1   **Asymmetric post-translational modifications regulate the intracellular distribution**

2   **of unstimulated STAT3 dimers**

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17   **Running title:** Asymmetric PTMs regulate latent STAT3 dimer localization

18   **Key words:** STAT3, bimolecular fluorescence complementation, dimerization, post-

19   translational modifications, acetylation

20   **Abbreviations:** BiFC, bimolecular fluorescence complementation; PTMs, post-

21   translational modifications

22   **Contribution:** BC is responsible for most microscopy studies, and RV for cloning,
23   mutagenesis and flow cytometry studies, as well as part of microscopy analyses. CSA
24   and AMR are responsible for the first optimization experiments with the BiFC system
25   and LIF experiments, a small part of which are shown in Suppl. Fig. 1. FM contributed
26   to the microscopy, flow cytometry and immunoblotting at different stages of the project.
27   CR and VM contributed to the analysis, presentation and interpretation of data. FH had
28   the original idea, designed the experiments, arrange the final figures and analyzed the
29   data, coordinated the project and wrote the manuscript.

30   **Conflicts of Interest:** The authors declare no potential conflicts of interest.

31   **Abstract**

32   Signal Transducer and Activator of Transcription 3 (STAT3) is a ubiquitous and

33   pleiotropic transcription factor that plays essential roles in normal development,

34   immunity, response to tissue damage and cancer. We have developed a Venus-STAT3

35   bimolecular fluorescence complementation (BiFC) assay that allows the visualization

36   and study of STAT3 dimerization and protein-protein interactions in living cells.

37   Inactivating mutations on residues susceptible to post-translational modifications

38   (K49R, K140R, K685R, Y705F and S727A) changed significantly the intracellular

39   distribution of unstimulated STAT3 dimers when the dimers were formed by STAT3

40   molecules that carried different mutations. Our results indicate that asymmetric post-

41   translational modifications on STAT3 dimers could constitute a new level of regulation

42   of STAT3 signaling.

43

44   **Significance**

45   The Signal Transducer and Activator of Transcription 3 (STAT3) transcription

46   factor plays key roles in development, immunity, cancer or response to stress or

47   damage. All previous studies on STAT3 dimerization work on a homogenous pool of

48   STAT3 molecules, where all STAT3 molecules are equally modified (i.e."symmetric").

49   However, this is highly unlikely in a complex intracellular environment, as post-

50   translational modifications do not necessarily occur with complete efficiency or

51   simultaneously. We demonstrate that asymmetric post-translational modifications

52   change the intracellular distribution of STAT3 dimers more strikingly than symmetric

53   ones. This could mean a new level of regulation of STAT3 activity, and therefore a new

54   possible therapeutic target. Our results could be highly relevant for other protein

55   complexes regulated by post-translational modifications beyond STAT3.

56

57 \body**Introduction**

58 The Signal transducer and activator of transcription 3 (STAT3) is a conserved

59 transcription factor that plays key roles in development, immunity, response to injury

60 and cancer (1,2). STAT3 homodimerization, post-translational modification (PTM) and

61 intracellular location are key events in its biological functions. STAT3 is canonically

62 activated by phosphorylation at Y705 upon stimulation with a variety of cytokines and

63 growth factors. However, unstimulated STAT3 also dimerizes, is found in the nucleus,

64 binds to DNA and controls the transcription of a specific set of genes, different from

65 phosphorylated STAT3 (1–5). STAT3 can be also found in the mitochondria, where it is

66 necessary for normal activity of the electron transport chain (6,7). This function is

67 independent of its activity as a transcription factor and Y705 phosphorylation, but

68 dependent on S727 phosphorylation (1,6,7). Other PTMs can regulate the behavior and

69 function of STAT3, such as acetylation at K49 or K685 (3,8,9) or dimethylation at K49

70 or K140 (10,11). Although dimethylation of the K49 or K140 residues is induced by

71 stimulation with cytokines and is favored by STAT3 phosphorylation, there is basal

72 K49 (but not K140) dimethylation in unstimulated STAT3 (10), and the same happens

73 with STAT3 acetylation (8,9). Literature on STAT3 generally assumes that STAT3

74 homodimers are formed by two identically modified molecules. However, this is highly

75 unlikely in a complex intracellular context, as PTMs do not occur in all the pool of

76 STAT3 molecules at the same time or with the same efficiency. Here, we aimed at

77 determining the relative contribution of residues K49, K140, K685, Y705 and S727 to

78 the dimerization and intracellular distribution of STAT3 homodimers.

