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# Matters Arising

## Arginine Methylation of STAT1: A Reassessment

An intriguing model was proposed stating that arginine 31 methylation of STAT1 enhances its DNA binding by reducing association with the specific inhibitor PIAS1, thus intensifying the growth-restraining activities of the interferons (Mowen et al., 2001). This finding may explain the interferon (IFN) insensitivity of cancer cells that accumulate high levels of the methyltransferase inhibitor methylthioadenosine (MTA).

Mowen et al. used monoclonal antibodies against dimethylarginine for immunoprecipitations to demonstrate methylation of STAT1 in vivo. However, coprecipitation with a methylated protein could not be excluded and the antibody did not function in Western blots to confirm the modification on STAT1 (Mowen and David, 2001). Thus, this approach does not prove STAT1 methylation, let alone differences in STAT1 methylation stoichiometry. Additionally, the authors reported mass spectrometry (MS) without providing sequencing data for peak identification and without negative controls. We performed three independent MS analyses using Strep-tagged STAT1 that was purified from stably transfected U3A cells (a STAT1-negative derivative of the cell line used by Mowen et al. in their Figure 2B), and the results were identical irrespective of IFN treatment of cells (for methods, see Supplemental Data at http://www.cell.com/cgi/content/full/119/5/587/DC1/). The MALDI spectra obtained with STAT1 after AspN cleavage, which yielded peptides with masses identical to those reported by Mowen et al., revealed singly charged mass peaks at *m/z* 1319.7 and *m/z* 1704.9, corresponding to the unmodified STAT1 fragments $^{29}$EIRQYLAQWL$^{38}$ and $^{29}$EIRQYLAQWLEKQ$^{41}$ (one missed cleavage), respectively (Figure 1A). Mass peaks which would match with R$^{31}$-methylated sequences (+14 mass units per methyl group) were not present. The amino acid sequences of the unmodified peptides were confirmed by nanoLC-ESI-MS/MS (Supplemental Figure S1 online). In addition, we synthesized R$^{31}$-unmethylated (*m/z* 1319.8 and 1709.9) and R$^{31}$-dimethylated (+28 mass units) peptides corresponding to the mentioned AspN fragments of STAT1. MALDI and ESI measurements (Figure 1B and Supplemental Figure S1 online) yielded comparable ion intensities irrespective of methylation, thus excluding the possibility that methylated peptides went undetected due to insufficient ionization. Considering the optimal peak intensities and the excellent signal-to-noise ratio of our MS analyses with the native material, we concluded that STAT1 is not methylated at R$^{31}$ to a significant extent. Based on the premise of rapid methyl turnover in IFNα-treated HeLa cells made by Mowen et al., such cells were metabolically labeled for 3 hr by using L-[*methyl*-$^{3}$H]-methionine before STAT1 was immunoprecipitated. Subsequent fluorography of two independent experiments revealed methylation of several proteins but not of STAT1 (Figure 2 and Supplemental Figure S2).

Mowen et al. used an R$^{31}$A mutant to mimic STAT1 methylation. We examined the mutant in a STAT1-negative background and found loss of function in contrast to authors of the Mowen et al. study who used cells expressing endogenous STAT1 and reported gain of function (Supplemental Figure S3; Shuai et al., 1996). We discovered that the IFN-induced association with Importin-α5 required a conserved STAT1 N domain of 130 residues (Figure 3A), explaining previously reported nuclear translocation defects (Strehlow and Schindler, 1998). Alanine mutation of single (E$^{29}$, R$^{70}$, E$^{111}$) or multiple surface residues ([H$^{81}$, R$^{84}$, K$^{85}$, R$^{88}$][K$^{110}$, E$^{111}$, R$^{113}$, K$^{114}$]) in the N domain, which itself is devoid of transport activity (Begitt et al., 2000), did not affect nuclear import (data not shown), indicating an unconventional nuclear targeting function. In contrast, mutations affecting a buried invariant salt bridge network (W$^{4}$, W$^{37}$, E$^{39}$, E$^{112}$, Vinkemeier et al., 1998) grouped around R$^{31}$ (including mutation of R$^{31}$ to A or K) abolished IFN-induced nuclear import (Supplemental Figures S4 and S5). Moreover, these mutations caused N-terminal degradation and defective dephosphorylation (Supplemental Figure S6), another malfunction seen also after amino-terminal deletions (Supplemental Figure S6). Fusion of STAT1-R$^{31}$A to GST, a stabilized derivative used by Mowen et al., neither remedied defective dephosphorylation (Shuai et al., 1996) nor restored nuclear import (Figure 3B). We

