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# Evasion from proteasomal degradation by mutated Fos proteins expressed from FBJ-MSV and FBR-MSV osteosarcomatogenic retroviruses

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**Abstract**

c-Fos proto-oncoprotein is highly unstable, which is crucial for rapid gene expression shut-off and control of its intrinsic oncogenic potential. It is massively degraded by the proteasome *in vivo* in various situations. Although there is evidence that c-Fos can be ubiquitinylated *in vitro*, the unambiguous demonstration that ubiquitinylation is necessary for recognition and subsequent hydrolysis by the proteasome *in vivo* is still lacking. Moreover, genetic analysis have also indicated that c-Fos can be addressed to the proteasome *via* different mechanisms depending on the conditions studied. c-Fos has been transduced by two murine osteosarcomatogenic retroviruses under mutated forms which are more stable and more oncogenic. The stabilization is not simply accounted for by simple deletion of a C-terminal c-Fos destabilizer but, rather, by a complex balance between opposing destabilizing and stabilizing mutations. Though mutations in viral Fos proteins confer full resistance to proteasomal degradation, stabilization is limited because mutations also entail sensitivity to (an) unidentified proteolytic system(s). This observation is consistent with the idea that Fos-expressing viruses have evolved gene expression controls that avoid high protein accumulation-linked apoptosis.


*Keywords:* c-Fos; Oncogene; Retrovirus; Protein degradation; Proteasome; Ubiquitin

## 1. Introduction

c-Fos proto-oncoprotein is a transcription factor contributing to the formation of the AP-1 transcription complex. The latter is a key regulator of major biological processes such as cell proliferation, differentiation, organogenesis, memory formation, apoptosis, response to stress, etc. [1–5] and its activity is also crucial in many tumorigenesis processes [2,3,5,6]. Within AP-1, c-Fos heterodimerizes owing to leucine zipper/leucine zipper interactions with a variety of partners, the best known being the Jun family members. It is expressed constitutively in a few cell types and at low or undetectable levels in most cell types where it can be rapidly and transiently induced by a variety of stimuli [5,7]. Accumulation of c-Fos outside its physiological windows of expression is sufficient for cell transformation [8] and induction of benign bone tumors in transgenic mice [8–11]. To ensure appropriate levels and timing of expression, c-*fos* gene is subjected to numerous cooperating transcriptional and post-transcriptional controls [5].

c-*fos* gene has originally been found in mutated forms transduced by two mouse retroviruses, FBJ-MSV and FBR-MSV [12], inducing aggressive and lethal osteosarcomas [7]. Both v-Fos proteins show a higher intrinsic oncogenic potential than c-Fos, that of v-Fos$^{FBR}$ being the strongest *in vitro* and *in vivo* [5,7,13]. Furthermore, the mutations present in the coding and non-coding regions permit the viral genes to evade the exquisitely controlled transcriptional and post-transcriptional mechanisms operating on c-*fos* [5,14]. This leads to constitutive expression of viral Fos proteins, which is crucial for their oncogenic activity. Of particular interest here, mutated viral proteins show a reduced turnover [14–16] and therefore accumulate to higher levels. Interestingly, slowed down destruction does not correspond to just slowed down proteasomal degradation but results from combined and opposing effects of the various mutations which confer both resistance to the

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proteasome and sensitivity to a protease(s) not operating on c-Fos. Alteration of v-Fos protein turnover is discussed in the more general context of retroviral gene expression.

