

Published in final edited form as:

Oncogene. 2012 May 31; 31(22): 2761–2772. doi:10.1038/onc.2011.452.

BAG-1 interacts with the p50-p50 homodimeric NF- κ B complex: implications for colorectal carcinogenesis

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Abstract

Understanding the mechanisms that promote aberrant tumour cell survival is critical for the determination of novel strategies to combat colorectal cancer. We have recently shown that the anti-apoptotic protein BAG-1, highly expressed in pre-malignant and colorectal cancer tissue, can potentiate cell survival through regulating NF- κ B transcriptional activity. In this study, we identify a novel complex between BAG-1 and the p50-p50 NF- κ B homodimers, implicating BAG-1 as a co-regulator of an atypical NF- κ B pathway. Importantly, the BAG-1-p50 complex was detected at gene regulatory sequences including the epidermal growth factor receptor [*EGFR*] and COX-2 [*PTGS2*] genes. Suppression of BAG-1 expression using siRNA was shown to increase EGFR and suppress COX-2 expression in colorectal cancer cells. Furthermore, mouse embryonic fibroblasts derived from the NF- κ B1 [p105/p50] knockout mouse were used to demonstrate that p50 expression was required for BAG-1 to suppress EGFR expression. This was shown to be functionally relevant as attenuation of BAG-1 expression increased ligand activated phosphorylation of EGFR in colorectal cancer cells. In summary, this paper identifies a novel role for BAG-1 in modulating gene expression through interaction with the p50-p50 NF- κ B complexes. Data presented led us to propose that BAG-1 can act as a selective regulator of p50-p50 NF- κ B responsive genes in colorectal tumour cells, potentially important for the promotion of cell survival in the context of the fluctuating tumour microenvironment. As BAG-1 expression is increased in the developing adenoma through to metastatic lesions, understanding the function of the BAG-1-p50 NF- κ B complexes may aid in identifying strategies for both the prevention and treatment of colorectal cancer.

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Conflict of interest: The authors declare no conflict of interest.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in many industrialised countries (Cancer Research UK, 2007); understanding the mechanisms that promote colorectal tumour cell survival is critical for the identification of novel strategies for the prevention and treatment of this important cancer.

BAG-1 was first discovered when identifying novel Bcl-2 and glucocorticoid receptor interacting proteins (Takayama *et al.*, 1995; Zeiner and Gehring, 1995 respectively). BAG-1 has since been shown to interact with a wide variety of molecular targets and consequently has been shown to regulate a number of key cellular processes such as proliferation, cell signalling, transcription, differentiation and apoptosis (reviewed in Townsend *et al.*, 2005). Characterised by the BAG domain (BD), BAG-1 is a member of a family of related proteins of which there are at least six in humans (reviewed in Takayama and Reed, 2001). The *BAG-1* gene encodes multiple isoforms, generated from alternate translation of a single mRNA (Packham *et al.*, 1997; reviewed in Sharp *et al.*, 2004). The BAG-1L isoform is found to be localised within the nucleus, BAG-1M is both cytoplasmic and nuclear. BAG-1S, although reported to be located predominantly within the cytoplasm (Brimmell *et al.*, 1999; Packham *et al.*, 1997; Takayama *et al.*, 1998; Yang *et al.*, 1998), is both nuclear and cytoplasmic in colorectal epithelial cells (Barnes *et al.* 2005). These differences in subcellular localisation are thought to be important for biological function, for example the cytoplasmic BAG-1S interacts with Raf-1 to promote cell survival (Song *et al.*, 2001), whereas, the nuclear BAG-1L plays a role in transcription through interacting with DNA (Niyaz *et al.*, 2001) and transcription factors (Cutress *et al.*, 2003; Froesch *et al.*, 1998, reviewed in Gehring *et al.*, 2009).

BAG-1 is over-expressed in a number of cancers, with studies focusing on breast (Brimmell *et al.*, 1999), lung (Rorke *et al.*, 2001), colon (Clemo *et al.*, 2008) and oral squamous cell carcinoma (Shindoh *et al.*, 2000; Wood *et al.*, 2009). In the colon, nuclear BAG-1 has been associated with shorter overall patient survival (Kikuchi *et al.*, 2002). It is therefore of interest that, when studying BAG-1 expression in colorectal tumour tissue, we recently demonstrated that BAG-1 increased NF- κ B transcriptional activity (Clemo *et al.*, 2008); activation of NF- κ B having been associated with resistance to therapy (Arlt *et al.*, 2002; Weldon *et al.*, 2001).

NF- κ B was first discovered in 1986 as a nuclear factor binding to the intronic kappa-light-chain enhancer element in B cells (Sen and Baltimore, 1986). NF- κ B is a family of transcription factors that regulate a number of biological processes including inflammation, apoptosis and cell proliferation (reviewed in Chen and Greene, 2004). In mammals there are five members in the NF- κ B family; RelA (p65), c-Rel, RelB, NF- κ B1 (p105/p50) and NF- κ B2 (p100/p52) (reviewed in Ghosh *et al.*, 1998; Xiao *et al.*, 2006). The NF- κ B family share a conserved rel homology domain in their N-terminus, which is required for dimer formation, DNA binding and interaction with the inhibitor of NF- κ B (I κ B) (Gilmore and Temin, 1988; Kieran *et al.*, 1990; reviewed in Perkins, 2007). p65, RelB and c-Rel contain a transactivation domain (TAD) which is necessary for the activation of transcription (Kamens *et al.*, 1990; reviewed in Perkins, 2007; Ryseck *et al.*, 1992; Schmitz and Baeuerle, 1991). NF- κ B1 (p105/p50) and NF- κ B2 (p100/p52) both lack this TAD but have been shown to induce transcription when bound to other co-activating proteins, for example Bcl-3 (Franzoso *et al.*, 1992, reviewed in Beinke and Ley, 2004; Schmitz and Baeuerle, 1991).

In unstimulated cells, NF- κ B is predominantly cytoplasmic through interaction with inhibitors of NF- κ B (I κ Bs) (Baeuerle and Baltimore, 1988; reviewed in Lin *et al.*, 2010). Upon stimulation (such as by cytokines, bacterial products and growth factors) (reviewed in

Pahl, 1999), NF- κ B can be activated by two main pathways. These are the canonical and non-canonical pathways (otherwise referred to as the ‘classical’ and ‘non-classical’). In response to stimulation, NF- κ B dimers translocate into the nucleus and bind to DNA at specific κ B sites (reviewed in Lin *et al.*, 2010). In addition to the canonical and non-canonical pathways, p50-p50 NF- κ B homodimers are generated as part of an atypical NF- κ B pathway (reviewed in Pereira and Oakley 2008). Although not as extensively studied as some of the other NF- κ B dimers, evidence from the literature shows that the p50-p50 homodimers are localised to the nucleus in unstimulated or “resting” cells where they bind to DNA and repress transcription (Hou *et al.*, 2003; Zhong *et al.*, 2002). In stimulated cells, the ability of the p50-p50 homodimers to act as transcriptional activators or repressors is dependent on the co-factors that interact with the NF- κ B complex at the DNA (reviewed in Glass and Rosenfeld, 2000). As the p50-p50 NF- κ B homodimeric complexes can bind κ B elements with high affinity, it is suggested that the p50-p50 homodimers may impact on the function of the transcriptionally active heterodimeric NF- κ B complexes (reviewed in Pereira and Oakley 2008).

