Evidence for Homodimerization of the c-Fos Transcription Factor in Live Cells Revealed by Fluorescence Microscopy and Computer Modeling

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ABSTRACT

The c-Fos and c-Jun transcription factors, members of the activator protein-1 (AP-1) complex, form heterodimers and bind to DNA via a basic leucine zipper, and regulate the cell cycle, apoptosis, differentiation, etc. Purified c-Jun leucine zipper fragments could also form stable homodimers, whereas c-Fos leucine zipper homodimers were found to be much less stable in earlier in vitro studies. The importance of c-Fos overexpression in tumors and the controversy in the literature concerning c-Fos homodimerization prompted us to investigate Fos homodimerization. FRET and molecular brightness analysis of fluorescence correlation spectroscopy data from live HeLa cells transfected with fluorescent protein-tagged c-Fos indicated that c-Fos formed homodimers. We developed a method to determine the absolute concentrations of transfected and endogenous c-Fos and c-Jun, which allowed us to determine dissociation constants of c-Fos homodimers (K_d=6.7±1.7 μM) and c-Fos–c-Jun heterodimers (on the order of 10-100 nM) from FRET titrations. Imaging fluorescence cross-correlation spectroscopy and molecular modeling simulations confirmed that c-Fos homodimers were stably associated and could bind to the chromatin. Our results establish c-Fos homodimers as a novel form of the AP-1 complex, which may be an autonomous transcription factor in c-Fos overexpressing tissues, and could contribute to tumor development.

Introduction

Activator protein 1 (AP-1) is a transcriptional regulator composed of members of the Fos, Jun and ATF families of DNA binding proteins (1, 2). c-Fos and c-Jun regulate a variety of processes including proliferation, differentiation, apoptosis and oncogenesis (3). They function as dimers binding to the promoter/enhancer regions of numerous mammalian genes (4). Their DNA binding domain is composed of a leucine zipper promoting dimerization and a basic region, which binds with high affinity to a specific 8 bp long DNA sequence (5, 6).

In addition to forming stable heterodimers with c-Fos (7-9), c-Jun can also homodimerize as revealed in vitro by electrophoretic mobility shift assay (EMSA) (8), and bind to DNA as a homodimer, although with lower affinity than the heterodimer (8, 10). In contrast, the c-Fos homodimer was found to be unstable in vitro, and thus c-Fos has been thought to interact with DNA only by forming heterodimers with c-Jun (9, 11, 12). The instability of the c-Fos dimer is thought to be due to repulsion between its negatively charged residues in the leucine zipper (6). The wild type c-Fos zipper showed no homoassociation at a concentration of 0.1 μM according to EMSA (13). O'Shea and coworkers estimated the K_d of the c-Fos leucine zipper homodimer to be 3.2 μM and 5.6 μM at 0 and 25 °C, implying that failure to detect c-Fos dimerization by others was probably due to low protein concentrations (14). It was shown by EMSA that a single amino-acid change in the leucine zipper is sufficient to allow a truncated c-Fos to homodimerize and bind to its DNA response element (15). Thermal melts on different leucine zipper dimers revealed that thermal stability increases from c-Fos–c-Fos through c-Fos–c-Jun to c-Jun–c-Jun (16).

c-Fos expression and activation can be induced by growth factors, cytokines or neurotransmitters via G-protein coupled receptors, MAPK, cAMP- or Ca^{2+}-dependent signaling pathways (17-19). c-Fos overexpression occurs in several pathological conditions, which can

have both proliferative and antiproliferative effects. c-Fos was overexpressed in some tamoxifen resistant human breast tumors (20), and highly overexpressed in malignant oral tissues (21). It could also contribute to hepatocarcinogenesis (22). In a murine skin carcinogenesis model c-Fos was shown to be required for malignant tumor conversion (23). c-Fos can be up-regulated via the thyroid hormone nuclear receptor α1, which is a tumor inducer in intestinal tumorigenesis (24). Conversely, c-Fos overexpression inhibited cell-cycle progression and stimulated cell death in hepatocytes (25). It also activated apoptosis in colorectal carcinoma cells in a p53 dependent manner (26).

