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*Keratinocyte Sensitization to TNF-induced NF- $\kappa$ B activation by the E2 regulatory protein of Human Papillomaviruses*

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10 running title : Activation of NF $\kappa$ B by Human Papillomavirus E2 protein

## **Summary**

Human Papillomavirus life cycle requires extensive manipulation of cell signaling to provide conditions adequate for viral replication within stratified epithelia. In this regard, we show that the E2 regulatory protein of  $\alpha$ ,  $\beta$  and  $\mu$ -HPV genotypes enhances TNF-induced activation of NF $\kappa$ B. This activation is mediated by the N-terminal domain of E2, but does not rely on its transcriptional properties. It is independent of the NF $\kappa$ B regulator Tax1BP1, which nevertheless interacts with all the E2 proteins. E2 specifically activates NF $\kappa$ B pathways induced by TNF, while IL1-induced pathways are not affected. E2 stimulates the activating K63-linked ubiquitination of TRAF5, and interacts with both TRAF5 and TRAF6. Our data suggest that E2 potentiates TNF-induced NF $\kappa$ B signaling mediated by TRAF5 activation through direct binding. Since NF $\kappa$ B controls epithelial differentiation, this activity may be involved in commitment of infected keratinocytes to proliferation arrest and differentiation, both required for implementation of the productive viral cycle.

## **Main Text**

Human papillomavirus (HPV) infect squamous epithelia of the skin (cutaneous HPV) or mucosa of the genital or oral tractus (mucosal HPV). They are responsible for asymptomatic infections or induce benign proliferative lesions which possibly evolve toward malignant progression only for “high risk” HPV (HR-HPV).

Infection initiates in the basal proliferative cells where the E6 and E7 early proteins interfere with cell cycle controls, thereby inducing cell proliferation in supra-basal layers. High levels of viral DNA replication, synthesis of capsid proteins, and virus particles assembly are confined to suprabasal and terminally differentiated keratinocytes (Doorbar, 2006). The early protein E2 binds to specific sites within the viral genome to ensure its mitotic segregation in basal infected cells, as well as the regulation of viral transcription and replication (Desaintes & Demeret, 1996; McBride *et al.*, 2006). In addition, the E2 proteins of mucosal HR-HPV directly impact on cell proliferation by inducing a G2/M arrest (Bellanger *et al.*, 2005) and apoptosis (Thierry & Demeret, 2008).

Upon viral infection, the host initiates a cell response mediated by NF $\kappa$ B. This transcription factor leads to the expression of a large panel of cytokines as well as cell cycle and survival genes. Accordingly, many viral pathogens developed strategies to modulate NF $\kappa$ B signaling in order to enhance viral replication, host cell survival, and evasion to immune responses (Hiscott *et al.*, 2001). Surprisingly, little is known about the regulation of NF $\kappa$ B by HPV. Viral oncoproteins have been reported to induce either attenuation or enhancement of NF $\kappa$ B signaling depending on cellular context (James *et al.*, 2006; Nair *et al.*, 2003; Nees *et al.*, 2001; Spitkovsky *et al.*, 2002), and a clear picture of the role of NF $\kappa$ B in the natural history of HPV infection and HR-HPV associated carcinogenic progression is lacking.

To address this issue, we analyzed the impact on NF $\kappa$ B signaling of the regulatory E2 proteins from cutaneous HPV of  $\alpha$  (HPV 3),  $\beta$  (HPV 5, 8, 9) or  $\mu$  (HPV 1) types, and mucosal  $\alpha$  types Low-Risk (LR-HPV 6, 11) or HR (HPV16, 18, 33, 39). NF $\kappa$ B activation was monitored through the activity of a luciferase reporter gene

under the control of NF $\kappa$ B in HaCaT keratinocytes, with an RNA polIII-directed Renilla reporter used as internal control. NF $\kappa$ B activity was measured either without or with TNF treatment (20 ng/ml) for 20 hours, as it plays a central role in response to viral infection. The E2 proteins were expressed as GFP-fusion proteins which have  
90 been shown to keep functions, stability and sub-cellular distribution of the untagged E2 polypeptides (Bellanger *et al.*, 2001; Blachon *et al.*, 2005). All GFP-E2 proteins enhanced TNF-induced NF $\kappa$ B activation, when compared to control GFP-expressing cells (Figure 1-a). Of note, the E2 proteins from cutaneous  $\beta$  or  $\mu$ -HPV (HPV1, 5, 8, 9) activated NF $\kappa$ B reporter to the highest levels. In contrast, E2 proteins had no significant effect on NF $\kappa$ B reporter activity without TNF, indicating that E2 did not induce constitutive NF $\kappa$ B signaling (supplemental Figure 1).