79 **Material and Methods**

80 We developed a suit of plasmids to study STAT3 homodimerization in living cells,

81 based on bimolecular fluorescence complementation (BiFC)(12). Briefly, the cDNA

82 sequence of STAT3-alpha was fused to the sequence of two complementary, non-

83 fluorescent fragments of the Venus protein (Venus 1, amino acids 1-157; and Venus 2,

84 amino acids 158-238)(Fig. 1A), and inserted in a pcDNA 3.3 TOPO backbone

85 (Invitrogene). When STAT3 dimerizes, the Venus fragments are brought together and

86 reconstitute the fluorophore, the fluorescence being proportional to the amount of

87 dimers (Fig. 1A). Deletion mutants lacking the C-terminus (DelCT) were produced by

88 PCR-mediated subcloning using full-length Venus-STAT3 BiFC constructs as

89 templates. The original lysine (K) residues on positions 49, 140 and 685 were replaced

90 by arginine (R) residues, the tyrosine (Y) residue on position 705 by phenylalanine (F)

3

91   and the serine (S) residue on position 727 by alanine (A)(Fig. 1A). Supplementary

92   Table I shows the primers used for cloning and mutagenesis were. Detailed methods for

93   site-directed mutagenesis, cell culture and transfection, fluorescence microscopy, flow

94   cytometry and immunoblotting were described elsewhere (12). All BiFC constructs

95   were deposited in Addgene (https://www.addgene.org/). HeLa human cervix

96   adenocarcinoma cells and HEK293 human embryonic kidney cells were acquired from

97   ATCC (references CRM-CCL-2 and CRL-1573, respectively), Leukemia inhibitory

98   factor (LIF) from R&D systems (MN, USA), and Stattic from Selleckchem (TX, USA).

99

100 **Results**

101 Transfection of HEK293 or HeLa cells with the wild-type (WT) pair of Venus-

102 STAT3 BiFC constructs led to successful expression of the chimeric proteins V1-

103 STAT3 and V2-STAT3 (Figs. 1B and 1C, Suppl. Fig. 1). Fluorescence was primarily

104 cytoplasmic in both cell lines, with low but visible nuclear signal (Fig. 1C, Suppl. Fig.

105 1B). Incubation with Leukemia Inhibitory Factor (LIF, 100 ng/ml) induced STAT3

106 phosphorylation and translocation to the nucleus in HEK293 and HeLa cells (Suppl.

107 Fig. 1B-C), but it did not enhance STAT3 dimerization (Fig. 1B, Suppl. Fig. 1D).

108 Incubation with STAT3 inhibitor Stattic (5 µM) or removal of the C-terminus

109 containing the SH2 domain partially prevented STAT3 dimerization (Fig. 1B),

110 consistent with previous reports (13,14). On the other hand, single or double

111 Y705F/S727A phosphoresistant mutants did not decrease fluorescence (Fig. 1B). These

112 results support relevant evidence indicating that STAT3 dimerization is actually

113 independent of phosphorylation (1,5,14). The behavior of the Venus-STAT3 BiFC

114 system is therefore consistent with previous reports for tagged STAT3.

115 In order to analyze the intracellular location of unstimulated STAT3 homodimers,

116 we classified cells qualitatively in three categories that are mutually exclusive (their

117 sum is 100% of cells), according to the relative intensity and location of the

118 fluorescence signal (Fig. 1C-1D): 1) predominantly in the cytoplasm (e.g. WT pair), 2)

119 predominantly in the nucleus (e.g. upon LIF induction, Suppl. Fig. 1B), or 3)

120 homogeneously distributed through nucleus and cytoplasm (e.g. Y705F pair). We also

121 determined the percentage of cells with mitochondrial signal or intracellular inclusions

122 (Suppl. Fig. 2). Although changes in patterns of STAT3 dimer distribution were

123 observed in several symmetric BiFC pairs, only Y705F and S727A pairs induced

4

124   significant increases in the percentage of cells with homogeneous nucleocytoplasmic

125   fluorescence or inclusions, respectively (Fig. 1D).