Previously, in colorectal tumour cells, BAG-1 has been shown to regulate NF- κ B transcriptional activity (Clemo *et al.*, 2008), although the mechanism has not been described. In the current study we establish that BAG-1 can interact with p50-p50 NF- κ B homodimers at a consensus κ B site and importantly, using the *EGFR* gene as an example, we reveal that the BAG-1-p50 complex can regulate gene expression. Hence this paper identifies a novel role for BAG-1 as a co-regulator of gene expression through interaction with the p50-p50 NF- κ B complexes, and suggests a potentially important role for this complex in colorectal carcinogenesis.

Materials and methods

Cell line and cell culture conditions

The human colorectal carcinoma-derived cell line HCT116 was obtained from the American Type Culture Collection (Rockville, USA). The human colorectal carcinoma-derived HCA7 colony 29 cell line (herein referred to as HCA7) was a gift from Dr. S. Kirkland (Imperial College London, UK). The NF- κ B^{+/+} and ^{-/-} MEF cell lines were a gift from J. Caamano (Birmingham University, UK).

RNAi

Cells were reverse transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK) with small interfering RNAs (siRNAs) from Applied Biosystems (Warrington, UK) targeting BAG-1 or a negative control sequence (50nM) as described previously (Clemo *et al.*, 2008) or from Dharmacon, (Lafayette, USA) targeting NF- κ B1, murine BAG-1 or a negative control siRNA (25nM siGENOME SMARTpool).

DNA transfection

Cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK) with pIRESneo2 expression plasmids encoding BAG-1L or BAG-1S, or a pRSV NF- κ Bp50 expression plasmid. The empty pIRES neo2 or pRSV plasmids were used as the negative controls.

BAG-1SNLS and BAG-1SNES fusion proteins

The BAG-1S isoform was cloned into a pIRESneo2 fused to either a nuclear localisation signal (NLS) or a nuclear exit signal (NES) using primers 5'-GTAGCTAGCGAAGAGATGGTGGACCTCCAAAAGAAGCTGGAGGAGCTGGAGCTGAATCGGAGCCAGGAGGTG for BAG-1SNES and

GGTAGCTAGCGAAGAGATGCCAAAAAGAAGAGAAAGGTAATCGGAGCCAG
GAGGTG for BAG-1NLS; common reverse primer was 5'-
ATGAGGATCCTCACTCGGCCGAGGGCAAAGT.

NF- κ B reporter assays

Growing cells were transiently transfected with either the NF- κ B reporter plasmid pNF- κ B-TA-luc or with the control plasmid pTA-luc (Clontech, Oxford, UK) including the pRL-SV40 renilla plasmid (Promega, Southampton, UK) using Lipofectamine 2000 following the manufacturer's protocol. Following lysis, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Southampton, UK) according to the manufacturer's instructions.

Immunoblotting

Whole cell lysates were prepared and subjected to immunoblotting as previously described (Williams *et al.*, 1993) using antibodies against BAG-1 (G3E2; kind gift from G. Packham, Southampton University, UK) and NF- κ B1 (E10: sc-8414; Santa Cruz Biotechnology, Ca, USA).

Immunofluorescence

Proteins were visualised as previously described (Barnes *et al.*, 2005). BAG-1 was visualised using the polyclonal anti-BAG-1 (TB3) antibody which recognises all three BAG-1 isoforms (kind gift from G. Packham, Southampton University, UK).

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Clemo *et al.*, 2008); Formalin-fixed, paraffin embedded normal human large intestinal sections were obtained from the Department of Histopathology, Bristol Royal Infirmary, Bristol, UK with local Ethics Committee approval. NF- κ B1 was detected using the E10 antibody (sc-8414; Santa Cruz Biotechnology, Ca, USA), BAG-1 was detected using the TB3 antibody (G. Packham, Southampton University, UK).

Preparation of nuclear protein extracts

A Nuclear Extraction Kit (Active Motif, Rixensart, Belgium) was used as per manufacturer's instructions. The protein concentration of the nuclear fractions was determined using the Bio-Rad DC Protein assay kit (Bio-Rad, Hertfordshire, UK).

Oligonucleotide-pulldown Assay

The assay was essentially carried out following manufacturer's instructions (Santa Cruz Biotechnology, Ca, USA), using NF- κ B binding oligonucleotide sequences (wild-type: 5'-AGTTGAGGGGACTTTCCCAGGC-3; mutant: 5'-AGTTGAGGCGACTTTCCCAGGC-3'). However, the binding buffer used was 8.5mM HEPES pH7.9; 1mM KCl, 1mM MgCl₂, 1mM DTT, 7.5% Ficoll, 1mg/ml BSA and 1-4 μ C [dIdC].

Electrophoretic Mobility Shift Assay (EMSA)

The EMSA was carried out using standard techniques as previously described (Smartt *et al.*, 2003). For supershift assays, 1 μ l of BAG-1 (G3E2; Kind gift from G. Packham, Southampton University, UK) or NF- κ B antibody (NF- κ B1; sc-114; p65: sc-372 and NF- κ B2: sc-298; Santa Cruz Biotechnology, Ca, USA) was used.

Quantitative Reverse Transcription Polymerase Chain Reaction (Q-RT-PCR)

Total RNA extraction and comparative quantitative real-time polymerase chain reaction (Q-RT-PCR) was performed as previously described (Clemo *et al.*, 2008). QuantiTect Primer Assays and primers for *EGFR* and *PTGS2* were obtained from Qiagen Ltd, (Crowley, West Sussex, UK).

Co-immunoprecipitation

Preparation of immunoprecipitating antibody—All immunoprecipitating antibodies were obtained from Santa Cruz Biotechnology (Ca, USA); BAG-1 antibody (C16: sc-939), NF- κ B antibodies (NF- κ B1: E10; sc-8414x; p65: F6; sc-8008x) and the IgG antibody (sc-2027) as an irrelevant control. 5 μ g of antibody was suspended in 25% Immunoprecipitation Matrix slurry (IP Matrix; Santa Cruz Biotechnology, Santa Cruz, California, USA)

Preparation of cell lysate—Cell pellets were suspended in immunoprecipitation lysis buffer supplemented with protease inhibitors (Complete Mini Protease Inhibitors, Roche Diagnostics Ltd, W.Sussex, UK), sonicated in a Bioruptor UCD-200 Waterbath Sonicator (Diagebode Inc, Sparta, New Jersey, USA) and then resuspended in the lysis buffer with protease inhibitors. Immunoprecipitation was carried out essentially as previously described (Arhel *et al.*, 2003).