The untagged version of E2 from HPV 5, 11 and 18 enhanced TNF-induced activation of the NF $\kappa$ B reporter similarly as their GFP fusion counterparts, indicating that the GFP tag does not exaggerate the observed NF $\kappa$ B activation (Figure 1-b, left  
100 panel). The expression of untagged E2 proteins was monitored through activation a synthetic E2-responsive promoter (Thierry *et al.*, 1990) (Figure 1-b, right panel). Overall, these results establish that the E2 proteins of various HPV potentiate TNF-induced NF $\kappa$ B activation.

We next determined whether NF $\kappa$ B activation could be assigned to a specific domain of the E2 protein. The N-terminal domain, central Hinge and C-terminal domains of HPV5, 11 and 18 E2 proteins were expressed separately in fusion with GFP, and their effect on the activation of NF $\kappa$ B reporter was analyzed. For the three E2 proteins, expression of the N-terminal domain enhanced TNF-induced NF $\kappa$ B activation, whereas the Hinge and C-terminal domains had no effect (Figure 1-c). The  
110 transactivation-dead point mutant I77A of HPV 18 E2 (Desaintes *et al.*, 1999) enhanced TNF-induced NF $\kappa$ B activation with an efficiency similar to that of the wild-type protein, indicating that N-terminal domain of E2 mediates NF $\kappa$ B activation independently of its transcriptional activity (Figure1-d).

The E2 proteins from HPV16 and 18 have been shown to interact with Tax1BP1 (Wang *et al.*, 2009b), which is a known regulator of NF $\kappa$ B signaling (Jouno *et al.*, 2009). Despite this interaction could be detected with the whole range of E2 proteins (see below), silencing of Tax1BP1 through Si-RNA did not relieve HPV5 or

18 E2-mediated NF $\kappa$ B activation (Figure 2-a). Tax1BP1-directed Si-RNA reduced the level of ectopically expressed Tax1BP1 (Figure 2-b), and lowered E2-dependent transcription for HPV16 or 18 E2, but not for HPV5 E2 (supplemental figure 2). Altogether, these results suggest that enhancement of TNF-induced NF $\kappa$ B activation by E2 in HaCaT keratinocytes is not mediated by E2 binding to endogenous Tax1BP1. However this interaction affects transcriptional activation by some of the E2 proteins, as previously described for BPV1E2 (Wang *et al.*, 2009b).

We then analyzed whether E2 could stimulate other NF $\kappa$ B signaling pathways, as those triggered by IL1 $\beta$ . Expression of E2 had no effect on NF $\kappa$ B activation upon IL1 $\beta$  treatment (20 ng/ml for 20 hours), in contrast to TNF-induced NF $\kappa$ B activation (Figure 2-c). Our results suggest that the E2 protein specifically activates TNF-induced NF $\kappa$ B signaling.

130 NF $\kappa$ B signaling involves different intermediates, such as TRAF6 for IL1-induced pathways and TRAF2 or TRAF5 for TNF-induced pathways (Lomaga *et al.*, 1999; Tada *et al.*, 2001). The TRAF proteins are E3 ubiquitin ligases catalyzing the non-degradative K63-linked polyubiquitination of NF $\kappa$ B signaling factors, which plays crucial role to recruit and activate downstream factors (Chen & Sun, 2009). We examined TRAF ubiquitination patterns in the presence or absence of HPV18 E2 using HA-tagged ubiquitin with only one lysine available for polyubiquitin chain formation at position 29 (K29 ubiquitin), 48 (K48 ubiquitin) or 63 (K63 ubiquitin). As shown in Figure 3-a, the polyubiquitination of TRAF5 and 6 was enhanced upon HPV18 E2 coexpression, whereas changes were minimal for TRAF2. The activating K63-linked ubiquitination of TRAF6 was detected in the absence of E2, in accordance to previous report (Lamothe *et al.*, 2007). It was not further increased upon E2 expression, in line with the lack of activation of TRAF6-dependent NF $\kappa$ B pathways by E2. In the presence of E2, TRAF6 exhibited enhanced K29- and K48- polyubiquitination, which essentially target proteins for degradation (Xu *et al.*, 2009). In contrast, for TRAF5, E2 expression enhanced the activating K63-polyubiquitination, concurrently to K29-polyubiquitination (Figure 3), suggesting that E2 enhanced TRAF5 activation.

