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[28] Identification of Ubiquitin Ligase Substrates by *In Vitro* Expression Cloning

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Abstract

The number of identified E3 ubiquitin ligases has dramatically increased in recent years. However, the substrates targeted for degradation by these particular ligases have not been easily identified. One reason for the inability of matching substrates and ligases is the finding that E3 recognition elements in substrates are often poorly defined. This minimizes the likelihood that bioinformatic approaches will lead to the identification of E3 substrates. For example, the multi-subunit complex the anaphase promoting complex (APC) is an E3 that recognizes destruction boxes (RXXLXXXXD/N/E) or KEN motifs within substrates (Glotzer *et al*., 1991; Pfleger and Kirschner, 2000). However, many proteins that contain either a potential destruction or a KEN motif are not recognized by the APC *in vitro* or *in vivo*, suggesting that there are other, less well-defined characteristics of substrates that contribute to their ability to serve as APC substrates (Ayad, Rankin, and Kirschner, unpublished observations). Aside from bioinformatic approaches of identifying APC substrates, several groups have also attempted to use affinity techniques to discover novel APC substrates. This has not been widely successful, because many APC substrates are not abundant. Also, as is the case with many ligase-substrate interactions, the affinity of substrates for the APC is likely to be very low. All these considerations have motivated a search for other techniques to

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assist in identifying substrates of this particular E3 ligase. Here, we describe the use of *in vitro* expression cloning to identify novel APC substrates.

In Vitro Expression Cloning

One technique that has led to the discovery of several APC substrates is *in vitro expression cloning* (IVEC; reviewed in Lustig *et al*., 1997). IVEC has been used successfully in our laboratory to identify substrates of both kinases and proteases that are activated in a cell cycle–dependent manner (McGarry and Kirschner, 1998; Stukenberg *et al*., 1997; Zou *et al*., 1999). In this approach, small pools of cDNAs (50–300 clones each) are translated in an *in vitro* expression reaction in the presence of ^{35}S methionine and cysteine, and the radiolabeled reaction products are incubated in cellular extracts containing the kinase or degradation activity of interest. As described later, we generally have used *Xenopus* embryonic extracts, which can be manipulated to enter particular cell-cycle states. More recently, we have used extracts of somatic cells synchronized in particular stages of the cell cycle or treated to activate particular signaling pathways (Wan *et al*., 2001). In all cases, the ability to compare the same protein pools incubated in control extracts greatly reduces the likelihood of identifying inherently unstable proteins (in the case of degradation screens) or constitutively phosphorylated proteins (in screens for kinase substrates). In addition, identifying positive clones from within small pools is generally straightforward and has been described in detail elsewhere (Lustig *et al*., 1997).

We have typically used *Xenopus* cDNA libraries originally created to assist in identifying components of developmental pathways when carrying out IVEC screens. These libraries were constructed by cloning cDNAs from distinct developmental stages, such as egg, blastula, and neurula, into an expression vector, pCS2^{+} (Turner and Weintraub, 1994), which allows *in vitro* transcription under the control of commercially available polymerases (e.g., SP6 from Promega). We have also used cDNAs from adult mammalian brain (Promega) to search for kinase and protease substrates. Because the kinase assays were already described in a prior *Methods in Enzymology* chapter (Lustig *et al*., 1997), we will mainly concentrate on the degradation screens that have led to the identification of several mitotic and G1 substrates of the APC (Fig. 1).

Identification of Mitotic APC Substrates Using *Xenopus* Egg Extracts

The APC must be activated to target substrates for degradation. Activation requires phosphorylation of core APC subunits and association with either one of two proteins, Cdc20 or Cdh1 (Peters, 2002), which confer