Chromatin immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) was carried out as previously described (Kaidi *et al.*, 2007) using BAG-1 antibody (C16; sc-939) and NF- κ B1 antibody (H119; sc-7179) or a species matched IgG control (Santa Cruz Biotechnology (Ca, USA)). Primers flanking the NF- κ B1 binding sites at the *EGFR* promoter (Thornburg *et al.*, 2003) were (forward, 5'-GGGGACCCGAATAAAGGAGCAGTT-3'; reverse, 5'-CTGAGGAGTTAATTTCCGAGAGGGG-3') and the *PTGS2* promoter (forward, 5'-CTGTTGAAAGCAACTTAGCT-3'; reverse, 5'-AGACTGAAAACCAAGCCCAT-3', Deng *et al.*, 2003). *Re-ChIP*: For the Re-ChIP assay, the complexes were eluted from the primary immunoprecipitation followed by re-immunoprecipitation with a second antibody.

Statistical analysis

Statistical analyses were carried out using a Student's t-test in Microsoft Excel and represented as: * = P < 0.05; ** = P < 0.01; *** = P < 0.001

Results

Nuclear localisation of BAG-1 is important for the regulation of TNF- α -induced NF- κ B transcriptional activity

As the BAG-1L isoform had been shown to significantly enhance TNF- α -induced NF- κ B transcriptional activity (in colorectal carcinoma cells, Clemo *et al.*, 2008, as well as adenoma and human embryonic kidney cell lines, data not shown), initial experiments were carried out to investigate the importance of the nuclear localisation of BAG-1 for regulating NF- κ B activity. Using the BAG-1S isoform, (common to the C terminus of BAG-1L containing the BAG domain but does not have an endogenous nuclear localisation signal, Brimmell *et al.*, 1999), fusion proteins were made introducing either a nuclear localisation (BAG-1SNLS) or nuclear exit signal (BAG-1SNES) (kind gift, G. Packham, Southampton University, UK) thus limiting the expression of the exogenous protein to either the nucleus or the cytoplasm respectively. The localisation of the fusion proteins was confirmed in HCA7 cells (as these cells have an increased cytoplasmic volume compared to HCT116 cells, facilitating the

visualisation of subcellular compartments, figure 1Ai). Expression of endogenous BAG-1 protein was found to be both nuclear and cytoplasmic, whereas the BAG-1SNES fusion protein was concentrated in the cytoplasm and the BAG-1SNLS protein in the nucleus. The ability of the fusion proteins to potentiate TNF- α -induced NF- κ B transcriptional activity was tested using a luciferase reporter assay, the results are summarised in figure 1B. A highly significant increase in TNF- α -induced NF- κ B transcriptional activity was detected in HCT116 cells when BAG-1S was fused to a NLS when compared to BAG-1S fused to a NES (figure 1B), despite the fact that expression of the BAG-1SNES protein was higher than the BAG-1SNLS protein (figure 1C). (Of note, it remains possible that the small but significant increase in NF- κ B transcriptional activity observed in the TNF- α treated BAG-1SNES transfected cells is due to the induction of the endogenous nuclear BAG-1L and 1M isoforms in the transfected cells and previously reported, Hinnit *et al.*, 2010). Taken together, these results demonstrate that TNF- α -induced NF- κ B activity is enhanced by the nuclear localisation of the BAG-1 protein and justifies further investigation into the mechanism by which BAG-1 protein can potentiate NF- κ B transcriptional activity in the nucleus.

BAG-1 interacts with the p50-p50 homodimeric NF- κ B complex

Previously it has been established that the nuclear localised BAG-1 is able to bind DNA and regulate transcription of reporter constructs (Zeiner *et al.*, 1999; Niyaz *et al.*, 2001). Furthermore, in prostate and breast cancer cells BAG-1 has been shown to interact with nuclear hormone receptors and regulate their transcriptional activity (Froesch *et al.*, 1998; Liu *et al.*, 1998). Therefore, because of the ability to bind DNA, we investigated whether BAG-1 could be isolated in association with an NF- κ B-DNA binding motif in colorectal cancer cells. Initially, we performed an oligopulldown assay using the consensus NF- κ B binding sequence GGGGACTTTCCC, and were able to demonstrate that BAG-1 could be detected at the κ B binding site in HCT116 cells treated with TNF- α (100ng/ml) (figure 2A). To investigate whether BAG-1 was associated with the NF- κ B complexes themselves, we initially treated HCT116 cells with a number of different NF- κ B activators to maximise NF- κ B signalling in the cells. HCT116 cells treated with IL-1 β (10ng/ml) resulted in an approximate 10-fold induction of the NF- κ B reporter construct as compared to four-fold induction by TNF- α (100ng/ml) and less than two-fold induction with LPS (10ng/ml, data not shown). Therefore treatment with IL-1 β (10ng/ml) was used to stimulate NF- κ B DNA binding in subsequent experiments. Within the literature, cytokine stimulation of colonic epithelial cells has predominantly been associated with increased binding of the p65-p50 heterodimer and p50-p50 homodimer NF- κ B complexes using an electrophoretic mobility shift assay (EMSA) (Inan *et al.*, 2000). Importantly both p65-p50 heterodimers and p50-p50 homodimers have high affinity for the consensus κ B sequence used (Duckett *et al.*, 1993). Addition of specific antibodies raised against individual NF- κ B subunits allowed the complexes in the stimulated cells to be identified (figure 2B, lanes 4-6). A recombinant p50 protein (Rhp50) was added to indicate the position of the p50-p50 homodimers (lane 7). Addition of the NF- κ B2 [p100/p52] antibody (which acts as a negative control as p52 NF- κ B complexes weakly associate with the GGGGACTTTCCC binding motif) did not supershift any of the bands (lane 6).

To identify whether BAG-1 interacted with an NF- κ B transcriptional complex in colorectal epithelial cells, the BAG-1 antibody G3E2 was incubated with the nuclear lysate and radiolabelled oligonucleotide (figure 2C). Addition of the antibody resulted in a consistent decrease of the p50-p50 homodimer band and no loss of the p65-p50 heterodimeric band. Of note, contrary to expectation, this suggested that BAG-1 associated with the p50-p50 homodimeric NF- κ B transcriptional complex and not the canonical p65-p50 NF- κ B

heterodimeric complex, raising the interesting possibility that BAG-1 could be regulating NF- κ B activity through interaction with the p50-p50 homodimer NF- κ B complex.