Because c-Fos but not c-Jun is overexpressed in many different types of tumors, we were interested whether at higher concentrations c-Fos could form stable homodimers and bind to DNA in live cells. Förster resonance energy transfer (FRET) can be used to assess distances between two fluorophores in the range of 2–10 nm (27, 28), whereas fluorescence cross-correlation spectroscopy (FCCS) can demonstrate the co-mobility of two molecules (29-31). Using these methods previously, we demonstrated heterodimerization and chromatin binding of c-Fos and c-Jun, and described the conformation of their complex in live cells (7, 32). It was shown in our lab (DKFZ) by imaging FCCS that mobility and protein-protein interaction maps of c-Fos and c-Jun were correlated (33).

Here we performed FRET measurements on fluorescent protein-tagged c-Fos molecules by confocal microscopy and flow cytometry to examine whether c-Fos could form homodimers. We developed a method combining fluorescence correlation spectroscopy and immunofluorescence to assess the concentrations of both the fluorescently labeled and unlabeled, endogenous c-Fos and c-Jun in cells. This allowed us to determine the K_d of c-Fos homodimers and c-Fos–c-Jun heterodimers in live HeLa cells by FRET titrations. We found that the K_d of the c-Fos homodimer is more than one order of magnitude higher than that of the heterodimer. To

our knowledge this is the first report on the determination of the K_d of transcription factors from FRET titrations in live cells. Imaging FCCS measurements revealed co-diffusion of stable c-Fos homodimers and their binding to chromatin. Our molecular dynamics simulations support that Fos homodimers can form, bind to DNA and remain stable over the time span of the simulation (500 ns). This novel homodimeric form of c-Fos may act as an autonomous transcriptional regulator.

MATERIALS AND METHODS

Cell culture, plasmid construction and transfection of Hela cells

Cell culture, plasmid construction and transfection have been described elsewhere (34). Detailed information on these procedures and plasmids is in the Supplement.

Confocal microscopic and flow cytometric FRET analyses

Confocal microscopic images were collected by using a Zeiss LSM 510 microscope. Flow cytometric measurements were carried out on a Becton Dickinson FACSAria III instrument. Details of data acquisition, and FRET analysis on a pixel-by-pixel or cell-by-cell basis have been described earlier (34) and are detailed in the Supplement.

Fluorescence correlation spectroscopy and calibration of fluorescence intensity as a measure of absolute concentration

In FCS (35), molecules diffuse across a subfemtoliter (<1 μm^3) detection volume defined by a focused laser beam. This causes fluorescence fluctuations, which are analyzed to derive dynamic parameters of the studied molecules. FCS measurements were performed on a modified Olympus FluoView 1000 confocal microscope based on an inverted IX-81 stand with an

UPlanAPO 60× NA 1.2 water immersion objective. The FCS extension (Steinbeis Transfer Unit for Biophysical Analytics, Heidelberg, Germany) equipped with two avalanche photodiodes (Perkin-Elmer, Wellesley, MA) is attached to the confocal scanning unit. Fluorescence of EGFP was excited by the 488 nm line of an Ar ion laser, and detected between 500–550 nm. To allow FCS measurements at high EGFP concentrations (up to 25 μM), laser illumination was dimmed by a neutral density filter (OD 1) to yield P=0.2 μW at the sample. FCS measurements on live HeLa cells were performed in eight-well chambered coverglass plates (NUNC). Points for FCS measurements were selected from confocal images. From each sample n~30 cells were measured at room temperature, and 6×8 s runs per cell were recorded. Fluorescence autocorrelation functions were calculated online by an ALV-5000E hardware correlator card (ALV Laser, Langen, Germany). Autocorrelation curves were fitted to a two-component 3D diffusion model with triplet correction by using the program QuickFit3.0 (36):

$$G(\tau)=\frac{1-T+Te^{-\tau/\tau_{tr}}}{1-T}\frac{1}{N}\left[r_1\left(1+\frac{\tau}{\tau_1}\right)^{-1}\left(1+\frac{\tau}{S^2\tau_1}\right)^{-1/2}+r_2\left(1+\frac{\tau}{\tau_2}\right)^{-1}\left(1+\frac{\tau}{S^2\tau_2}\right)^{-1/2}\right] \qquad (1)$$

where τ is the lag time, T denotes the triplet fraction, τ_{tr} is the triplet correlation time, τ_1 and τ_2 are the diffusion times of the fast and slow species (average dwell times of molecules in the detection volume), r_1 and $r_2 = 1-r_1$ are the fractional amplitudes of the two components, N is the average number of molecules in the detection volume and S is the aspect ratio of the ellipsoidal detection volume. To assess the aggregation state of EGFP-labeled proteins, the molecular brightness or fluorescence per particle, F/N, was calculated and compared with monomeric EGFP.