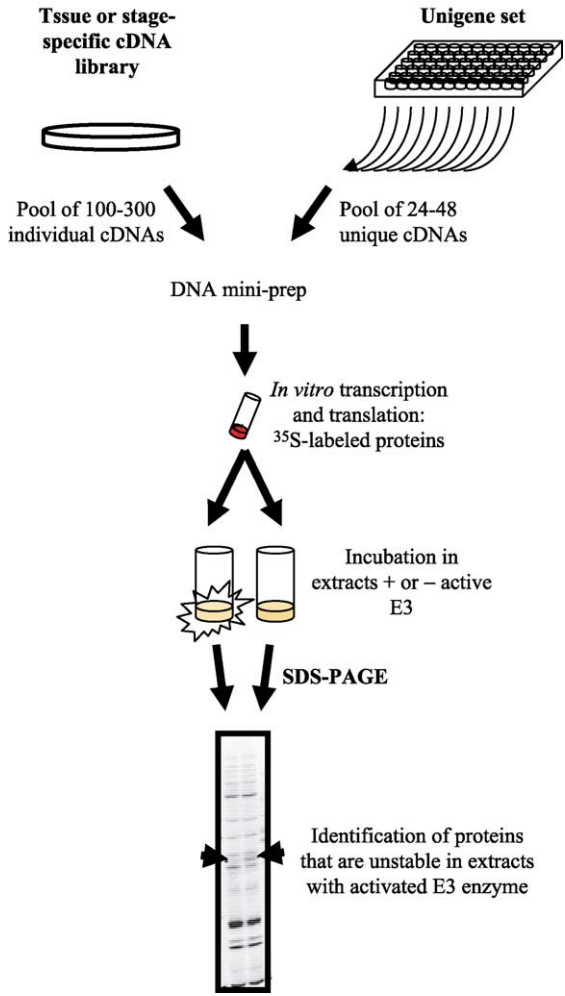

FIG. 1. Schematic illustration of *in vitro* expression cloning (IVEC). Small pools of *in vitro* transcribed and translated radiolabeled proteins are incubated in extracts that have been treated to activate the E3 enzyme of interest. The proteins that are specifically ubiquitinated by the activated E3 are degraded in the extract and can be identified when the pools are run on SDS-PAGE gels adjacent to the same pool incubated in control extracts. See text for details.

substrate specificity to the complex. In the early *Xenopus* embryo, there is little or no Cdh1 expression and no apparent Cdh1-dependent APC activity. The APC^{CDC20} form of the APC is predominant (Lorca *et al*., 1998) and only active during mitosis (not interphase), because Cdc20 association with

the APC is increased in the presence of high mitotic kinase activity (Kotani *et al.*, 1999). These observations provided the rationale for conducting a screen designed to identify proteins degraded when the APC^{Cdc20} is active (mitosis) relative to when it was inactive (interphase).

Xenopus Egg Extract Preparation

Interphase extracts (Murray, 1991) are prepared from activated *Xenopus* eggs presoaked in freshly made cycloheximide (Sigma; 100 mg/ml stock in dimethyl sulfoxide is diluted to a final concentration of 100 μg/ml) to prevent translation of cyclin B from endogenous RNA, which would eventually drive the extract into mitosis. The eggs can be released from a metaphase arrest either using ionophore (A23187; Sigma) at 200 ng/ml final concentration or by using an electrical activation chamber as described in Murray (1991). Extracts are supplemented either with sucrose (300 m*M* final concentration) or glycerol (4% final concentration), stored frozen in liquid nitrogen, and thawed at room temperature or on ice before use. A cocktail (1:1:1 ratio) of energy mix (150 m*M* creatine phosphate, 20 m*M* ATP, pH 7.4, 2 m*M* EGTA, pH 7.7, and 20 m*M* $MgCl_2$, stored at –20°), cycloheximide (0.1μg/ml), and 0.1 μg/ml ubiquitin is added to the extracts (1 μl cocktail:21 μl of preactivated extract) immediately before use. To generate mitotic extracts, interphase egg extracts are incubated with a nondegradable form of cyclin B to drive the extract into mitosis (to yield mitotic extracts) or a buffer control (interphase extracts). The extracts contain all of the accessory activities, including ubiquitin, E1 and E2 enzymes, and proteasomes, required for the efficient ubiquitination and degradation of E3 substrates. Extracts driven into mitosis by the addition of recombinant nondegradable cyclin contain activated APC capable of initiating degradation of several mitotic proteins.