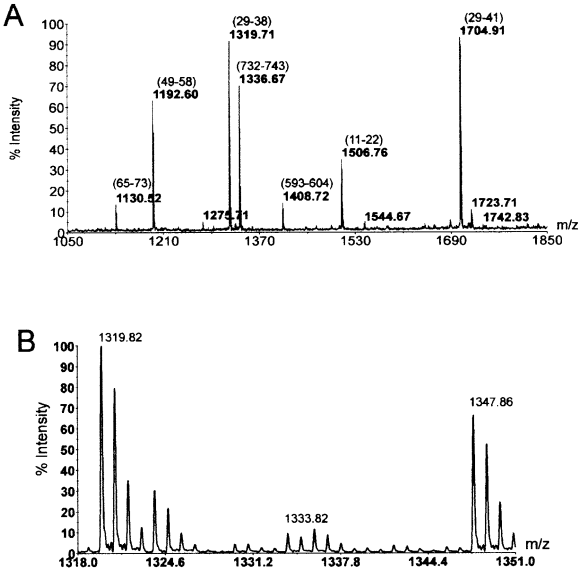


Figure 1. MALDI Mass Analyses of STAT1 R$^{31}$ Methylation

(A) Peaks with *m/z* 1319.71 and 1704.91 correspond to the unmodified AspN fragments 29–38 and 29–41, respectively. STAT1 was purified from U3A cells.

(B) Peaks with *m/z* 1319.8 and 1347.9 correspond to the unmodified and R$^{31}$-dimethylated AspN fragment 29–38, respectively. An equimolar mixture of synthetic peptides was used.

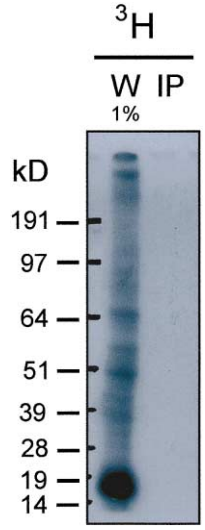


Figure 2. Absence of Methylation of STAT1

HeLa cells were incubated with the methyl donor L-[*methyl*-$^3$H]methionine in the presence of protein synthesis inhibitors. The gel was loaded with 1% of the whole-cell extract (W) or the immunoprecipitate obtained with a STAT1-specific antibody (IP). Shown is the methylation pattern as determined by fluorography after 2 weeks exposure.

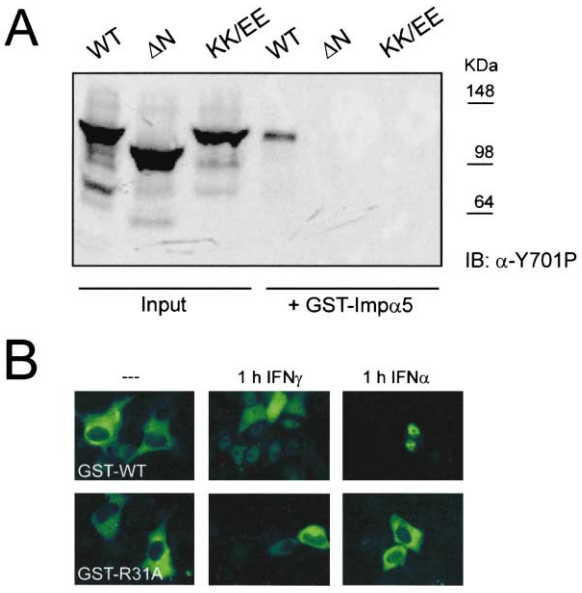


Figure 3. Mutations Affecting the N Domain Structure Preclude Nuclear Import of STAT1

(A) Lysates from IFNγ-treated U3A cells expressing STAT1-GFP fusion proteins (WT, wild-type; ΔN, STAT1 lacking residues 1–126; KK/EE, import mutant KK410/413 to EE; Meyer et al., 2002) were normalized with respect to tyrosine-phosphorylated STAT1 (input) and incubated with sepharose-immobilized GST-Importin-α5. Tyrosine-phosphorylated STAT1 in the input and bound to Importin-α5 was detected by Western blotting.