To confirm the interaction between the BAG-1 and p50 proteins, a co-immunoprecipitation assay in HCT116 cells transfected with a p50 expression plasmid was performed. The p50 expressing HCT116 cell lysates (figure 2D ii) were precipitated with anti-p65, NF- κ B1 or BAG-1 antibodies and interrogated by Western analysis using the mouse anti-BAG-1 antibody (figure 2Di). As expected, the anti-BAG-1 antibody precipitated all BAG-1 isoforms as indicated by strong bands at 36, 46 and 50kDa (lane 6). The nuclear localised BAG-1 isoforms (predominantly BAG-1L) were also precipitated with the anti-NF- κ B1 antibody (lane 4) but not with the anti-p65 antibody (lane 2), further implicating BAG-1 as a p50 binding protein. In addition, the reverse precipitation was carried out; p50 was precipitated by the anti-p65 and BAG-1 antibodies in HCT116 cells transfected with a BAG-1 expression plasmid (data not shown). Importantly, the BAG-1-p50 interaction was confirmed when the immunoprecipitation was repeated with endogenous protein expression (in untransfected HCT116 cells; figure 2E). Using the anti-BAG-1 antibody to interrogate bound protein, the anti-NF- κ B1 antibody precipitated the nuclear BAG-1L isoform (lane 2), whereas BAG-1 protein was not precipitated by the antibody raised against p65 (lane 4). These results confirm that BAG-1 interacts with the p50 but not the p65 NF- κ B protein in the HCT116 colorectal carcinoma derived cell line.

The potential significance of an interaction with NF- κ B1 (p50) in the colon is emphasised by the work of the Giardina group. They showed that p50-p50 homodimers were differentially expressed in the normal crypt, being the predominant complex in the non-proliferating cells (Inan *et al.*, 2000). In accordance with these findings, we have found that NF- κ B1 is highly expressed at the top of normal colonic crypt but is also expressed at the bottom of the crypt coincident with nuclear BAG-1 expression (figure 3A). NF- κ B1 represents the predominant NF- κ B complex in stimulated colorectal cancer cells (figure 3B) and both BAG-1 and NF- κ B1, are also expressed in all cell lines investigated *in vitro* (figure 3C). This data supports a role for NF- κ B1 in tissue homeostasis in the colon, and emphasises the potential significance of the novel finding that BAG-1 interacts with NF- κ B1 in colorectal tumour cells.

Suppression of NF- κ B1 signalling increases apoptosis in HCT116 cells

As BAG-1 has been previously shown to promote tumour cell survival through regulation of NF- κ B activity (Clemo *et al.*, 2008), we wished to investigate whether NF- κ B1 expression was implicated in colorectal tumour cell survival. HCT116 cells were transfected with either BAG-1 or NF- κ B1 siRNA and apoptosis measured 48 h after treatment with TNF- α (100ng/ml), results are summarised in figure 4. Data shows that not only does loss of BAG-1 increase apoptosis in TNF- α treated cells consistent with the previous report (figure 4A, Clemo *et al.*, 2008), but that suppression of NF- κ B1 also promotes cell death in TNF- α treated cells (figure 4B). Taken together, this data implicates a role for both BAG-1 and NF- κ B1 in promoting tumour cell survival.

The BAG-1-p50 complex can be detected at the promoter of the *EGFR* and *PTGS2* genes

The previous EMSA allowed the detection of the BAG-1-p50-p50 homodimeric NF- κ B complex at a κ B consensus sequence in colorectal carcinoma derived cells. However to establish whether the complex was functional in the cells, the potential of BAG-1 to modulate p50-p50 regulated gene expression was investigated. A focused miniarray performed on mRNA obtained from HCT116 cells transfected with BAG-1 siRNA and treated with TNF- α (100ng/ml) or IL-1 β (10ng/ml) had identified *EGFR* as possible BAG-1 regulated genes (data not shown). Furthermore, as the expression of the *EGFR* gene had

previously been described as regulated by p50-p50 homodimeric NF- κ B complexes (Thornburg *et al.*, 2003), *EGFR* was chosen for further study. To investigate whether the BAG-1-p50 complex could be detected at the *EGFR* promoter, we carried out a chromatin immunoprecipitation [ChIP] assay using antibodies against both BAG-1 and NF- κ B1 (figure 4C). Recovered DNA underwent PCR analysis using primers flanking the κ B sites in the *EGFR* promoter (Thornburg *et al.*, 2003, refer to methods section); the PCR products were detected by gel electrophoresis. RNA pol II was precipitated as a positive control for binding to the *EGFR* promoter (lane 4). DNA complexes were precipitated with the control mouse and rabbit IgG antibodies to account for non-specific interaction of the antibodies (lanes 3 and 5). Importantly, a positive interaction between both BAG-1 and NF- κ B1 within the same region of the *EGFR* promoter was detected in HCT116 cells (figure 4C).

To determine whether BAG-1 and p50 were part of the same DNA complex at the *EGFR* promoter, a re-ChIP was carried out (figure 4D). In HCT116 cells, DNA complexes were firstly immunoprecipitated with the NF- κ B1 antibody and then underwent a second immunoprecipitation using the BAG-1 antibody. DNA was recovered and underwent PCR analysis using the *EGFR* promoter primers; PCR products were identified using gel electrophoresis (figure 4D). Protein DNA complexes were not detected using the anti-IgG antibody ruling out non-specific binding of the antibodies (lane 3 and 4). Detection of a PCR product (lane 5) demonstrated that BAG-1 and p50 are indeed part of the same DNA complex at the *EGFR* promoter. In addition, as results from the focused microarray also identified the COX-2 gene (*PTGS2*) as a possible BAG-1 regulated gene, it was of interest to show that the BAG-1-p50 complex could also be detected at the *PTGS2* promoter (figure 4C and D) providing further evidence for the binding of the BAG-1-p50 homodimeric NF- κ B complex to gene regulatory sequences.

BAG-1 regulates EGFR expression in colorectal cancer cells

To investigate the consequence of the interaction of BAG-1 with the p50-p50 homodimers at the gene promoter, we were able to show that EGFR is a transcriptional target of BAG-1 by studying the mRNA abundance of *EGFR* by Q-RT-PCR in cells with increased or suppressed BAG-1 expression (figure 5Ai). Importantly, although the potentially repressive BAG-1-p50 complex was detected at the *EGFR* promoter in unstimulated HCT116 cells (figure 4), basal expression of EGFR was detected (figure 5Aii) suggesting the complex may be involved in limiting expression of the protein, rather than completely inhibiting it. Results show that suppression of BAG-1 protein expression using RNAi resulted in an increase in *EGFR* mRNA abundance (~1.7 fold; figure 5Ai) and protein (figure 5Aii). Conversely, expression of exogenous BAG-1L suppressed *EGFR* mRNA (figure 5Ai) and protein (figure 5Aii). These data establish BAG-1 as a repressor of *EGFR* expression in colorectal cancer cells.

BAG-1 can promote PTGS2 gene expression in colorectal cancer cells

To determine whether the BAG-1:p50 complex is commonly associated with gene repression we also investigated the effect of loss of BAG-1 expression on *PTGS2* expression (shown to have BAG-1-p50 complexes at its promoter, figure 4C and D). *PTGS2* mRNA abundance was investigated by Q-RT-PCR in HT29 (COX-2 expressing) cells transfected with BAG-1 siRNA (figure 5B). Interestingly, data show that suppression of BAG-1 protein expression resulted in a decrease in *PTGS2* mRNA abundance (~0.6 fold), suggesting that BAG-1 actually potentiates expression of the *PTGS2* gene. This is in contrast to the increased *EGFR* expression on suppression of *BAG-1* in the HT29 cells (~1.5 fold, figure 5B). Taken together, this study suggests that BAG-1 can either repress or enhance gene expression, dependent on the target gene.