Preparation of Substrate Pools and Degradation Assays

Pools of radiolabeled *in vitro* transcribed and translated cDNAs are prepared. The cDNA libraries are maintained as frozen glycerol stocks, each stock containing a mixture of bacterial strains representing 100–300 discrete cDNA clones. These stocks are used to inoculate liquid bacterial cultures, and small-scale (1–3 ml culture) plasmid preparations from each pool are isolated using a Qiagen spin Miniprep kit (50 μl total per pool). Subsequently, 1 μl of each pool (0.2–0.5 μg/μl DNA) is incubated in a TNT-coupled transcription–translation reaction containing ^{35}S methionine (6 μl total reaction volume, 1 μl DNA:5 μl TnT mix containing rabbit reticulocyte lysate from Promega; we now routinely use trans-^{35}S-label from ICN, Cat#510064, 1175Ci/mmol) at 30° for 1.5 h (we have recently

found that TNT Gold^{Tm} from Promega provides optimal translation). A small volume (1.0 μl) from each pool of *in vitro* translated ^{35}S-labeled proteins is aliquoted into two wells of a 96-well microtiter plate on ice (Costar Thermowell 96-well plate); to one is added 5 μl of the mitotic extract; to the other, 5 μl of the control interphase extract. Once all of the reaction mixes are assembled, the plate is sealed (Microseal A film, MSA-5001; MJ research), moved to room temperature, and both experimental and control reactions are allowed to proceed for 1 h. The reactions are then stopped by the addition of 40 μl of SDS-containing sample buffer (125 m*M* Tris-Cl, pH 6.8, 2% (w/v) sodium dodecyl sulfate, and 10% (w/v) glycerol) supplemented with 1 m*M* DTT. The samples are then heated to 95° for 5 min in a PCR machine (MJ Research PTC-100 programmable thermocycler), and 5 μl of each stopped reaction mixture resolved by SDS-PAGE. Pools containing ^{35}S-labeled proteins present in interphase extracts but missing in mitotic extracts are easily identified. An example of such a degradation screen is shown in Fig. 1. The same pool incubated in control extract is resolved in an adjacent lane of the SDS gel to facilitate identification of positive clones. We routinely use gradient gels (most often 10–15% acrylamide to allow the resolution of proteins of a wide variety of molecular weights).

Three novel APC substrates were isolated from an identical mitotic APC screen: the DNA replication inhibitor geminin, the separase inhibitor securin, and the chromokinesin Xkid (Funabiki and Murray, 2000; McGarry and Kirschner, 1998; Zou *et al*., 1999). All three proteins contain destruction boxes and were subsequently confirmed to be *bona fide* APC substrates *in vitro* and *in vivo*. Interestingly, in the case of securin, *in vitro* expression cloning was the only successful means of identifying this protein, despite our efforts to purify it using conventional chromatography. This illustrates the advantage of *in vitro* expression cloning over standard biochemical techniques when substrates are not abundant or are only transiently expressed during the cell cycle (Zou *et al*., 1999).

In Vitro *Transcription/Translation Mix*	In Vitro *Transcription/Translation Reaction*
200 μl Reticulocyte lysate from Promega	5 μl *in vitro* transcription/translation mix
16 μl TNT buffer from Promega	1 μl Miniprep DNA from pool
32 l ^{35}S-methionine-cysteine	30°, 1.5 h
trans-label ICN (1175 Ci/mmol)	
8 μl methionine-cysteine free	
amino acid mix from Promega	
8 μl Rnasin from Promega	
8 μl SP6, T7, or T3 enzyme	
128 µ*l* dh20	
400 μl total	

Identification of G1 APC Substrates Using *Xenopus* Egg Extracts

There is no apparent G1 phase in the cell cycle in the early *Xenopus* embryo. Work from our laboratory has indicated that the APC present in interphase *Xenopus* egg extracts is inactive but can be activated by the addition of recombinant or *in vitro* translated Cdh1, the G1 activator of the APC (Fang *et al*., 1998; Pfleger and Kirschner, 2000). This finding facilitated our search for proteins specifically targeted for degradation by the G1 form of the APC. We searched for proteins that are unstable in extracts containing Cdh1-activated APC using a strategy similar to the one used to identify substrates of the mitotically activated form of the APC. How this specific screen was performed is described in the following.