## 2. c-Fos is an unstable protein mostly degraded by the proteasome

Most, if not all, unstable cellular proteins studied thus far are physiologically degraded by the proteasome, which is the major intracellular protein destruction machinery [17]. Most often, but not always [18], the addressing to the proteasome requires conjugation of multiubiquitin chains to protein substrates [19–21]. Accordingly, the proteasome has been shown to play a crucial role in c-Fos destruction in mouse fibroblasts during the $G_0$-to-G1 phase transition [22,23], in a lymphoma cell line undergoing apoptosis [24] as well as in exponentially growing cells [14]. Lysosomes and calpains have also been suggested to contribute to c-Fos breakdown [25–28]. However, this contribution is, at best, minor and could affect c-Fos destruction only within the cytoplasm where the latter protein can be retained for significant periods of time and/or under specific conditions [29,30]. Determining the actual influence of these two proteolytic systems on c-Fos accumulation in living cells deserves further work.

One important point to take into consideration when addressing c-Fos turnover is that it is not yet clear whether its ubiquitinylation is required for proteasomal degradation. Apparently divergent observations can thus be found in the literature. On the one hand, c-Fos can be ubiquitinylated by purified or semi-purified ubiquitinylation enzymes *in vitro* [31,32] and is slightly stabilized in hamster mutant cell line (*E36-ts20*) thermosensitive for the E1 ubiquitin-activating enzyme of the ubiquitin pathway when cultured at the restrictive temperature [32]. On the other hand, an *Escherichia coli*-produced c-Fos is an extremely sensitive and specific substrate for purified 26S proteasome *in vitro* (unpublished results), as c-Jun is [33], and c-Fos is not stabilized [23]in a mouse embryo fibroblast cell line (Balb/C *A31N-ts20* cells) also thermosensitive for the E1 ubiquitin-activating enzyme. The phenotype of the latter cell line is however leaky [34] and, although stabilization of proteins such as c-Jun or p53 is efficient upon culture at the non-permissive temperature, it is at present not possible to rule out that c-Fos belongs to a subset of proteins still ubiquitinylated due to residual E1 activity. In conclusion, the formal and definite proof of requirement upon ubiquitinylation for c-Fos destruction by the proteasome is not yet available for several reasons. First, c-Fos-ubiquitin conjugates have, thus far, not been described *in vivo*. Second, should such conjugates be observed, it would then be necessary to demonstrate that multiubiquitin chains are necessary for binding to and subsequent proteolysis by the proteasome [18]. This is all the more important that recent works have demonstrated that multiubiquitin chains might alter enzymatic and effector functions of several proteins independently of degradation [35]. Analysis of non-ubiquitinylable c-Fos mutants, most probably through the mutation of the possible ubiquitin acceptor lysines, is thus required to solve this issue. A critical review on ubiquitinylation requirement for protein degradation by the proteasome can be found in Verma and Deshaies [18].

Even though the proteasome plays a major part in c-Fos degradation in the situations where the latter protein destruction has been studied, there is genetic evidence that the mechanisms of addressing are not always the same. For example, c-Fos C terminus plays a major role in the degradation occurring upon constitutive expression in asynchronously growing cells ([36] and our unpublished data), only a modest stabilization is observed upon its deletion in cells undergoing a $G_0$-to-G1 transition [22]. More precisely, using site-directed mutagenesis and an expression system reproducing c-*fos* gene transient expression in transfected cells, we have analyzed the turnover of c-Fos mutants in synchronized mouse embryo fibroblasts. c-Fos contains three PEST motifs which are sequences rich in Pro, Glu, Asp, Ser, and Thr that have been proposed to constitute protein instability determinants [37,38]. One of them, comprising the C-terminal 20 amino acids, has even been proposed to be the major determinant of c-Fos instability [36,37]. In fact, our data showed no role for the two internal PEST motifs in c-Fos instability. However, deletion of the C-terminal PEST region led to only a 2-fold stabilization of the protein. Further dissection of c-Fos C-terminal region showed that the degradation-accelerating effect is not contributed by the whole PEST sequence but by a short PTL tripeptide which cannot be considered as a PEST motif and which can act in the absence of any PEST environment. Interestingly, the PTL motif is conserved in other members of the *fos* multigene family. Nevertheless, its contribution to protein instability is restricted to c-Fos suggesting that the mechanisms whereby the various Fos proteins are broken down are, at least partially, different. Finally, even though the PEST motif-containing C terminus of c-Fos is crucial for instability in asynchronously growing cells, it is worth noting that the implication of the PEST motif *per se* in rapid protein degradation has not yet been demonstrated formally. More generally speaking, we do not feel that the PEST hypothesis, although elegantly proposed on the basis of a computational analysis and, since, widely admitted, has, thus far, been substantiated by unambiguous experimental proofs (see discussion of Acquaviva *et al*. [22] for more information).