To facilitate K_d determinations, we developed a method to assess absolute concentrations from fluorescence intensity, similar to that described in (37). In the first step we determined the detection volume, V_{eff} of the microscope by using a 130 nM Alexa 488 solution as a standard.

From its autocorrelation function τ_d and S were determined by fitting, and the lateral radius ω_{xy} and the axial radius ω_z were calculated with the following equations:

$$\omega_{xy} = \sqrt{4D\tau_d}, \omega_z = \omega_{xy} S, \quad (2)$$

where τ_d is the measured diffusion time of the dye, which measures mobility and is inversely proportional to the diffusion coefficient and D=435 $\mu m^2/s$ is its diffusion coefficient of Alexa 488 at 22.5 °C (38). The effective detection volume is:

$$V_{eff} = \pi^{3/2} \omega_{xy}^2 \omega_z. \quad (3)$$

From autocorrelation curves with EGFP the particle numbers N were determined. From these, molar concentrations c were calculated as

$$c = N / \left(N_A V_{eff} \right), \quad (4)$$

where N_A is Avogadro's number. Before every FCS measurement the fluorescence intensity F was measured with the imaging detector (photomultiplier tube) of the confocal microscope at the site of FCS measurement; thus, *c vs. F* calibration lines were generated (Fig. 4B).

To facilitate comparison of measurements on different days we used fluorescent beads. The fluorescence intensity of 6-μm green calibration beads (bead with 1% relative intensity from the InSpeck Green Microscope Image Intensity Calibration Kit, Molecular Probes, Life Technologies, Inc.) was used to normalize EGFP fluorescence. Confocal sections in the equatorial plane of the beads were recorded on the same day as the FCS calibration, using identical instrument settings. Average fluorescence intensity per pixel in the central area of the beads was obtained. Using the calibration curve in Fig. 4B, the local intensity of the bead at its center corresponded to an EGFP concentration of $c_{bead\ unit}^{confocal}$ ~15.4±0.7 μM (average±SEM of five experiments).

We could also transfer the concentration calibration to flow cytometric measurements. In the microscopic calibration described above, the local fluorescence intensities at a pixel of the sample and the bead are compared. In contrast, in flow cytometry the total intensity of the whole cell and the bead are measured. Therefore, the ratio of the cellular and bead volumes had to be taken into account. The volume of HeLa nuclei (where Fos and Jun are localized) is ~13.6-times larger than that of the beads as determined by confocal microscopic 3D sectioning and using the Imaris software (Bitplane AG, Zurich). In addition, the different detection efficiencies of the spectra of the bead and EGFP arising from different band pass filters in the flow cytometer and the confocal microscope differed by a factor of 13. Taking these factors into account, the total intensity of a bead corresponded to an EGFP concentration of $c_{bead\ unit}^{flow\ cyt}$ ~1.2 µM (distributed in a HeLa nucleus) in flow cytometric experiments. If the localization of the protein is not perfectly nuclear, we can correct for this as well. From confocal microscopic sectioning we determined the nuclear and cytoplasmic fractions of Fos^{215}-EGFP (the protein we used for FRET titrations), which were 85±5% and 15±5%, independent of expression level (see Suppl. Fig. 3). With this correction, a bead unit of ~1.0±0.1 µM was used for calculating the nuclear concentration of Fos^{215}-EGFP in flow cytometric experiments.

Determination of the absolute concentration of endogenously expressed Fos and Jun

With regular immunofluorescence assays only the relative amounts of endogenous and transfected proteins can be assessed. By knowing the absolute concentration of the transfected proteins, the endogenous concentration can also be determined. Therefore, we combined the immunofluorescence assay with the results of FCS-based EGFP concentration calibration to assess the endogenous concentrations of Fos and Jun in HeLa cells.

Immunofluorescence labeling was carried out as follows. After washing 3× with PBS, cells were fixed with 3.7% formaldehyde (4˚C, 10 minutes), permeabilized with 0.25% Triton and 0.1% TWEEN/TBS (room temperature, 30 min) and blocked with 2% BSA with 0.1% TWEEN/TBS (room temperature, 30 min). Cells were then incubated with mouse anti-c-Fos (Merck, Whitehouse Station, New Jersey, USA) or mouse anti-c-Jun monoclonal antibody (Millipore, Billerica, Massachusetts, USA) at 20 μg/ml concentration (room temperature, 1 hour), followed by incubation with NL-637-DAMIG polyclonal secondary antibody (R&D Systems, Minneapolis, Minnesota, USA) at 50 μg/ml for 1 hour at room temperature in the dark. Between consecutive steps cells were washed 3× with PBS. The applied antibody concentrations were chosen based on titrations (Suppl. Fig. 4A-C); the used concentrations are close to saturation values.