BAG-1 fails to regulate EGFR expression in NF- κ B1 knock out mouse embryonic fibroblasts

To establish whether this regulation of *EGFR* expression by BAG-1 is dependent on the interaction with the p50-p50 homodimeric NF- κ B complex, EGFR protein expression was studied in NF- κ B1 knock out [NF- κ B1^{-/-}] mouse embryonic fibroblasts (MEFs) and compared to the response in age matched wild type [NF- κ B1^{+/+}] MEFs (a kind gift from J. Caamano Birmingham University, UK). NF- κ B1^{+/+} and NF- κ B1^{-/-} MEFs were transfected with 25nM of control scrambled or murine BAG-1 [mBAG-1] siRNA and the level of EGFR expression determined by Western analysis (figure 5C). Results show that suppression of BAG-1 expression in NF- κ B1^{+/+} MEFs lead to an increase in EGFR protein expression (a 60% decrease in BAG-1 expression lead to a 66% increase in EGFR protein expression). In contrast, this response was attenuated in the NF- κ B1^{-/-} MEF cells resulting in no detectable up-regulation of the EGFR protein expression (a 57% suppression of BAG-1 expression caused a less than 1% increase in the EGFR protein expression). These results further demonstrate that suppression of BAG-1 expression increases EGFR protein levels and demonstrate that the regulation of EGFR expression by BAG-1 is mediated via a p50 dependent mechanism.

The BAG-1-p50 complex suppresses epidermal growth factor signalling in colorectal cancer cells

To determine whether the regulation of EGFR expression by BAG-1 is sufficient to modulate signalling through the EGF pathway, HCT116 cells were transfected with 50nM of control scrambled or BAG-1 siRNA and treated with EGF (10ng/ml), results are summarised in figure 5D. Western analysis using a phosphor-specific antibody was used to demonstrate activation of the EGFR, which could be blocked by addition of the EGFR inhibitor [10 μ M CL-387.785] confirming specificity of the assay (lanes 3,5,8 and 10). Activation of the EGFR was detected on addition of 10ng/ml EGF to the cultures 72h after siRNA transfection (5 min (lane 2) and 10 min (lane 4) after addition of the EGF ligand) which was blocked by addition of the EGFR inhibitor (lanes 3 and 5). Strikingly, when BAG-1 expression is suppressed by siRNA (lanes 6-10) there is increase phosphorylation of the EGFR on addition of EGF (5 min (lane 7) and 10 min (lane 9) after addition of 10ng/ml EGF). These data reveal an increase in the phosphorylation, and hence activation, of the EGFR in the presence of the ligand when BAG-1 protein expression is suppressed by siRNA. This is reflected in an increase in phospho-ERK (a downstream target of the EGF signalling pathway) when BAG-1 expression is suppressed (data not shown), confirming that the regulation of EGFR expression by BAG-1 is sufficient to significantly attenuate signalling through the pathway.

Taken together this study has identified for the first time that BAG-1 interacts with the p50-p50 homodimeric NF- κ B complex, and that these complexes can be detected on the promoters of genes known to be important in colorectal carcinogenesis, including *EGFR* and *PTGS2*. Of note, although the role of both EGF and COX-2 signalling are well established in colorectal carcinogenesis, we propose that it is the combined effect of the BAG-1-p50 complex on the expression of a number of genes that is important for promoting colorectal tumour survival. This finding has implications not only for normal tissue homeostasis but suggest that BAG-1-p50 NF- κ B complexes could play a fundamental role in the de-regulation of key signalling pathways in colorectal carcinogenesis.

Discussion

This investigation has established that nuclear localisation of BAG-1 is important in the regulation of NF- κ B activity, identifying BAG-1 as a novel binding partner of the p50-p50

homodimeric NF- κ B complex. The function of BAG-1 as a regulator of gene expression through interaction with p50-p50 NF- κ B complexes has potentially significant implications for colorectal carcinogenesis. Although not as extensively studied as some of the other NF- κ B dimers, p50-p50 homodimers are important signalling complexes generally recognised as repressors of transcription due to the lack of a transactivation domain (reviewed in Piera and Oakley, 2008). There have been several reports of p50-p50 homodimers repressing key NF- κ B associated genes, including TNF- α and IL-8 (Baer *et al.*, 1998; Tong *et al.*, 2004). However, there are also reports of p50-p50 homodimers acting as transcriptional activators. Studies have shown p50-p50 homodimers to increase the expression of the NF- κ B target genes such as IL-6 in melanoma cells and IL-10 in macrophages (Cao *et al.*, 2006; Karst *et al.*, 2009). It appears that the ability of the p50-p50 homodimeric NF- κ B complex to act as a transcriptional activator or repressor is dependent on the co-factors that interact with the NF- κ B complex at the DNA (reviewed in Glass and Rosenfeld, 2000). For example, a study by Zhong *et al.* used a co-immunoprecipitation assay to demonstrate that p50 interacted with HDAC-1 in unstimulated murine pre-B cells, resulting in transcriptional silencing of the IL-8 and TNF- α genes (Zhong *et al.*, 2002). On the other hand, the p50-p50 homodimers have been shown to become transcriptional activators when interacting with the nuclear BCL-3 protein (Bours *et al.*, 1993; Fujita *et al.*, 1993). As a co-chaperone, it is of interest to hypothesise that either by stabilising the p50-DNA complexes or influencing the recruitment of other co-factors, BAG-1 may be able to either repress and/or trans-activate gene expression.

In the colon, consistent with previous reports (Inan *et al.*, 2000), we found NF- κ B1 to be expressed throughout the normal colonic crypt. Interestingly, BAG-1 has been described as predominantly nuclear at the bottom of the crypt and associated with colorectal epithelial cell survival (Clemo *et al.*, 2008; shown in figure 3; Barnes *et al.*, 2005). Taken together with evidence from the literature, we would propose that the nuclear localisation of BAG-1 in association with p50-p50 homodimeric NF- κ B complexes is able to regulate gene expression potentially involved in preserving the proliferating compartment of the colonic crypt. Consistent with this hypothesis, the increased expression of BAG-1 in colonic tumour cells would promote the pro-survival function of the p50-p50 homodimeric NF- κ B complexes, identified as the predominant NF- κ B complexes in stimulated colorectal tumour cells (figure 3B) and as such high BAG-1 expression would contribute to cell survival and hence be associated with poor therapeutic response in colorectal cancer patients (as reported by Kikuchi *et al.*, 2003). As NF- κ B signalling, which is up-regulated in response to cytotoxic treatments, is accountable for many tumours being resistant to current cancer therapies (reviewed in Nakanishi and Toi, 2005), the emerging role of BAG-1 in the regulation of NF- κ B function has potentially significant therapeutic implications. Although NF- κ B is a crucial player in many cellular processes, targeting the BAG-1-p50 complex may provide a mechanism for inhibiting the tumour-promoting/survival function of NF- κ B whilst still retaining many of the normal physiological processes. Interestingly, a previous study reported that another member of the BAG family (BAG-3) could promote DNA binding of NF- κ B subunits mediated via the conserved BAG domain (Rosati *et al.*, 2007). Furthermore, a recent study in HeLa cells reported suppression of BAG-1 to attenuate NF- κ B activation, although in this report they found BAG-1 to promote NF- κ B activity through down-regulation of I κ B α leading to the nuclear accumulation of NF- κ B (Maier *et al.*, 2010). Therefore, it is of interest to speculate that the BAG proteins may have a multi-factorial mode of action, able to modulate the function of NF- κ B at more than one stage in the signalling pathway, whether dependent on cell type or stimulus remains to be determined. However, because of the potential role of p50-p50 homodimeric complexes in colorectal tissue homeostasis (Inan *et al.* 2000), we would propose that the recruitment of BAG-1 to the p50-p50 homodimeric NF- κ B complex may have a particularly important selective advantage for the survival of colorectal tumour cells.