Activation of the G1 Form of the APC Complex

Recombinant Cdh1 is produced in SF9 cells and added to concentrated interphase extracts, prepared as described previously, to activate the endogenous APC. In our case, the ratio of interphase egg extracts to recombinant Cdh1 was 20:1 and yielded a 0.4 μM final concentration of Cdh1 in the extract. The Cdh1 or buffer-supplemented extract is incubated at room temperature for 20 min (to allow APC activation) and then returned to ice to assemble the degradation reactions. Again, small pools of *in vitro* transcribed and translated proteins are aliquoted into wells of a microtiter plate and gently mixed separately with the two extracts. The degradation assays are initiated by shifting the plates to room temperature and stopped after 60 min by the addition of SDS sample buffer. *In vitro* translated ^{35}S-labeled Cdc20 is used as a positive control, because Cdc20 is degraded specifically when APC is activated by Cdh1 in *Xenopus* egg extracts.

We were able to isolate two novel APC^{CDH1} substrates using this screening protocol, both of which contained KEN motifs. The first substrate, Tome-1, regulates the G2-M transition in both *Xenopus* egg extracts and somatic cells (Ayad *et al*., 2003; Ayad and Kirschner, in preparation) and is a *bona fide in vitro* substrate of APC^{CDH1}; mutation of the KEN sequence in Tome-1 inhibits its degradation in Cdh1 supplemented *Xenopus* egg extracts. Tome-1 is also degraded by means of the APC *in vivo*, because its levels are lowest during G1 (Ayad *et al*., 2003). The second novel APC^{CDH1} substrate identified is also degraded during G1 and will be described in detail elsewhere (Rankin *et al*., manuscript in preparation). A third APC^{CDH1} substrate has been identified using a modification of this screening method in which a unigene set of *Drosophila* cDNAs was used instead of pools of *Xenopus* cDNAs (Fig. 2; Ooi and Kirschner, unpublished observations).

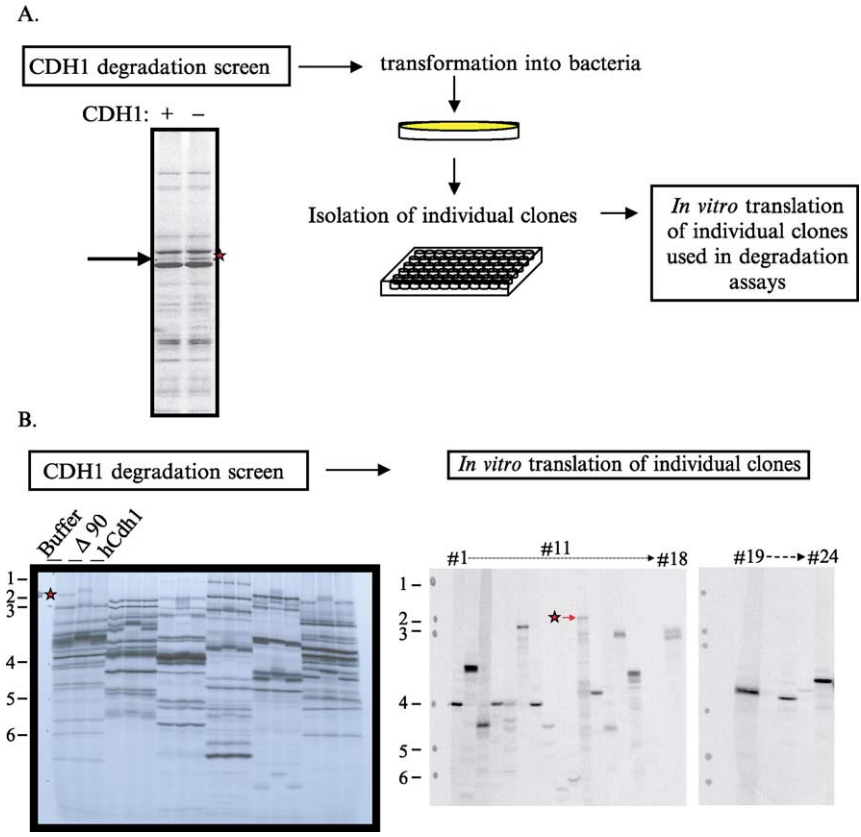

FIG. 2. Sib selection versus isolating positive clones from unigene set. (A) SDS-PAGE of pools of *in vitro* translated proteins incubated in *Xenopus* egg extracts supplemented with Cdh1 or buffer (+ or −, respectively). Once the desired pool is identified, a sib selection procedure is performed to isolate the single clone of interest labeled with star. (B) Alternately, if a unigene set of cDNAs is used, sib selection is not necessary because the single clone can be identified simply by matching the molecular weight of *in vitro* translation products by SDS-PAGE. An example of this technique is shown for a protein degraded in *Xenopus* egg extracts supplemented with Cdh1 and stable in extracts supplemented with buffer or a nondegradable version of cyclin B (Δ90). The band of interest is labeled with a star and has a slightly lower electrophoretic mobility than the #2 molecular weight standard.