Flow cytometric measurements were performed on a FACSAria III flow cytometer. The green EGFP signal (I^{green}) was excited at 488 nm and emission was detected between 515-545 nm, while the red NL637 signal (I^{red}) was exited at 633 nm and emission was detected through a 655 LP filter.

The amount of Fos-EGFP was determined by comparing its I^{green} fluorescence signal to that of the green bead used for concentration calibration. The red signal I^{red} of the NL637-DAMIG antibody used for immunofluorescence labeling is proportional to the total amount of Fos: the endogenous Fos in the non-transfected sample, and the endogenous Fos + Fos-EGFP in the transfected one. The concentrations of endogenous Fos ($c_{Fos\text{-}endogen}$) and Fos-EGFP ($c_{Fos\text{-}EGFP}$) were calculated from the green and red signals using transfected and non-transfected cells as:

$$c_{Fos-EGFP} = \frac{I^{green}_{transfected}}{I^{green}_{bead}} \times c^{flow\,cyt}_{bead\,unit} \qquad (5)$$

$$\frac{c_{Fos-EGFP}+c_{Fos-endogenous}}{c_{Fos-endogenous}}=\frac{I^{red}_{transfected}}{I^{red}_{non-transfected}} \quad (6)$$

$$c_{Fos-endogenous}=\frac{c_{Fos-EGFP}}{\left(I^{red}_{transfected}/I^{red}_{non-transfected}\right)-1}=\frac{\left(I^{green}_{transfected}/I^{green}_{bead}\right)\times c_{bead\,unit}}{\left(I^{red}_{transfected}/I^{red}_{non-transfected}\right)-1} \quad (7)$$

with $c^{flow\,cyt}_{bead\,unit}$ ~1.2 μM in our measurements. I^{red} signals were collected from fixed cells (required by the immunofluorescence labeling protocol), whereas I^{green} signals were measured in non-fixed live cells (from the same transfected population) to avoid deterioration of EGFP fluorescence due to fixation. The concentration of endogenous and EGFP-tagged Jun was determined using the same principle.

Calculation of dissociation equilibria from FRET data

To assess the K_d of Fos homodimers and Fos-Jun heterodimers we carried out FRET titration experiments. In the derivation of dissociation equilibria we assumed that the heterodimer was more stable than the Fos homodimer. Therefore, in the case of Fos-Jun association we neglected the presence of Fos homodimers (assuming they were not present at lower concentrations). The law of mass action for heterodimer formation is:

$$[F][J]/[FJ]=K_d^{FJ} \quad (8)$$

where square brackets denote concentrations of free monomers and heterodimers, and K_d^{FJ} is the dissociation constant of the heterodimeric complex. The total concentration of Fos or Jun can be written as the sum of the concentrations of free monomers F and J and heterodimers FJ:

$$[F]_t=[F]+[FJ];\quad [J]_t=[J]+[FJ] \quad (9)$$

The concentration of the heterodimer is:

$$[FJ]=\frac{1}{2}\left([F]_t+[J]_t+K_d^{FJ}-\sqrt{[F]_t^2-2[F]_t\times\left([J]_t-K_d^{FJ}\right)+\left([J]_t+K_d^{FJ}\right)^2}\right) \quad (10)$$

The measured FRET efficiency between EGFP- and mRFP1-labeled proteins is an average value stemming from FRET-producing and non-FRET-producing donor molecules. Free donors or donors associated with endogenous unlabeled protein give zero FRET. Only donors forming a complex with an acceptor have a positive contribution to FRET (Fig. 5). Thus, we need to calculate the concentration of Fos-Jun dimers labeled with both donor and acceptor. The total concentrations of Fos and Jun are:

$$[F]_t = [F_D]_t + [F_e]_t \text{ and } [J]_t = [J_A]_t + [J_e]_t, \quad (11)$$

where the indexes *D*, *A* and *e* refer to donor-tagged, acceptor-tagged and endogenous molecules. The fractions of donor-tagged Fos (p_D) and acceptor-tagged Jun (p_A) are:

$$p_D = \frac{[F_D]_t}{[F_D]_t + [F_e]_t} \text{ and } p_A = \frac{[J_A]_t}{[J_A]_t + [J_e]_t} \quad (12)$$