We next examined whether E2 could act through direct binding to the TRAF proteins, using the SPICA assay (Sensing of Protein-protein Interactions by

150 Complementation Assay). This assay is based on the reconstitution of *Gaussia princeps* luciferase activity upon coexpression of interacting proteins in fusion with two inactive fragments of the enzyme (Cassonnet *et al.*) Briefly, reconstituted Gaussia activity is expressed as a Ratio of Luminescence, RL. RL is given by the Gaussia luciferase activity obtained upon coexpression of protein pairs divided by the sum of luciferase activity of each single protein expressed against the corresponding empty vector. Using this assay, the binding to Tax1BP1 was detected with all E2 proteins. In addition, the E2 proteins were found to interact with both TRAF5 and TRAF6, but not with TRAF2 (figure 3-b). Such binding potentially underlies the effect of E2 on the ubiquitination pattern of TRAF 5 and 6. Altogether, our findings suggest  
160 that E2 potentiates a TNF-induced NF $\kappa$ B signaling pathway mediated by TRAF5 through direct interaction.

In this study, we present evidence that the HPV E2 protein stimulates NF $\kappa$ B activity upon TNF induction, through its N terminal domain but independently of its transcriptional functions. The E2 protein functions primarily as a regulator of viral genome transcription, replication and segregation through binding to the viral genome. Our study indicates that E2 activates the transcription of NF $\kappa$ B target genes upon TNF induction independently of its transcriptional functions. In addition, this activity is independent of the Tax1BP1 protein, although this factor is involved in the regulation of NF $\kappa$ B signaling (Journo *et al.*, 2009; Shembade *et al.*, 2007) and binds to E2  
170 (Wang *et al.*, 2009b) and this study). This interaction has been shown to take part to the transcriptional activation properties of BPV1 E2 (Wang *et al.*, 2009b), which was observed here for HPV16 and 18 E2 proteins.

NF $\kappa$ B activation is conserved throughout 11 HPV genotypes representative of diverse HPV tropism and pathogenicity, indicating that it is a basic process of HPV infection. The activation of NF $\kappa$ B by TNF has a unique role in the control of epithelial differentiation. TNF induces both an arrest in cell proliferation and the activation of keratinocyte differentiation markers (Basile *et al.*, 2003; Basile *et al.*, 2001), thereby being an essential factor inducing epithelial differentiation. In the course of viral cycle, the sensitization to TNF-induced NF $\kappa$ B activation by E2 may be required to activate  
180 the differentiation program of infected keratinocyte, which is necessary for the late phase of the viral cycle. The E2 protein is known to alter the transcription of

differentiation markers through cooperation with cellular transcription factors as C/EBP (Hadaschik *et al.*, 2003) or through direct binding to cellular promoter (Oldak *et al.*, 2004). Our study indicates that E2 possibly stimulates keratinocyte differentiation through activation of NF $\kappa$ B target genes. During viral cycle, E6 and E7 expression prevent cell cycle exit and differentiation of infected keratinocytes. The E2 protein would overcome these effects by inducing the transcription of keratinocyte differentiation genes through multiple mechanisms, including sensitization to TNF-induced NF $\kappa$ B activation.

190            Since TNF induces a cell cycle arrest in keratinocytes, E2 activity would also provide adequate conditions for high levels of viral DNA replication, which has been shown to occur in pseudo G2-arrested cells in the supra-basal layer of infected epithelia (Wang *et al.*, 2009a). Of note, the  $\beta$  and  $\mu$ -skin genotypes produce lesions with high virus content when compared to lesions associated with  $\alpha$  genital HPV. The high level of NF $\kappa$ B activation by E2 of cutaneous HPV would thus correlate with viral yield, in line with its role in viral DNA replication

              Several lines of evidence point to a role of E2 in the carcinogenic conversion associated with HR-HPV. In case of HR-mucosal HPV, E2 induces a G2/M arrest through inactivation of the Anaphase Promoting Complex (Bellanger *et al.*, 2005),  
200            inducing genomic instability, thus favoring cell immortalization. In addition, E2 from HR cutaneous HPV8 exhibits intrinsic oncogenic potential when expressed in the skin of transgenic mice (Pfefferle *et al.*, 2008). E2-induced skin-tumors are associated to impaired wound-healing and chronic inflammation (Pfefferle *et al.*, 2008). Since NF $\kappa$ B signaling is a major mediator of inflammation, sustained activation of NF $\kappa$ B by E2 could have a role in such chronic inflammation, and thus participate to HPV-associated carcinogenesis. This could add to E6 and E7 immortalizing properties during natural infection .