(B) HeLa cells transiently expressing wild-type or R$^{31}$A mutant STAT1-GFP fused to the C terminus of GST were left untreated or stimulated with IFNα or -γ for 60 min. GST-STAT1 was detected in fixed cells by GFP fluorescence. IFNα stimulation was performed in HeLa cells that additionally coexpressed Tyk2 and IFNα receptor 1.

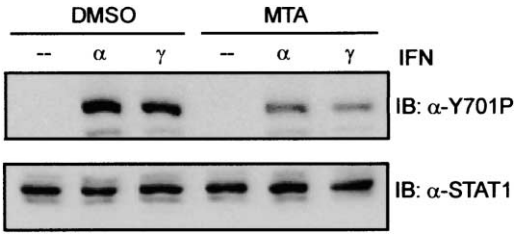


Figure 4. MTA Inhibits Tyrosine Phosphorylation of STAT1

MCF-7 cells were incubated for 3 hr with medium containing carrier (DMSO) or MTA (0.3 mM). Subsequently, the cells were left untreated or stimulated with IFNα or -γ for 30 min as indicated. Shown are Western blot results of whole-cell extracts with antibodies against tyrosine-phosphorylated STAT1 (α-Y701P) or total STAT1 (α-STAT1).

concluded that mutation of R$^{31}$ destabilized and inactivated the N domain.

According to Mowen et al., inhibitory effects on IFNα-induced transcription of the methyltransferase inhibitor MTA can be attributed specifically to the reduced arginine methylation of STAT1. Yet, MTA has been shown to adversely affect a variety of biological processes (Williams-Ashman et al., 1982), as evidenced by its influence on numerous enzymatic reactions. Used at the concentration reported by Mowen et al., it considerably diminished the IFN-induced tyrosine phosphorylation of STAT1 (Figure 4). This was seen for several cell lines, various MTA concentrations, and even in the absence of the STAT1 N domain, thus ruling out the participation of R$^{31}$ (Supplemental Figures S7A and S7B). The proportionate decrease in DNA binding activity provides a simple explanation for the reduced transcriptional activity of STAT1 during MTA treatment (Supplemental Figures S7C and S7D). Analogous observations were made also for STAT6 (Chen et al., 2004). In addition, we found that MTA inhibited also the dephosphorylation of the kinase p38, and MTA strongly reduced transcription of a STAT1-independent NF-κB reporter gene (Supplemental Figure S8).

In summary, our examination provided evidence that contradicts previous results that stated methylation of arginine 31. Thus, alternative explanations to STAT1 methylation need to be explored in order to understand the molecular mechanisms that underlie the reduced interferon sensitivity of many tumor cells.

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**Selected Reading**

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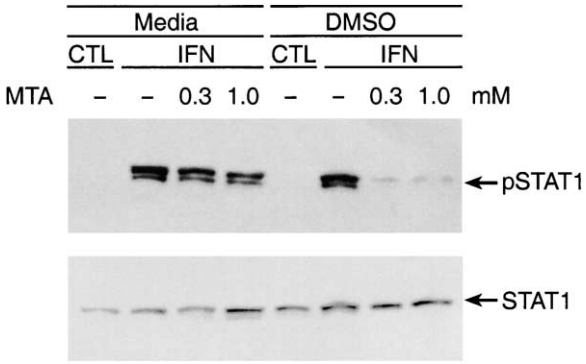


Figure 1. Effect of MTA on STAT1 Tyrosine Phosphorylation

MTA was dissolved either in DMSO or directly in tissue culture medium. MTA was added to the cells 1 hr prior to stimulation with IFNβ (1,000 u/ml) for 30 min. Cell lysates were probed with p(Y701)STAT1 or STAT1 antisera.