Identification of APC Substrates Using Somatic Cell Extracts

We have found *Xenopus* egg extracts invaluable for studying the early embryonic cell cycle and identifying novel APC substrates. However, we realize that many investigators do not have access to *Xenopus laevis* but

would like to use *in vitro* expression cloning to identify E3 ligase substrates. Those investigators who have access to tissue culture cells can also perform *in vitro* expression cloning screens. Recently, we have developed highly concentrated somatic cell extracts that recapitulate *in vitro* degradation of various APC substrates. Using extracts from mink lung epithelial cells or HeLa cells, we have demonstrated that Sno-N and Skp-2 are APC substrates *in vitro*, respectively (Wan *et al*., 2001; Wei *et al*., 2004). Recently, we have been able to recapitulate degradation of APC substrates using frozen extracts as described later (when freezing extracts, it is imperative to do so as quickly as possible using liquid nitrogen and to store the extract at -80°).

Preparation of Somatic Cell Extracts

To prepare concentrated Hela cell extracts from mitotic or G1 cells, 1 to 2 liters of cells grown in suspension are first synchronized with a thymidine/nocodazole block. Cells are grown at 37° in 5% CO_2 in DMEM supplemented with 10% fetal calf serum and 100 μg/ml each penicillin and streptomycin. Thymidine is added to a final concentration of 2 m*M* from a 100$\times$ sterile stock solution for 24 h to asynchronous Hela cells. The cells are released from this block by washing with medium twice and returning them to the tissue culture incubator for 3 h (thymidine release). Nocodazole is then added (330 n*M* final concentration) for 11 h (maximum is 12 h; less is better). Hela S3 cells are generally grown in spinner flasks (Bellco μ-carrier 1000 ml spinner flasks; cells are inoculated at 2×10^5 cells/ml; final concentration at harvesting is 5×10^5 cells/ml). To prepare extracts, cells are pelleted (1000 RPM, 290*g* for 5 min in Sorvall RC3C), and washed three times with PBS. Subsequently, half of the cells are saved on ice (nocodazole-arrested cells for the preparation of mitotic extracts) and the remainder resuspended in culture medium and returned to the tissue culture incubator for 4 h. After 4 h, these cells are pelleted again and washed three times with PBS. These are now a source of G1 cells for the preparation of extracts. As much PBS as possible is removed from both pellets (the nocodazole-released G1 cells and the nocodazole-arrested cells left on ice for 4 h), and the cells are resuspended in swelling buffer (Swelling buffer = 20 m*M* Hepes, pH 7.7, 5 m*M* $MgCl_2$, 5 m*M* KCl, 1 m*M* DTT, ATP regenerating system, and protease inhibitor tablet from Pierce, EDTA-free Complete tablets; 0.75 ml buffer:1 ml packed cells; typical volumes were 2–3 ml of packed cell volume). The samples are frozen and thawed twice (Liquid Nitrogen/30° water bath), then passed through a 20.5-gauge needle twice (1-ml syringes are used; we ice syringes and needles beforehand). The resulting lysates are spun at 5000 RPM

(2655*g*) for 5 min in the cold room in an Eppendorf centrifuge. The supernatants are then collected and spun for an additional 30 min (at top speed, 20,000*g*, 4°, Eppendorf). Finally, the supernatants from the mitotic and G1 cell extracts are carefully isolated (avoiding the top lipid-containing layer) and used in degradation assays that are assembled on ice. These degradation assays are assembled similarly to those detailed previously using *Xenopus* egg extracts. One microliter of *in vitro* translated ^{35}S-labeled substrate is mixed with 20 μl extract (mixed thoroughly but not too vigorously, avoiding frothing) and 1 μl of a cocktail of energy mix, cycloheximide, and ubiquitin (1:1:1 ratio as above) on ice in a 96-well microtiter plate. The reactions are started by shifting the plate to room temperature. Four microliters of each of the reactions are added to 10 μl sample buffer at various time points (usually 60–120 min) to stop the reaction. The stopped reactions are heated to 95° and separated by SDS-PAGE. Using this protocol, we have identified several proteins that are degraded in G1 extracts but not mitotic extracts. The cell cycle role of these proteins is currently being investigated (Rape and Kirschner, unpublished observations).