Through interacting with the EGFR promoter, the BAG-1-p50 complex was shown to repress signalling through the EGF pathway. However, EGF signalling has been reported to promote cell survival and proliferation through activating the RAS-MAPK and PI3K-AKT pathways (reviewed in Yarden, 2001). These reports suggest that high levels of EGF signalling would be beneficial for promoting colorectal carcinogenesis, and hence an apparent paradox exists as BAG-1 is up-regulated in colorectal cancer tissue and can inhibit EGFR gene and protein expression. However, there are conditions where, by driving proliferation, EGFR expression could be detrimental to cancer cells (for example when attempting to adapt and survive in response to a changing microenvironment within the developing tumour, Kaidi *et al.*, 2007). However, it is equally important to note that in the colon, activating mutations of *K-ras* commonly occur in approximately 50% of colorectal cancers (Bos *et al.*, 1987), and as a consequence tumour cells have constitutive MAPK activation and may not require EGF signalling for cell survival (reviewed in Jiang *et al.*, 2009). Furthermore, there are situations where high EGFR expression may be unfavourable to the cells. For example, EGFR has been shown to sequester the insulin receptor substrate-1 (IRS-1), which is an essential component of the insulin-like growth factor type 1 receptor (IGF-1R), implying that high EGFR expression could inhibit the IGF-II survival signalling pathway (Knowlden *et al.*, 2008). Inhibition of EGFR expression by BAG-1 could therefore actually promote cell survival through the IGF-II pathway. We suggest that many of the stress induced signalling pathways stimulated by the microenvironment in a developing tumour, such hypoxia or energy deprivation, could increase the formation of BAG-1-p50 complexes resulting in suppression of genes involved in proliferation (such as EGFR) and promoting genes involved in survival and adaptation (such as the COX2/prostaglandin pathway).

In conclusion, this paper identifies a novel role for BAG-1 as a co-regulator of gene expression through interaction with the p50-p50 NF- κ B complexes. Data presented have led us to propose that BAG-1 can act as a selective regulator of p50-p50 NF- κ B responsive genes in colorectal tumour cells, important for promoting cell survival in the context of the fluctuating tumour microenvironment. As BAG-1 expression is increased in the developing adenoma through to metastatic lesions, understanding the function of the BAG-1 p50-NF- κ B complexes may aid in identifying novel strategies for both the prevention and treatment of colorectal cancer.

Acknowledgments

This work was funded by a Cancer Research UK programme grant, the Citrina Foundation, The Wellcome Trust and by the John James Bristol Foundation.

We thank the Medical Research Council for providing an Infrastructure Award to establish the School of Medical Sciences Cell Imaging Facility at the University of Bristol.

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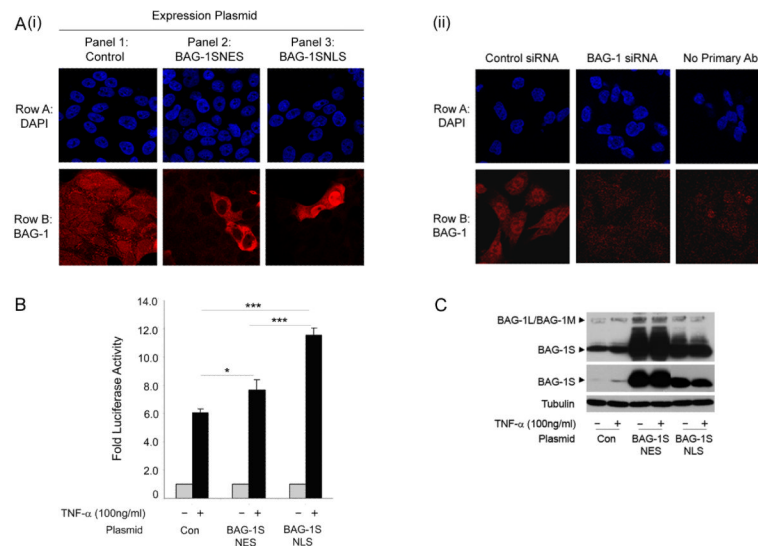


Figure 1. Enforced nuclear localisation of the BAG-1S isoform increases TNF- α induced NF- κ B transcriptional activity

(A) (i) Confocal immunofluorescence microscopy was used to show the localisation of BAG-1 in HCA7 cells transfected with the control IRES plasmid, BAG-1SNES or BAG-1SNLS expression plasmids. Row A: DAPI staining showing the cell nuclei; Row B: Alexa Fluor-546 TRITC fluorescence showing BAG-1 staining. It is important to note that endogenous BAG-1 expression could not be detected in panel 2 or panel 3 as the intensity of the confocal microscope was decreased in order to visualise the localisation of the exogenous fusion proteins. (ii) The specificity of the antibody was confirmed in cells transfected with the control negative / BAG-1 siRNA, as previously reported (Clemo *et al.*, 2008) Row A: DAPI staining showing the cell nuclei; Row B: Alexa Fluor-546 TRITC fluorescence showing BAG-1 staining. (B) Transcriptional activation of the NF- κ B luciferase reporter in HCT116 cells transfected with control plasmid, BAG-1SNES or BAG-1SNLS expression plasmids and treated with TNF- α (100ng/ml) for 16 hours. The results shown are representative of three separate experiments done in triplicate (\pm standard deviation). Statistical analysis was carried out using a Student's t-test * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. (C) Western analysis confirmed over-expression of the BAG-1SNES and BAG-1SNLS fusion proteins (middle panel shows lower exposure to demonstrate expression of the exogenous BAG-1S fusion proteins). With TNF- α treatment, an increase in endogenous BAG-1 expression was detected. Equal loading was confirmed by α -tubulin. Of note, expression of exogenous BAG-1SNES or BAG-1SNLS proteins increased expression of the endogenous BAG-1L/BAG-1M proteins (as previously reported, Hinnit *et al.*, 2010).