The concentration of doubly labeled Fos_D-Jun_A dimers is:

$$[F_D J_A] = [FJ] \times p_D \times p_A = [FJ] \times \frac{[F_D]_t}{[F_D]_t + [F_e]_t} \times \frac{[J_A]_t}{[J_A]_t + [J_e]_t} \quad (13)$$

We denote the FRET efficiency in the complex of a single donor-tagged Fos and an acceptor-tagged Jun by E_0. The measured apparent FRET efficiency E_{meas} can be written as:

$$E_{meas} = \frac{[F_D J_A]}{[F_D]_t} \times E_0 + \frac{[F_D]_t - [F_D J_A]}{[F_D]_t} \times 0 = \frac{[F_D J_A]}{[F_D]_t} \times E_0 \quad (14)$$

where $[F_D J_A]$ is the concentration of complexes of donor-tagged Fos with acceptor-tagged Jun, and $[F_D]_t$ is the total concentration of donor-tagged Fos (without respect to being monomeric or

in a Fos-Jun complex). $[F_D]_t - [F_D J_A]$ is the concentration of donor-tagged Fos not complexed with acceptor-tagged Jun (Fos-EGFP in monomeric form or complexed with endogenous Jun), contributing zero FRET efficiency. By introducing the acceptor-to-donor expression ratio $N_A/N_D = [J_A]/[F_D]$ (Suppl. eq. S8) and combining equations 10, 13 and 14, the measured FRET efficiency can be expressed as:

$$E_{meas} = \frac{[F]_t + [J]_t + K_d^{FJ} - \sqrt{[F]_t^2 - 2[F]_t\left([J]_t - K_d^{FJ}\right) + \left([J]_t + K_d^{FJ}\right)^2}}{2[F]_t[J]_t} \times [F_D]_t \frac{N_A}{N_D} E_0, \qquad (15)$$

By substituting Eq. 11 and the expression for the N_A/N_D ratio into Eq. 15 we get the formula used in the nonlinear fit (see Eq. S12 in the Supplement) with variables $[F_D]_t$ and N_A/N_D.

For calculating Fos-Fos equilibria, we have to take into account Fos-Jun formation as well. Since the heterodimer is more stable, we make the simplifying assumption that all Jun molecules present are in complex with Fos at the high Fos concentrations where Fos homodimerization takes place, leaving no free Jun. We can write the following equilibrium equation:

$$\begin{aligned} &[F][F]/[FF] = K_d^{FF}, \\ &[J]_t = [FJ], \quad [F]_t = [F] + [FJ] + 2[FF] \end{aligned} \qquad (16)$$

where K_d^{FF} is the dissociation constant of the Fos homodimer, *FF* and *FJ* denote the homo- and the heterodimer. The amount of Fos homodimer can be expressed as:

$$[FF] = \frac{1}{8}\left(4[F]_t - 4[J]_t + K_d^{FF} - \sqrt{8K_d^{FF}[F]_t - 8K_d^{FF}[J]_t + \left(K_d^{FF}\right)^2}\right) \qquad (17)$$

Fos molecules can be labeled with donor, acceptor or can be unlabeled, and only homodimers containing both a donor and an acceptor will produce FRET (Fig. 5). The fraction of such double-labeled pairs follows a multinomial distribution, and equals

$$p_{D,A} = 2 p_D p_A \tag{18}$$

where $p_D = [F_D] / \left([F_D]_t + [F_A]_t + [F_e]_t \right)$ and $p_A = [F_A] / \left([F_D]_t + [F_A]_t + [F_e]_t \right)$ are the donor and acceptor-tagged fractions of Fos. The measured FRET efficiency is:

$$E_{meas} = \frac{[F_D F_A]}{[F_D]_t} \times E_0 = \frac{[FF]\, p_{D,A}}{[F_D]_t} \times E_0 \tag{19}$$

Combining equations 17 and 19 (see also Eq. S14 in the Supplement) yields

$$E_{meas} = \frac{1}{4}\left(4[F]_t - 4[J_e]_t + K_d^{FF} - \sqrt{K_d^{FF}} \sqrt{8[F]_t - 8[J_e]_t + K_d^{FF}} \right) \frac{[F_D]_t \frac{N_A}{N_D} E_0}{[F]_t^2} \tag{20}$$

with

$$[F]_t = [F_D]_t + [F_A]_t + [F_e]_t = [F_D]_t \left(1 + \frac{N_A}{N_D} \right) + [F_e]_t \tag{21}$$

where $[F]_t$ is the total Fos concentration (including donor-tagged, acceptor-tagged and endogenous Fos, without respect to monomeric or dimeric state) and E_0 is the FRET efficiency between a donor-acceptor pair (this may be different from the E_0 of the heterodimer). These equations were used to determine the K_d of dimers from flow cytometric FRET experiments by nonlinear regression.