126   We made use of the unique properties of our BiFC system to determine the relative

127   contribution of each residue to the dimerization and intracellular distribution of

128   unstimulated STAT3 dimers. We combined all possible inactivating PTM mutations

129   with each other, but no combination had a consistent effect on STAT3 dimerization as

130   determined by flow cytometry (Suppl. Fig. 3). However, the intracellular distribution of

131   STAT3 homodimers was significantly altered by specific combinations of STAT3

132   molecules (Fig. 2A). Unlike the K49R symmetric pair, K49R asymmetric combinations

133   dominantly induced an increase in cells with homogeneous nucleocytoplasmic

134   fluorescence at the expense of cytoplasmic location (Fig. 2A), similar to the Y705F

135   symmetric pair. K140R- or K685R-containing pairs showed some tendency to shift

136   cytoplasmic location towards nucleus, but only the K140R+S727A combination

137   achieved significance. This phenotype was almost identical to the Y705F+S727A

138   asymmetric pair (Fig. 2A).

139   We then pooled and analyzed all results according to the number and type of PTM

140   mutations present in the each BiFC pair. Combinations carrying any one (asymmetric)

141   or two K-R substitutions (symmetric or asymmetric) significantly increased

142   mitochondrial translocation, while decreasing the percentage of cells with STAT3

143   dimers predominantly in the cytoplasm (Fig. 2B). Asymmetric combinations of one K-R

144   substitution and one phosphoresistant mutant also increased nuclear translocation, but

145   only 2xK-R combinations increased homogeneous nucleocytoplasmic distribution.

146   Combinations carrying any two phosphoresistant mutations (symmetric or asymmetric)

147   had no significant effect on cellular distribution of STAT3 homodimers (Fig. 2B).

148   These results indicate that specific asymmetric PTMs on STAT3 dimers can prevent

149   their nuclear import/export. This was later confirmed by pooling the data according to

150   whether the STAT3 pair was symmetric or asymmetric in their PTM mutations (Fig.

151   2C). We found that only asymmetric PTM mutant combinations increased

152   nucleocytoplasmic or nuclear distribution at the expense of decreasing cytoplasmic

153   localization of STAT3 homodimers. Asymmetric combinations were also sufficient to

154   produce an increase in mitochondrial localization of STAT3 dimers (Fig. 1C).

155   **Discussion**

156   Our results indicate that asymmetric PTMs could constitute a new level of
157   regulation of unstimulated STAT3 behavior and function. Most STAT3 molecules are
158   not phosphorylated in the absence of extracellular stimuli, and this proportion is
159   reversed shortly after cytokines bind to their corresponding membrane receptors (Suppl.
160   Fig. 1C). However, cells often show small amounts of phosphorylated STAT3 in resting
161   state (Suppl. Fig. 1C) and, conversely, cytokine-stimulated STAT3 induces the *de novo*
162   transcription of new STAT3 molecules that are not necessarily phosphorylated (1,2).
163   This indicates that unphosphorylated and phosphorylated STAT3 should coexist at
164   similar levels in many situations, and the literature presents evidence that this could be
165   equally true for other STAT3 PTMs induced by cytokines (8-10).

166   To the best of our knowledge, there is no direct empirical evidence in the literature
167   showing that asymmetric STAT3 dimers actually happen in living cells, and such
168   demonstration would be currently extremely difficult from a technical point of view,
169   even *in vitro*. Previous studies most often rely on systems that do not differentiate
170   between monomers and dimers (2,3,6-11), and/or that produce a single population of
171   STAT3 molecules, either mutated or normal (5,14,15). And yet, in the crowded and
172   diverse intracellular environment, the probability for two identical STAT3 molecules to
173   form a dimer (or for a dimer to be modified in both molecules simultaneously and in the
174   same residues) should be low, although it could certainly be enhanced by either total
175   absence or presence of stimuli. Our results point in this exciting direction, and open a
176   series of interesting questions. If asymmetric STAT3 dimers actually happen, do they
177   regulate specific sets of genes? Do they enable gradation of STAT3 transcriptional or
178   mitochondrial activities? And if they do not happen, how do cells manage to achieve
179   perfectly symmetrical STAT3 dimers with such high efficiency? Given the essential
180   roles of STAT3 in development, immunity, tissue stress and cancer, addressing these
181   questions could have important implications for the diagnosis, treatment and
182   understanding of a wide spectrum of human pathologies.