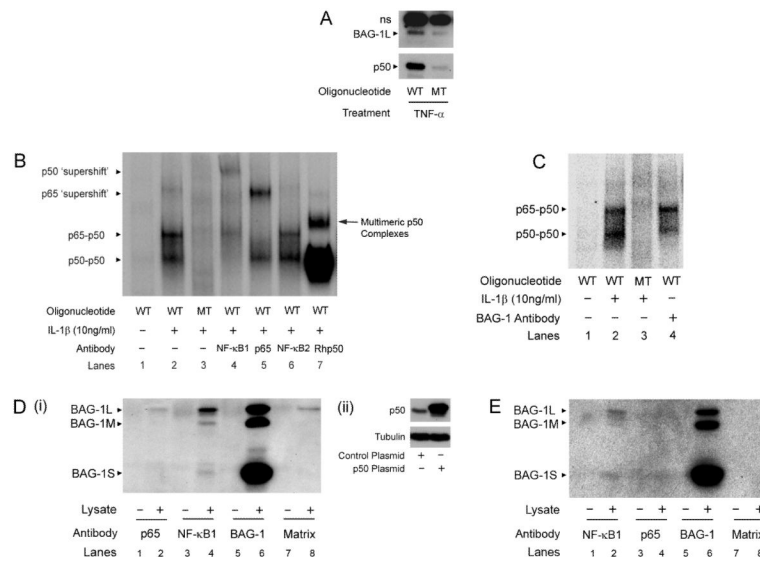


Figure 2. BAG-1 interacts with the p50-p50 homodimeric NF- κ B complex

(A) BAG-1 interacts with a consensus NF- κ B DNA binding site as shown by an oligonucleotide pull-down assay. ns denotes a non-specific band. HCT116 cells were transfected with the BAG-1L expression plasmid and treated with TNF- α (100ng/ml) for 16 hours. After cellular fractionation, the nuclear lysate was incubated with either the wild-type (GGGGACTTCCC, WT) or mutant (GGCGACTTCCC, MT) oligonucleotide. (B) IL-1 β (10ng/ml) treated HCT116 nuclear fractions were analysed by EMSA. Nuclear lysates were incubated with the mutant (MT) oligonucleotide to control for non-specific interactions (Lane 3). 1 μ g of NF- κ B1, p65 or NF- κ B2 antibody were incubated with nuclear lysate and wild-type (WT) oligonucleotide (Lanes 4, 5, 6). 0.5 μ g of recombinant human p50 (Rhp50) was incubated with the nuclear lysate and wild-type oligonucleotide to show the position of the p50-p50 homodimeric NF- κ B complex (Lane 7), and other multimeric complexes (Duckett *et al.* 1993). (C) HCT116 nuclear fractions were treated with IL-1 β (10ng/ml) for 16 hours. G3E2 BAG-1 antibody was added to the HCT116 nuclear lysate, followed by incubation with the wild-type (WT) radiolabelled oligonucleotide (Lane 4). Results are representative of three independent experiments. **BAG-1 interacts with the p50 protein.** (D) (i) HCT116 cells were seeded for 72 hours and transfected with the control or p50 expression plasmid (shown by Western analysis, ii). 48 hours following transfection, cells were lysed and proteins immunoprecipitated using antibodies against p65 (Lane 2), NF- κ B1 (Lane 4) and BAG-1 (Lane 6); (Lane 8 is the no antibody control). Western analysis identified BAG-1 protein interactions within the whole cell lysate. Results are representative of three separate experiments. (E) BAG-1 interacts with the p50 protein in untransfected HCT116 cells. Endogenous protein was immunoprecipitated as before. Western analysis was carried out to determine endogenous BAG-1 protein interactions within the whole cell lysate. Proteins were immunoprecipitated using antibodies against NF- κ B1 (Lane 2), p65 (Lane 4) and BAG-1 (Lane 6). Results are representative of three independent experiments.

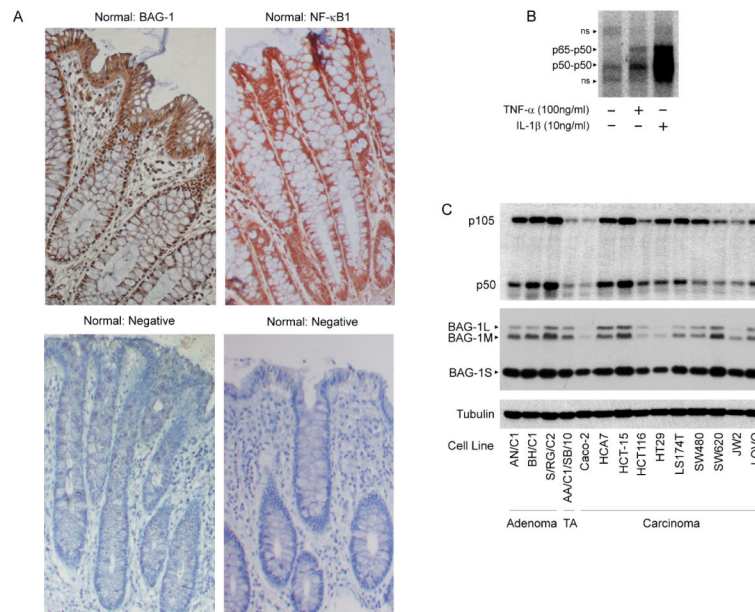


Figure 3. Expression of BAG-1 and NF- κ B1 in normal colorectal epithelium and cell lines (A) BAG-1 and NF- κ B1 expression in paraffin-embedded normal colorectal epithelial tissue. BAG-1 expression was detected using the BAG-1 (TB3; Brimmel *et al.*, 1999) antibody, NF- κ B1 expression was detected using the anti-NF- κ B1 (E10) antibody. Both were visualised using DAB (brown staining) and counter-stained with haematoxylin (blue staining), objective x10. (The negative control for staining is normal colonic epithelium without primary BAG-1 or NF- κ B1 antibody; objective x10). (B) DNA bound NF- κ B complexes in cytokine stimulated HCT116 cells. 3 μ g of HCT116 nuclear fractions were treated with TNF- α (100ng/ml) or IL-1 β (10ng/ml) for 16 hours and analysed by EMSA using the wild type [WT, GGGGACTTCCG] labelled oligonucleotide to detect active NF- κ B DNA binding complexes. Non-specific bands are denoted with ns. (C) Western analysis to determine NF- κ B1 and BAG-1 protein expression in a panel of colorectal cell lines (TA denotes transformed adenoma). Equal loading was confirmed by α -tubulin. Results are representative of three independent experiments.