## Response to Matters Arising

In their Matters Arising article, Meissner et al. present several experiments that call into question our conclusions from the results reported in our original manuscript (Mowen et al., 2001). We clearly acknowledge the importance of these new findings, but at the same time have reservations about the extent to which these results invalidate our conclusions of STAT1 arginine methylation.

We had employed monoclonal antibodies against dimethylarginine (DMA) to demonstrate methylation of STAT1 in vivo. Since the antibody was ineffective in Western blots, we performed immunoprecipiations, followed by anti-STAT1 immunoblotting. Specificity of the antibodies was demonstrated by the fact that only a methylated STAT1 N-terminal domain could successfully compete for binding to the DMA antibody. Other labs have in the meantime reproduced these findings for STAT1, STAT3, and STAT6 (Chen et al., 2004; Duong et al., 2004; Rho et al., 2001). However, despite the use of high-stringent immunoprecipitation condition, coimmunoprecipitation of STAT proteins with associated, arginine-methylated proteins can naturally not be definitively excluded in these experiments. However, Rho et al. reported the arginine methylation of STAT3 (Rho et al., 2001). This study not only used the DMA antibody for immunoprecipitation followed by STAT3 Western blotting but also successfully employed the DMA antibody for Western blotting of STAT3 immunoprecipitates to demonstrate STAT3 arginine methylation (Rho et al., 2001, Figure 2).

In the metabolic labeling experiments using L-[*methyl*-$^{3}$H]methionine, the incorporation of radioisotope is not restricted to arginine residues, as lysines as well as COOH termini can also be methylated. It would therefore be helpful to know whether the authors were able to detect radioisotope incorporation into other proteins known to be arginine methylated on a single residue (e.g., Sam68, CBP, EWS).

Dr. Vinkemeier's group further reports that in their hands the methylation inhibitor MTA blocks tyrosine phosphorylation of STAT1, which is in striking contrast to our findings. The authors further state that "… the same observations were also made for STAT6 (Chen et al., 2004)." Meissner et al. incubated the cells with MTA in DMSO, whereas we had solubilized MTA in tissue culture media. While somewhat unexpected, this difference in solvents used seems to make a significant difference (see Figure 1): when dissolved in DMSO, MTA does indeed block STAT1 tyrosine phosphorylation. However, when MTA is dissolved in media (as was done in our original studies to completely block IFNα/β-mediated ISG induction), no inhibition of STAT1 tyrosine phosphorylation is observed. While we cannot offer an explanation for these different, solvent-dependent effects of MTA, the finding nevertheless invalidates the notion that mere inhibition of STAT1 tyrosine phosphorylation accounts for the inhibitory effects of MTA on IFNα/β-induced gene transcription. In addition, the paper reporting STAT6 arginine methylation quoted in this context by the authors clearly shows that STAT6, *but not STAT1* tyrosine phosphorylation is inhibited by the methyltransferase inhibitors (Chen et al., 2004: Figure 6B). Lastly, MTA-mediated inhibition of STAT1 tyrosine phosphorylation cannot explain the increased PIAS association with hypomethylated STAT1 we and others have observed (Duong et al., 2004). The fact that MTA also blocks an NF-κB luciferase does not justify the conclusion that it is a nonspecific transcriptional inhibitor. The inhibitory effect of MTA on LPS-mediated NF-κB activation has been previously reported; however, the same paper provides clear evidence for significantly increased IL-10 production under these conditions (Hevia et al., 2004). We have also shown in our manuscript that c-*fos* induction by serum is not affected by MTA. Lastly, in a follow-up paper published in 2002 we had used different methylation inhibitors, which like MTA caused abrogation of IFNα/β-induced transcription without blocking STAT1 tyrosine phosphorylation (Zhu et al., 2002).

Our own mass spectrometry data, which had identified peptides of the appropriate molecular mass (Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/5/589/DC1/), and the concurring results we had obtained from the mutational analysis of STAT1 R31 may have mislead us into the conclusion that R31