects in vivo. For example, in isolated rat adipose cells, the stimulation of leptin secretion by insulin is inhibited by stimulation of β3-adrenergic receptors (8). Establishing which is the correct explanation is a fruitful area for further study.

Evidently, leptin is not stored in adipose cells or cell lines in culture, but rather is secreted as it is produced, leading to the suggestion that leptin secretion is regulated only by changes in transcription or translation (5–7). However, we observed a rapid increase in the rate of leptin secretion, even at times when the leptin content actually decreased. In addition, our morphological data suggest that insulin accelerated the movement of leptin out of the cell. Therefore, although leptin is not targeted to a stored pool of secretory vesicles, the secretion of leptin appears to be modulated by insulin. Insulin also stimulates secretion of adipin in cultured adipocytes (35, 36). It is possible that insulin increases the movement of cargo throughout the secretory pathway, although it is not known whether leptin and adipin are in the same transport vesicles after insulin treatment. We provide here the first evidence that, at this level of resolution, leptin is seen primarily in the ER as it is colocalized with a well established ER marker, calnexin (37, 38). Recent immunohistochemical studies of leptin localization are consistent with this localization, although the results from these studies have been interpreted as cytoplasmic staining (39–41). This discrepancy is probably technical in origin, possibly caused by the diffusion of the chromogen (39) and the low resolution of conventional immunofluorescence (40). Moreover, secreted proteins generally are cotranslationally inserted into the ER and then travel in vesicles to the cell surface. In 3T3-L1 adipocytes, leptin secretion is inhibited by brefeldin A, indicating that it is in this classical secretory pathway (5). Nonetheless, leptin staining within the Golgi or in secretory vesicles has not been detected. Although direct transport of cholesterol from the ER to the plasma membrane has been reported (42) and the integral membrane protein caveolin may also use this direct route (43), no examples are known of secretory proteins bypassing the Golgi complex on the way to the cell surface. It is tempting to speculate that leptin may be moving via a novel secretory pathway. However, it is more likely that because leptin is not glycosylated, it passes through the Golgi complex quickly without being concentrated there. Clearly, insulin affects leptin secretion from adipocytes by a mechanism that is distinct from the release of stored secretory vesicles.

Acknowledgments

We thank Carol Renfrew Haft for discussions and advice throughout this study, Steven Richards for technical assistance, and Paul Goldsmith for help with the antibody characterization.

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