In the analysis we also considered the presence of dark acceptor species due to imperfect maturation, and a cytoplasmic fraction of Fos (see Supplemental Material). These factors influence E_0, but not the value of K_d.

Single Plane Illumination Microscopy – fluorescence cross-correlation spectroscopy (SPIM-FCCS)

The SPIM-FCCS measurements were performed on an in-house built selective plane illumination microscope setup based on the design described in (39, 40). Data were analyzed using the software QuickFit 3.0. Details of the experimental setup and analysis are summarized in the Supplement.

Molecular dynamics simulation of Fos-Jun and Fos-Fos complexes

Two systems were submitted to molecular dynamics simulation. The first one is constructed from the Fos (139-198)_Jun (257-313) protein fragments associated to the DNA fragment as was deposited in the protein data bank (41). It was completed by adding the missing hydrogen atoms and closing the N- and C-terminal residues by the acetyl and N-methyl groups, respectively. The second system, a Fos (139-198)_Fos (139-198) was obtained from the first one using the Jun fragment as a template in a proper position for homology modeling of the Fos protein. Each of these systems was put in a dodecahedral box, solvated by the TIP3P explicit water model, neutralized by Na^+ ions, and further Na^+ and Cl^- ions were added to set the ionic strength to 0.15 M. They were then minimized, slowly heated to 310 K and after an 80 ns equilibration period they were submitted to a 500 ns constant particle number (123888 and 123870 for the Fos-Jun and Fos-Fos systems, respectively), constant pressure ($P=10^5$ Pa), constant temperature (T=310 K) production dynamics. For the simulations the AMBER99SB force field (42) and periodic boundary condition were used. Short range electrostatic and van der Waals interactions were calculated explicitly within a 1 nm cut-off. For the long range electrostatics the particle mesh Ewald method (43) was applied. A Berendsen barostat and thermostat (44) was used during this simulation. For the simulations the GROMACS packages were used (45, 46).

For control purpose molecular dynamics simulations on the Leu zipper region only of the c-Fos:c-Jun and c-Fos:c-Fos dimeric structures were also carried out using the same set-up protocol which is detailed above. The Leu zipper region we considered consisted of the 275-313 and 160-198 amino acid residues for the c-Jun and c-Fos fragments, respectively. Simulations were completed for both the wild type Leu zipper regions and the corresponding structures applying Leu280Asp, Leu294Asp virtual mutations in c-Jun- and Leu165Asp, Leu179Asp mutations in c-Fos protein fragments.

RESULTS

FRET microscopy implies Fos homodimerization

FRET is the radiationless transfer of energy from a donor fluorophore to a nearby acceptor (27, 28), which is often used to assess molecular distances. To measure the association of c-Fos molecules (referred to as Fos in the following sections), we used Fos and its C-terminal truncation mutant Fos^{215} tagged with ECFP (donor) or EYFP (acceptor) in confocal microscopic FRET experiments. Fos^{215} was prepared (32) to bring the FP-labeled C-termini of Fos and Jun to a similar distance from the leucine zipper to enhance FRET (Fig. 1). Images of donor, transfer and acceptor signals were recorded, and FRET efficiencies E between labeled proteins, as well as acceptor-to-donor molecular ratios N_A/N_D were calculated on a pixel-by-pixel or cell-by-cell basis.

Pixel-by-pixel FRET efficiency maps and histograms of representative cells are shown in Fig. 2. Cells cotransfected with full length Fos-ECFP+Fos-EYFP (top row) yielded a mean FRET efficiency of E=5.0±0.5% (n~30 cells, ±SEM); for Fos^{215}-ECFP+Fos^{215}-EYFP (2^{nd} row, n~30) it was 10.0±0.5%. The higher E between the truncated Fos^{215} molecules is probably due to the reduced distance between the shorter C-terminal regions (Fig. 1). We also measured the FRET

efficiency for the Fos-ECFP+Jun-EYFP and Fos^{215}-ECFP+Jun-EYFP samples (3rd and 4th rows, n~30), which was 7.9±0.4% and 15.0±1.1%. For these pairs we have shown heterodimer formation earlier by FCCS and FRET (7, 32, 34). Both Fos and Jun molecules showed strong nuclear enrichment. The negative control (ECFP coexpressed with EYFP as separate proteins) and the positive control (ECFP-EYFP fusion protein) had mean *E* values of 2.8±0.4% and 48.6±0.8% (5th and 6th rows, n~20). These proteins had a diffuse distribution in the whole cell. The FRET efficiency of the Fos-Fos (or Fos^{215}-Fos^{215}) samples was lower than that of the Fos-Jun or Fos^{215}-Jun heterodimers, but significantly higher than for the negative control, indicating that Fos formed homodimers in these cells.