              The TRAF2, 5 and 6 proteins are essential mediators of NF $\kappa$ B signaling. We found that E2 stimulates one or both of the K29- or K48- forms of polyubiquitination of  
210            TRAF5 and TRAF6 which target proteins to degradation (Xu *et al.*, 2009). E2 concurrently stimulates the activating K63-ubiquitination of TRAF 5, potentially underlying the enhancement of TNF-induced NF $\kappa$ B activation by E2. In addition, E2 binds to both TRAF5 and TRAF6, providing evidence that E2 could alter their

ubiquitination pattern through direct interaction. It is unclear at present how E2 stimulates the polyubiquitination of TRAF 5 and 6. It can be envisioned that E2 binding to the TRAF proteins could facilitate association with Ubiquitin-Activating enzymes or ubiquitin ligases, or could increase accessibility of the target lysines through conformational changes. Our data nevertheless suggest that E2 potentiates a signaling pathway mediated by TRAF5 through direct interaction.

220 In conclusion, we provide evidence that the E2 regulatory protein of human papillomaviruses manipulates specific cell signaling, as shown here with the activation of a TNF-induced NF $\kappa$ B pathway mediated by TRAF5. This effect is likely involved in commitment of infected keratinocytes to arrest in proliferation and differentiation, both required for implementation of the productive viral cycle within stratified epithelia. Given that cancers arise from initial precancerous productive lesions associated with HR-HPV, such activities are relevant for the carcinogenic progression of HR-HPV associated lesions.

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340

## Figures Legend

### Figure 1 : Stimulation of TNF-induced activation of NF $\kappa$ B by HPV E2 protein.

(a) HaCaT cells were transfected with NF $\kappa$ B reporter p-NF $\kappa$ B-Luc, p-polIII-Ren and the indicated GFP-E2 expressing plasmids. Luciferase activity was measured 24 hours post transfection, normalized to Renilla and given in Relative Luciferase Activity. (b) HaCaT cells transfected with NF $\kappa$ B- or E2- reporters and expression plasmids for the indicated untagged E2 proteins or pCIneo, and processed as in a. (c) HaCaT cotransfected with NF $\kappa$ B-reporter and expression plasmids for GFP fusions with N terminal (N), Hinge (H) or C terminal (C) domains of HPV5, 11, or 18 E2 proteins, then treated as in a. (d) HaCaT cotransfected with NF $\kappa$ B or E2 reporters and with expression plasmids for wild-type or I77A HPV18 E2 protein fused to GFP, then treated as in a. Error bars indicate the standard deviation of the mean of 2 (without TNF) to 8 (plus TNF) samples.