## 3. Stabilization of viral Fos proteins

As mentioned previously c-*fos* gene has been transduced in mutated forms by FBJ-MSV and FBR-MSV retroviruses. The mutations that have accumulated during and after the transduction event are however different,

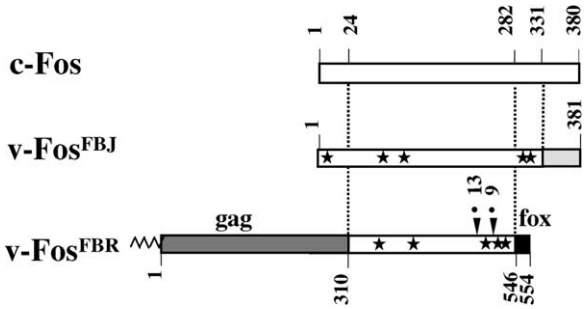


Fig. 1. Structure of c-Fos and v-Fos proteins. c-Fos sequences are represented as white bars whereas v-Fos$^{FBJ}$ and v-Fos$^{FBR}$ specific sequences are represented as colored bars. The numbers indicate amino acid positions. Stars correspond to point mutations, namely S15P, I67T, G110E, S286V, and D291N in v-Fos$^{FBJ}$ and V64E, E138V, I259S, R279G, and P280H in v-Fos$^{FBR}$. Triangles indicate deletions of 13 (amino acids 228–240 of c-Fos) and 9 amino acids (amino acids 260–268 of c-Fos) in v-Fos$^{FBR}$. The broken line at the N terminus of v-Fos$^{FBR}$ represents the myrystyl group.

including at the protein level [5,7]. v-Fos$^{FBJ}$ (381 amino acids; Fig. 1) contains five scattered point mutations and a frameshift that replaces the last 48 amino acids of c-Fos with an unrelated 49 amino acid-long tail [5]. v-Fos$^{FBR}$ is a 554 amino acid protein (Fig. 1), of which (i) 310 are encoded by the retrovirus Gag-derived sequence and replace the N-terminal 24 amino acids of c-Fos, (ii) 236 are derived from c-Fos, and (iii) 8 are derived from a genomic locus called *fox* and replace the C-terminal 98 amino acids of c-Fos. Five point mutations and two in-frame deletions of 13 and 9 amino acids are also scattered throughout the Fos moiety of the molecule. Finally, v-Fos$^{FBR}$ is cotranslationally myristoylated on the Gag N-terminal glycine, which becomes accessible to acylation after removal of the initiator methionine [39,40]. This modification is quantitative and stable over time and is crucial for the tumorigenic activity of the protein [13,39,41–44] (also see [14] for a review).

Pulse-chase experiments have shown that both v-Fos proteins are more stable than c-Fos. However, stabilization is limited and viral proteins keep residual instability since v-Fos$^{FBJ}$ and v-Fos$^{FBR}$ show half-lives of approximately 110 and 140 min, respectively, to be compared to the 50–55 min half-life of c-Fos in exponentially growing simian Cos-7 cells [14]. Moreover, degradation kinetics conducted in the presence of protease inhibitors showed that mutations both confer resistance to proteasomal degradation and sensitivity to another to-be-identified protease(s) which is resistant to all currently available cell-permeant protease inhibitors [14]. Interestingly, a fine genetic analysis of cellular and viral Fos proteins showed that the molecular phenotype of v-Fos$^{FBJ}$ and v-Fos$^{FBR}$ is not simply accounted by loss of a common C-terminal destabilizer of c-Fos but, rather, by a complex balance between opposing stabilizing and destabilizing mutations which are not the same in the two viral proteins [14].