Dimer formation depends on the concentrations of the interacting partners; therefore, we analyzed FRET in cells expressing various amounts of the proteins. We calculated average intensities in the whole nucleus for Fos or Jun, and in the whole cell for the positive and negative controls in single cells, and determined FRET on a cell-by-cell basis. This method allowed a rapid analysis of several hundred cells. Fig. 3A and B show the mean *E* value as a function of the acceptor-to-donor molecular ratio N_A/N_D for the Fos^{215}-ECFP+Fos^{215}-EYFP and Fos-ECFP+Fos-EYFP samples. Data were grouped into three subsets according to donor concentration based on the fluorescence intensity of the donor (low, medium, high). For both protein pairs, *E* increases from low to high donor concentrations, in accordance with the higher probability of complex formation. *E* also increases with increasing N_A/N_D because more acceptor-tagged Fos molecules are available for donor-tagged ones to form a complex. The plateau or in its absence the average of the highest *E* values are presented in Fig. 3C, which shows that at higher Fos concentrations the extent of homodimerization increased as expected. Similar to the pixel-by-pixel analysis, the mean FRET efficiencies of the Fos-Fos and Fos^{215}-Fos^{215} dimers are between those of the negative control and the respective Fos-Jun or Fos^{215}-Jun dimers.

The measured *E* values depend on the FRET efficiency in a single donor-acceptor complex determined by the dye-to-dye distance and orientation, and on the fraction of donors forming dimers with an acceptor. The length of the Fos^{215} molecule downstream of the dimerization domain is similar to that of Jun, thus, the dye-to-dye distances in the Fos^{215}-Fos^{215} and Fos^{215}-Jun complexes should be similar. However, in the case of Fos homodimers, only complexes of donor- and acceptor-tagged proteins yield FRET. The measured mean *E* value is a weighted average of non-FRET-ting and FRET-ting dimers. Thus, the mean FRET efficiency of the homodimer is expected to be lower than that of the heterodimer. This was taken into account in our subsequent analyses.

Calibration of fluorescence intensity to measure absolute EGFP concentration

The above FRET titrations curves demonstrated that FRET efficiency can be used to monitor the extent of homo- and heteroassociations quantitatively in our system. The stability of a complex is characterized by its dissociation constant, K_d. In the Materials and Methods section we outlined a method to determine the K_d of interacting proteins in live cells from FRET titration curves. This requires knowing the absolute concentrations of all interacting molecules: the transfected fluorescent, and the endogenous non-fluorescent ones. In subsequent measurements we used the EGFP-mRFP1 dye pair because of the higher photostability of EGFP compared to ECFP. First, we developed a calibration method to determine the concentration of fluorescent proteins. Confocal images of cells expressing free EGFP were taken, and autocorrelation curves (ACFs) were recorded at selected points of the images (Fig. 4A). From ACFs local dye concentrations were determined by nonlinear fitting yielding a calibration curve of EGFP concentration vs. fluorescence intensity per pixel, *c(F)* (Fig. 4B) (37). To make the concentration calibration portable and facilitate comparison of measurements on different days, we normalized

EGFP fluorescence by using a fluorescent bead as an intensity standard. The EGFP concentration corresponding to one bead unit was ~15.4±0.7 μM for our confocal microscope and 1.0±0.1 μM for the flow cytometer. For the latter calculation we took the nucleus-to-bead volume ratio (13:1) and the nuclear fraction of Fos^{215}-EGFP (85%) into account. This way, the molar concentration of EGFP-tagged protein could be assessed by simply comparing its intensity to that of the bead measured on the same day in the microscopic or flow cytometric setup without having to repeat the FCS calibration.