Practical Considerations

We used 10–15% gradient gels, because they optimally separated proteins in the size range in which we were interested (we rarely found very high molecular weight proteins in our pools); we routinely resolved 30–40 bands (from a 100 cDNA pool). After fixing these gels in a 5% methanol, 7.5% glacial acetic acid solution, and drying them, we exposed them to film for 1 week (we routinely exposed six gels to the same large film), and then proceeded to search for protein bands that were specifically absent in reactions containing Cdh1 (Figs. 1 and 2). Film gave higher resolution than phosphorimaging, and this was necessary in several cases. This initial phase of the screen in which approximately 1300 pools of cDNAs (mostly from neurula stage library, some from egg and blastula stages as well) lasted approximately one and a half months, including exposure to film. Routinely, one person could easily perform 50 *in vitro* transcription-translation and degradation reactions per day, as well as resolve these reactions by SDS-PAGE. After potential APC^{CDH1} substrates within a pool were identified, the degradation reactions were repeated with the positive pool to ensure reproducibility. Beyond this short initial screening phase, the sib selection phase lasted approximately 1 week. Sib selection has been described previously and involves subdividing a pool containing a cDNA encoding a putative substrate until a single clone can be attained (Lustig *et al.*, 1997). Once a single clone was isolated, control reactions were performed to

determine whether substrate degradation could be competed with a known APC substrate such as cyclin B. Finally, the construct encoding the positive substrate was retransformed into bacteria, and then the cDNA was sequenced to identify destruction boxes or KEN sequences.

As mentioned, we have begun to use the *Drosophila* unigene set in addition to other cDNA libraries as a source of cDNAs. The use of unigene sets will ultimately greatly simplify the analysis. First, in libraries that have not been normalized, many rare cDNAs are likely not to be represented, whereas others will appear multiple times. Therefore, in the past, we have identified the same substrates several times, although we failed to identify several previously characterized substrates. The second limitation involved the sib selection procedure. In several cases, we were unable to sib select cDNAs encoding large molecular weight proteins, perhaps because of the toxicity of these clones in bacteria, which might result in their underrepresentation in pools of bacterial transformants. Because the *Drosophila* unigene set is an arrayed set of individual cDNAs, we are able to identify positive clones without sib selection (Fig. 2). We are currently duplicating this technology to create pools of human and mouse cDNAs derived from unigene sets of cDNAs, which should obviate the need to perform sib selection.

Conclusions

The protocols we have discussed were mainly created to search for novel APC substrates. However, they can be easily adapted to identify substrates of other E3s. This can be accomplished by identifying conditions in which the particular E3 is active or inactive, perhaps by isolating cells from different phases of the cell cycle or distinct developmental stages. One major advantage of using the IVEC strategy for the identification of E3 substrates is that it can be done in complex cellular extracts. This might be particularly important if the signals or activities that target particular substrates for degradation might not be well characterized. For example, many SCF complex substrates must be phosphorylated to be recognized by the relevant F-box protein. Identification of the specific kinase might not be necessary if extracts can be prepared from cells isolated under the appropriate conditions. In terms of developmental control of proteolysis, we found that the homeobox transcriptional repressor Xom is degraded during a specific stage of *Xenopus* development by means of an SCF complex containing β-TRCP (Zhu and Kirschner, 2002). It is likely that other degradation reactions are similarly controlled during development, thereby providing the basis for various *in vitro* expression-cloning screens. It may also be possible to include dominant-negative versions of a particular E3

into an extract capable of supporting degradation and search for proteins that are stabilized in the presence of the dominant negative. Finally, we had the advantage of having some known APC substrates to use as internal controls. Therefore, if any substrates are known for an E3, they can be used to judge the robustness of the *in vitro* expression cloning screen.

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