183   **Acknowledgements**

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238

239   **Figure Legends**

240   **Figure 1. A Venus-STAT3 BiFC system allows the visualization and study of**
241   **STAT3 homodimers in living cells.** A, Venus BiFC fragments constituted by amino
242   acids 1-158 (Venus 1, V1) and 159-238 (Venus 2, V2) were fused to the N-terminus of
243   the STAT3 sequence in two independent constructs. K49, K140, K685, Y705 and S727
244   residues can be post-translationally modified, and were inactivated in both V1- and V2-
245   STAT3 constructs by site-directed mutagenesis. B, Wild-type Venus-STAT3 constructs
246   produced fluorescence in HeLa cells, and it was monitored by flow cytometry 24 hours
247   after transfection with BiFC constructs. Incubation with Leukemia Inhibitory Factor
248   (100 ng/ml) for 2 hours in the absence of serum or the presence of the indicated drugs or
249   mutant BiFC pairs (n=3, p<0.05). Results were normalized versus the Wild-type STAT3
250   pair (100%). C, Microscopy pictures of representative cell phenotypes in the different
251   symmetric combinations of BiFC Venus-STAT3 constructs (Incl, inclusions; Scale bar,
252   20µm). D, Percentage of cells displaying fluorescence predominantly in the Nucleus
253   (black bar), predominantly in the Cytosol (white bar), homogeneously distributed in
254   cytoplasm and nucleus (nucleocytoplasmic, grey bar), in the mitochondria or in non-
255   mitochondrial inclusions. Data are shown as the average ± SEM of n=12 (Wild-type,
256   WT) or n=3 (rest of combinations) independent experiments. Statistical analysis was
257   carried out by means of a One-way ANOVA followed by a Bonferroni test adjusted for
258   multiple comparisons. *, sign. vs the symmetric Wild-type STAT3 pair, p<0.05.

259

260   **Figure 2. Asymmetric STAT3 PTMs regulate intracellular distribution of STAT3**
261   **homodimers.** A, Intracellular distribution of fluorescence in asymmetric combinations
262   of Venus-STAT3 BiFC constructs (and the WT symmetric pair as reference). Data are
263   shown as the average of n=12 (Wild-type, WT) or n=3 (rest of combinations)

264   independent experiments ± SEM. Statistical analysis was carried out by means of a
265   One-way ANOVA followed by a Bonferroni test adjusted for multiple comparisons. *,
266   significant vs the symmetric Wild-type STAT3 pair, *, $p<0.05$, ** $p<0.01$. B-C, The
267   same original data, but pooled according to the number and nature of substitutions (B)
268   or the symmetry or asymmetry of substitutions (C) in the STAT3 homodimer, and
269   represented as box plots. The limits of the boxes represent the smallest and largest
270   values, the straight line represents the median, the dashed line represents the average,
271   and the dotted line represents the average for wild-type STAT3 pair. Statistical analysis
272   was carried out on the Average ± SEM of each pool of data (1xYF/SA:1xKR, n=6;
273   2xYF/SA, n=3; 2xKR, n=6; sym, n=5; asym, n=10). Statistical analysis was carried out
274   by means of a One-way ANOVA followed by a Bonferroni test adjusted for multiple
275   comparisons. *, significant vs the symmetric Wild-type STAT3 pair, *, $p<0.05$, **
276    $p<0.01$; #, significant vs 2xYF/SA substitution (in B) or the symmetric mutant pairs (in
277   C), #, $p<0.05$, ##, $p<0.01$.
278

A

C — STAT3 (N)
Venus 1 (1-158)

C — STAT3 (N)
Venus 2 (159-238)

→ STAT3 STAT3

STAT3 mutations

ΔCT

1	130	320	465	585	688	770
NTD	CCD	DBD	LD	SH2	TAD	

K49R   K140R   K685R   Y705F   S727A

B

% Fluorescent cells vs WT-STAT3 pair

WT-STAT3 pair, +LIF, +Stattic*, DelCT + WT*, Y705F pair, S727A pair, Y705F/S727A pair

C

Symmetric BiFC pairs

WT, K49R, K140R, K685R, Y705F, S727A incl

20 µm

D

Nucleus   Cytoplasm   Nucleocytoplasmic
Mitochondria   Inclusions

% Cells

WT   K49R   K140R   K685R   Y705F*   S727A

Symmetric STAT3 dimer Combinations