## 4. Concluding remarks

As previously mentioned, c-*fos* gene expression is restricted to precise physiological windows of expression *via* a number of transcriptional and post-transcriptional mechanisms (for references, see [5]). At the transcriptional level, those include the tight regulation of transcription initiation as well as that of elongation of nascent RNA transcripts whereas, at the post-transcriptional level, they include regulation of mRNA splicing, rapid mRNA and protein turnover, the control of the nuclear transport and post-translational modifications such as phosphorylations. It is thus remarkable that *fos* genes expressed from FBJ-MSV and FBR-MSV retroviruses have gained the capability of constitutive expression through the alteration of nearly all of these multiple transcriptional and post-transcriptional regulations (Table 1). It is, however, puzzling

Table 1
c-Fos and v-Fos genes regulation

| c-Fos | v-Fos$^{FBJ}$ | v-Fos$^{FBR}$ |
|---|---|---|
| Complex and tightly regulated promoter region | Constitutive retroviral LTR | Constitutive retroviral LTR |
| Negative transcription regulator element (FIRE) at the end of exon 1 | FIRE element present | FIRE element absent |
| Regulated pre-mRNA splicing | No intron | No intron |
| Highly unstable mRNA | Stabilized mRNA | Stabilized mRNA |
| Highly unstable protein | Stabilized protein | Stabilized protein |
| Transport of the protein into the nucleus regulated by extracellular signals | Constitutive transport of the protein into the nucleus | Constitutive transport of the protein into the nucleus |
| Numerous phosphorylations | Still phosphorylated but loss of the C-terminal phosphorylation region | Not phosphorylated |
| Dimerizes with Jun family members and other bZip transcription factors | Dimerizes with Jun family members and other bZip transcription factors | Dimerizes with Jun family members and other bZip transcription factors |
| Binds to AP-1 and CRE DNA motifs when dimerized with transcription partners | Binds to AP-1 and CRE DNA motifs when dimerized with transcription partners | No longer binds to AP-1 and CRE DNA motifs when dimerized with transcription partners |
| Stimulates AP-1-dependent transcription | Stimulates AP-1-dependent transcription | Cannot stimulate AP-1-dependent transcription |
| Not myristoylated | Not myristoylated | Myristoylated |

that the escape of v-Fos proteins from proteasomal degradation is partially compensated by their sensitivity to another proteolytic system(s), which limits stabilization, and, thereby, accumulation to high levels. High levels of oncogene expression is often pro-apoptotic [45] and may account for the difficulty in isolating stable cell lines expressing high levels of viral Fos proteins (our unpublished observations). It is thus tempting to speculate that the FBR-MSV and FBJ-MSV expression machineries have evolved to ensure controlled protein levels, corresponding to an optimal balance between pro-transformation and pro-apoptotic effects. Supporting this notion, transcription efficiency from viral LTRs is low as compared to that of the activated c-*fos* promoter (our unpublished observations) and viral FBR-MSV RNA is stabilized no more than 2–3-fold as compared to the highly unstable c-*fos* mRNA (our unpublished observations). At that stage, it is also worth mentioning that viral Fos protein accumulation is not only the consequence of gene expression alteration since the activity of both v-Fos$^{FBJ}$ and v-Fos$^{FBR}$ proteins is also most likely altered. In support to this idea, the main phosphorylation domain of c-Fos, which is located within the C terminus 20 amino acids is deleted in both proteins, and v-Fos$^{FBR}$ is quantitatively subjected to N-terminal myristoylation (see [14]). Further work is still required to decipher the relative contributions of quantitative and qualitative effects of mutations in both viral proteins.

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