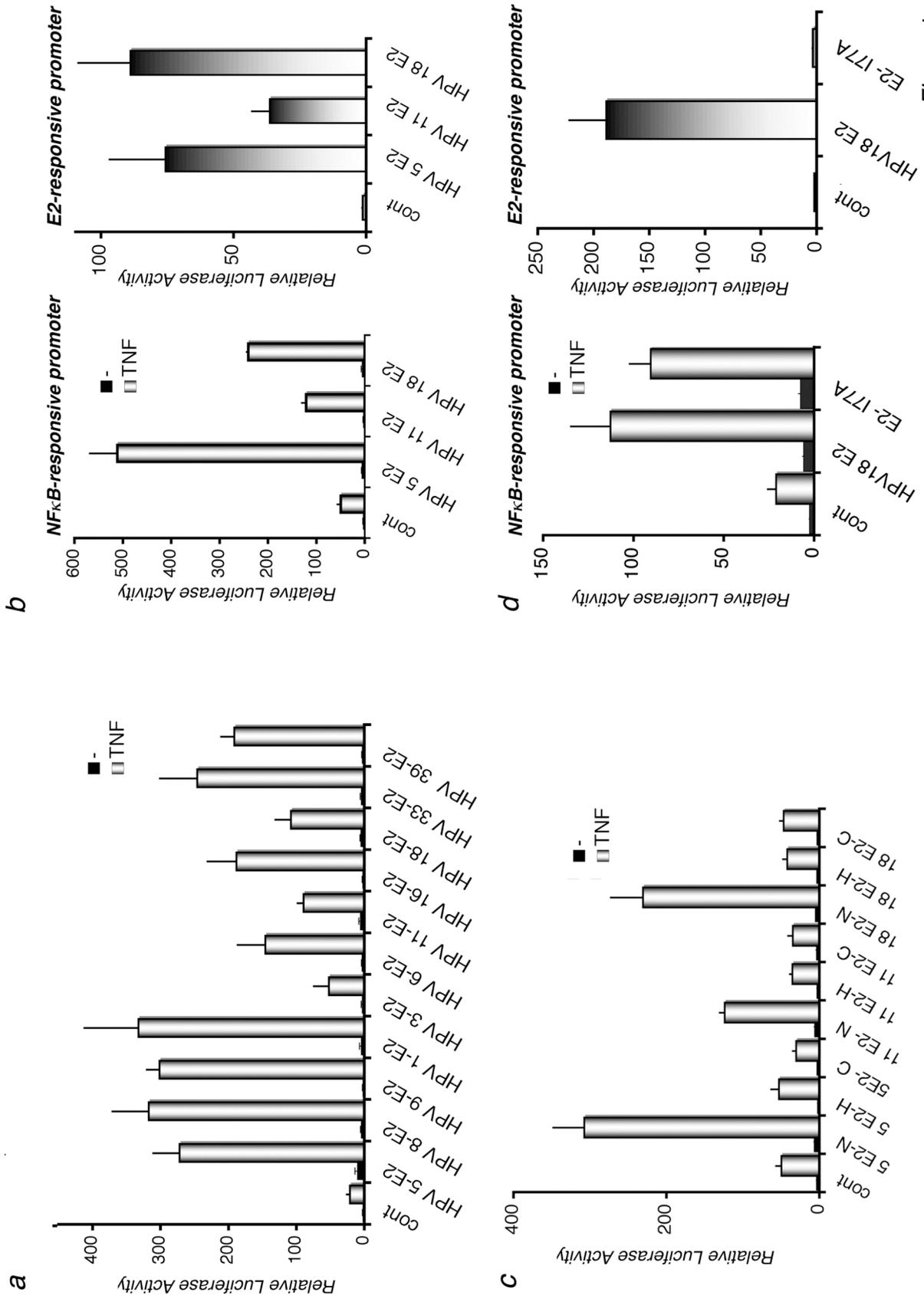
### Figure 2 : E2-mediated NF $\kappa$ B activation is independent of Tax1BP1 and specific to TNF induction.

(a) HaCaT were transfected with p-NF $\kappa$ B-Luc reporter, p-polIII-Ren, GFP-5 E2, GFP-18 E2 or GFP (control) expression plasmids, together with Tax1BP1 Si-RNA or GAPDH Si-RNA (cont). Luciferase activity was measured 20 hours post transfection ( mean of triplicate samples) (b) HaCaT cells were cotransfected with expression plasmid for cherry-Tax1BP1 and GAPDH-directed Si-RNA (control) or Tax1BP1-directed Si-RNA. Cherry-Tax1BP1 was detected with anti-DsRed Polyclonal antibody, anti- $\alpha$ tubulin was used to normalize for loading. (c) HaCaT cells transfected with p-NF $\kappa$ B-Luc, p-polIII Ren, and GFP (control), GFP-5 E2 or GFP-18 E2 expression plasmids were treated with TNF- $\alpha$  (20 ng/ml) or IL1 (20 ng/ml) before Luciferase activity was measured. Fold induction gives the relative luciferase activity of treated to untreated samples (means of duplicate samples).

### Figure 3 : The interplay of E2 with TRAF signaling intermediates.

(a) 293 T cells were transfected with GFP-TRAF2, GFP-TRAF5, or GFP-TRAF6 expression plasmids together with HA-Ubiquitin K29, K48 or K63 and mCherry-HPV18 E2 (+) or mCherry alone (-). Thirty hours post transfection, cell extracts (Habelhah *et al.*, 2004) were subjected to immunoprecipitation (IP) with rabbit anti-GFP antibodies. Immunoprecipitates were analyzed by western blots with anti-GFP, followed by anti-HA antibodies. Anti-GFP and anti-18E2 western blots were performed on cell extracts. (b) SPICA experiments were performed in 293 cells. Reconstituted Gaussia activity is expressed as a Ratio of Luminescence, RL. The threshold of positive interaction has been determined to be 3,5 and is indicated in the figure.

