**Supplementary Material For:**

Ubiquitin-fusion protein system: a powerful tool for ectopic protein expression in mammalian cells

Konstantin Matentzoglu and Martin Scheffner
*University of Konstanz, Konstanz, Germany*

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**Construction of the HA-puro-ubi vector**

To construct the HA-puro-ubi vector, a cDNA encoding human ubiquitin-K48R was PCR amplified with the oligos 5′-CACAGATCTAGTGCAGATTTT CGTCAAGAC-3′ and 5′-GCGGATCCCT TGTCATCGTCGTCCTTGATAG TCCATGCCAC CGCGGAGCCCT TAGCAC-3′ resulting in a BglII-ubi-Flag-BamHI fragment. The puromycin N-acetyltransferase cDNA was PCR-amplified from pIRESpuro (Clontech, Saint-Germain-en-Laye, France) with the oligos 5′-GGGGTACCAT GTACCCCATAC GATGTTCCAG ATTACGCTCT TGGCAATGACC GAGTACAAGC ACCAC-3′ and 5′-GCGGATCCTGCGGGTCATGCAGTGACC GCCGTCATG-3′ resulting in a KpnI-HA-puro-Flag-BamHI fragment. The KpnI-HA-puro-Flag-BamHI fragment was cloned into pcDNA3 (Invitrogen, Carlsbad, CA, USA) using the KpnI/BamHI sites within the multiple cloning site of pcDNA3. The resulting vector was then used to clone the BglII-ubiquitin-Flag-BamHI fragment into the BamHI site yielding the HA-puro-ubi vector, which was used as parental vector for the cloning of all other HA-puro-ubi constructs.

**Supplementary Figure 1.** Expression of EGFP or E6AP from HA-puro-ubi constructs is more efficient than from IRES-based constructs. (A) H1299 cells were transfected with pcDNA3-EGFP [driving the expression of the enhanced green fluorescent protein (EGFP) by a cytomegalovirus (CMV) promoter and of the puromycin resistance gene by the simian virus 40 (SV40) promoter], pcDNA3-EGFP-IRESpuro [transcription of the EGFP and the puromycin resistance orph open reading frames (ORFs) is driven by a CMV promoter resulting in a single transcript encoding both EGFP and the puromycin resistance marker protein; translation initiation of the puromycin resistance ORF is mediated by the internal ribosomal entry sites (IRES) element], and pcDNA3-HA-puro-ubi-Flag-EGFP. Twenty-four hours after transfection, puromycin was added (final concentration 4 μg/mL), and transfected cells were selected for more than 4 weeks. Cells were then analyzed for EGFP expression by flow cytometry. (B) HeLa cells were transfected with pcDNA3-IRESpuro expression constructs encoding Flag-tagged versions or wild-type E6AP (wt) or a catalytically inactive mutant of E6AP (C820A). In parallel, HeLa cells were transfected with expression constructs for HA-puro-Flag fusion protein forms of wild-type E6AP and E6AP-C820A. Twenty-four hours after transfection, an aliquot of the cells was prepared for Western blot analysis. The remaining cells were put under selection by addition of puromycin (final concentration 4 μg/mL). Ninety-six hours upon selection, ectopic expression of E6AP was analyzed by Western blot analysis.
Supplementary Figure 2. Expression of HA-puro-ubi does not induce p53 activation. U2OS cells (expressing endogenous wild-type p53) were transfected with the expression constructs indicated and levels of endogenous p53 determined 24 h (A) and 120 h (B) after transfection by Western blot analysis using a mouse monoclonal to p53 (DO1; Calbiochem, San Diego, CA, USA). As a positive control for induction of p53 stability, nontransfected U2OS cells were treated with 5 nM (final concentration) of ActinomycinD (parental U2OS plus ActD), which is known to induce p53 stabilization. Twenty-four hours upon transfection, p53 levels are enhanced independent of the expression construct used, indicating that the stress induced by the transfection procedure is sufficient to induce p53 stabilization. One hundred twenty hours after transfection (96 h of selection), p53 levels are no longer increased, indicating that none of the constructs used results in permanent activation of p53. Since cell lines (pooled clones) stably expressing HA-puro-ubi-Flag-EGFP can be readily established (see Supplementary Figure 1A), this indicates that at least under the conditions used, expression of HA-puro-ubi is not cytotoxic.