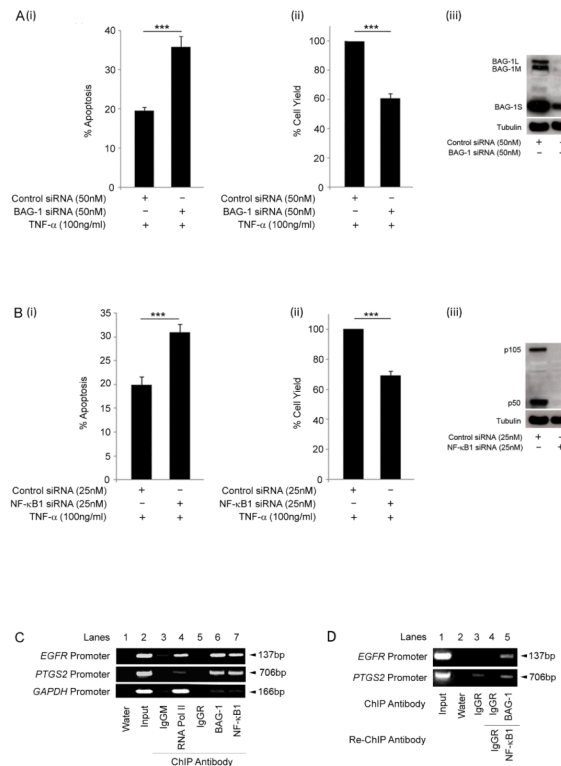


Figure 4. BAG-1 and NF- κ B1 promote cell survival in HCT116 cells

HCT116 cells were transfected with (A) control negative / BAG-1 or (B) control negative / NF- κ B1 siRNA; suppression of protein expression was confirmed by Western analysis (iii). Cells were treated with TNF- α (100ng/ml) for 16h and apoptosis (i) and attached cell yield (ii) assessed. The results shown are the mean of three independent experiments done in triplicate (\pm standard deviation). Statistical analysis was carried out using a Student's t-test * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. **BAG-1 and p50 are present at the same binding site on the EGFR and PTGS2 promoters.** (C) ChIP analysis of the EGFR, PTGS2 and GAPDH promoters in HCT116 cells using antibodies to immunoprecipitate the indicated proteins. The IgGM and IgGR antibodies are used as negative controls for non-specific binding of the antibody to the chromatin fragments. Results are representative of four independent experiments. **p50 and BAG-1 are part of the same DNA complex at gene promoters.** (D) Re-ChIP analysis of the EGFR and PTGS2 promoters in the HCT116 cell line using the indicated combination of antibodies.

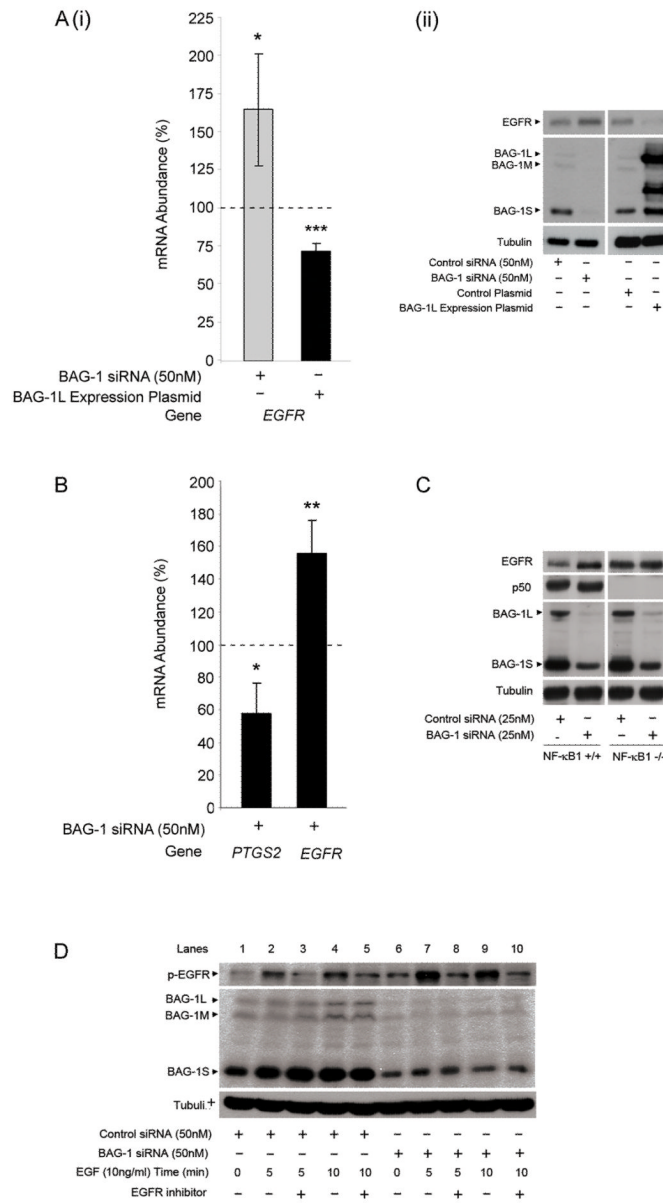


Figure 5. BAG-1 regulation of EGFR expression and function is dependent on NF-κB1 expression

(A) (i) HCT116 cells were transfected with control negative / BAG-1 siRNA or control plasmid / BAG-1L expression plasmid for 48 hours. Q-RT-PCR was carried out to show *EGFR* mRNA abundance; all mRNA values were normalised to the housekeeping gene, *TBP*. Data is presented as a percentage change of levels in control transfected cells, represented by the dotted line. Data points are of three independent experiments carried out in triplicate (\pm standard deviation). Statistical analysis was carried out using a Student's t-test * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. (ii) Western analysis to show regulation of EGFR protein +/- BAG-1 expression. Equal loading was confirmed by α -tubulin. Data is

representative of three independent experiments. (B) HT29 cells (positive for COX-2 expression) were transfected with control negative or BAG-1 siRNA for 48 hours. Q-RT-PCR was carried out to show *PTGS2* and *EGFR* mRNA abundance; all mRNA values were normalised to the housekeeping gene, *TBP*. Data is presented as a percentage change of levels in control transfected cells, represented by the dotted line. Data points are of three independent experiments carried out in triplicate (\pm standard deviation). Statistical analysis was carried out using a Student's t-test * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. (C) NF- κ B1^{+/+} and NF- κ B1^{-/-} MEF cells were transfected with 25nM of control siRNA or mBAG-1 siRNA. Western analysis was carried out to show the regulation of EGFR protein expression following suppression of the BAG-1 protein in NF- κ B1^{+/+} and ^{-/-} MEF cells. Equal loading was confirmed by α -tubulin. Results are representative of three independent experiments. (D) HCT116 cells treated with EGF (10ng/ml) for 72 hr after siRNA transfection either in the presence or absence of an EGFR inhibitor CL-387.785 (10 μ M) added 1h prior to EGF treatment. Activation of the EGFR upon EGF treatment was shown by the phosphorylation status of the receptor, specificity confirmed by the blockade with the EGFR inhibitor. Western blotting was used to confirm suppression of BAG-1 expression by siRNA. Equal loading was confirmed by α -tubulin.