Figure 1



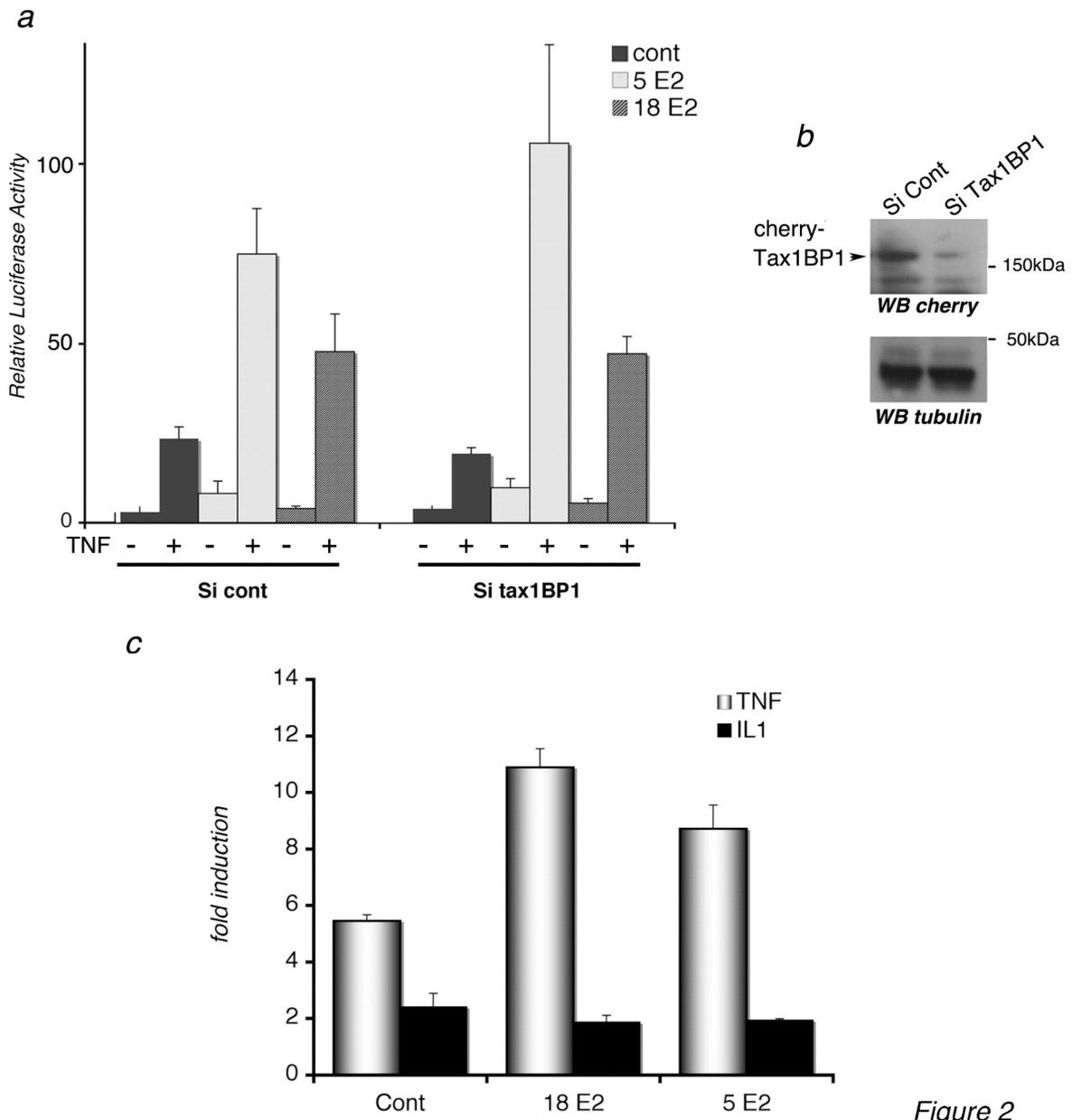


Figure 2

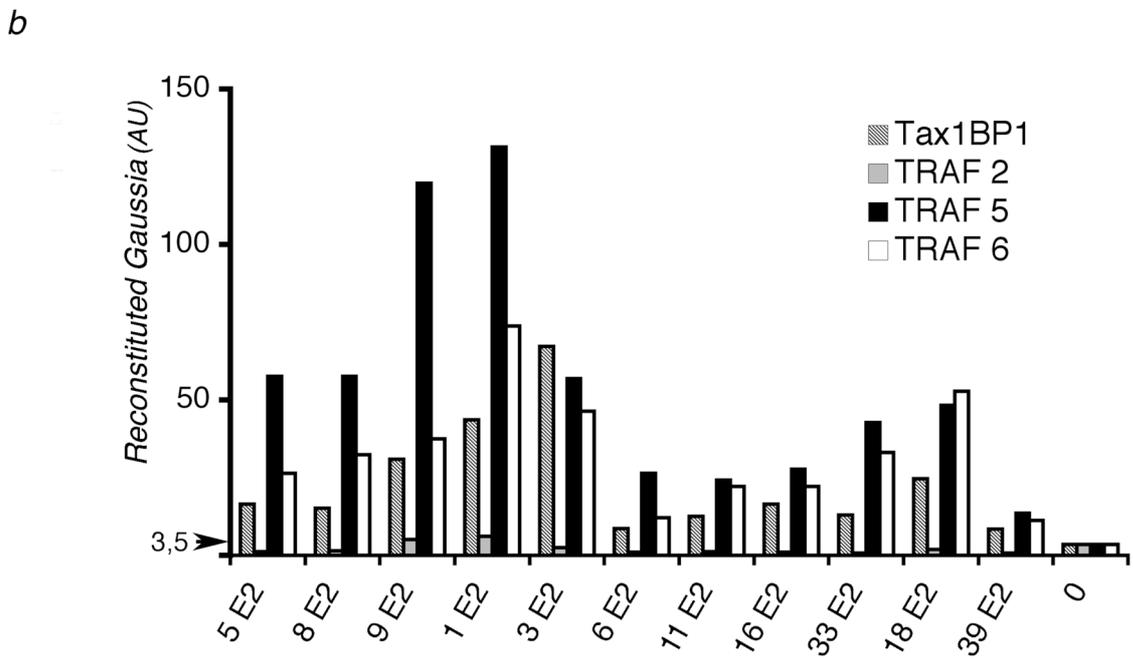
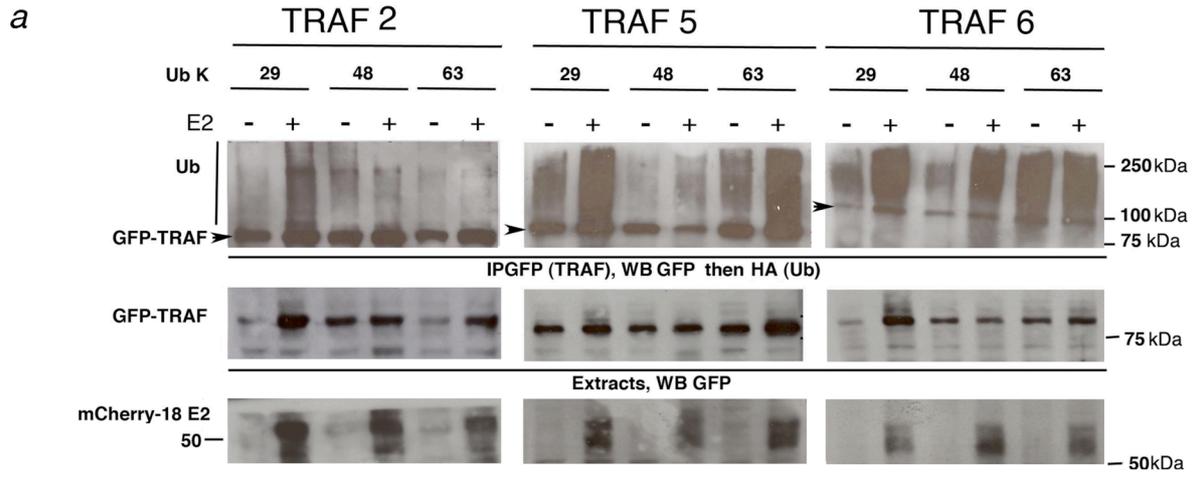


Figure 3