Determining the absolute concentrations of endogenous and transfected Fos and Jun

For calculating the K_d of dimers we also need to know the amount of endogenous Fos and Jun, since they can also form dimers with each other or with their fluorescent counterparts. First, we detected the green fluorescence signal of Fos-EGFP in transfected cells, and compared it to that of the calibration bead to determine the absolute concentration of transfected protein (Eq. 5 in the Materials, Suppl. Fig. S4 D-G). Then we used immunofluorescence labeling, paired with far red channel flow cytometry, to detect the total Fos pool in non-transfected and in Fos-EGFP transfected samples. The immunofluorescent signal of the non-transfected sample is proportional to the endogenous Fos concentration, while that of the transfected one corresponds to the sum of the endogenous and transfected amounts. Thus, using the known concentration of Fos-EGFP, we deduced the average concentration of endogenous Fos (113±11 nM) and Jun (94±10 nM) in HeLa cells (see Eq. 7 in the Materials). Our procedure combining immunofluorescence and EGFP-tagged protein expression can be generally used to assess the absolute concentration of any endogenously expressed non-fluorescent protein.

Determination of the dissociation constant of Fos-Jun heterodimers and Fos homodimers in live cells using flow cytometric FRET data

We wanted to determine the dissociation constants of homo- and heterodimers from FRET titrations. Therefore, we derived the expressions of FRET efficiency in terms of the concentrations of donor-tagged, acceptor-tagged and unlabeled, endogenous proteins of interest and the K_d's (eqs. 15, 20 and supplementary equations S12, S14). Flow cytometric FRET experiments were carried out on large cell populations expressing EGFP- and mRFP1-tagged proteins at various concentrations. Cell-by-cell values of FRET efficiency (E), donor concentration $\left[F_D\right]_t$ (derived from the FRET-corrected I_D donor intensity by comparison to beads), and acceptor-to-donor molecular ratio (N_A/N_D) data were determined. To characterize heterodimers, cells were cotransfected with Fos^{215}-EGFP+Jun-mRFP1 or Jun-EGFP+Fos^{215}-mRFP1 pairs. The extent of association depends on the concentrations of both molecules. To create functions with a single variable, cells were grouped into classes with approximately constant N_A/N_D ratios. E values of selected N_A/N_D groups were plotted as a function of the donor concentration (Fig. 6A,C). The FRET efficiency increases with increasing N_A/N_D ratio as expected. Figures 6B,D display the theoretical values of the maximal FRET efficiencies at various N_A/N_D ratios (Eq. S13 in the Supplement), which could be measured if all possible donor-acceptor complexes were formed (association were complete). The function increases linearly up to N_A/N_D=1 where it reaches E_0, the FRET efficiency between a single donor-acceptor pair, and remains constant at higher N_A/N_D ratios. E vs. N_A/N_D data were fitted to the model function describing the heterodimerization process (Eq. S12 in the supplement), taking the presence of FP-tagged and unlabeled endogenous Fos and Jun into account. The apparent K_d values derived from the fits varied with varying N_A/N_D for both pairs between 10 and 370 nM.

We received smaller apparent K_d-s where Fos was present in excess (larger Fos:Jun ratios) (Fig. 6E).

Fos homodimerization was studied by measuring the FRET efficiency between Fos^{215}-EGFP and Fos^{215}-mRFP1 (Fig. 6F). Here, homodimers can contain donor-acceptor, donor-donor, and acceptor-acceptor tagged protein pairs (plus dimers containing endogenous Fos); thus, the dependence of E on the N_A/N_D ratio is different than for heterodimerization (Fig. 6G). The theoretical E_{max} value approximates the value of E_0 asymptotically as N_A/N_D tends to infinity (Eq. S15 in the Supplement). Curves were fitted taking into account both the homoassociation of Fos (in all combinations of donor-tagged, acceptor-tagged and endogenous molecules) and its heteroassociation with endogenous Jun (Eq. S14 in the supplement). In the fits the K_d and E_0 values were linked for data sets with different N_A/N_D values, yielding K_d=6.7±1.7μM and E_0=9.5 ± 0.8% for the homodimerization process. When different N_A/N_D groups were fitted independently, K_d and E_0 ranged between 5.4-9.7 μM and 9.1-11.9%. As expected, the K_d of the Jun-Fos^{215} heterodimer is much smaller than that of the Fos homodimer, which means that Fos homodimerization will be significant only in case of its selective overexpression. At equal Fos and Jun concentrations the formation of Fos-Jun heterodimers is more probable than the formation of Fos homodimers.

Fluorescence brightness and slow diffusion indicate Fos homoassociation and DNA binding

FCS was not only used for concentration calibration, but also as an additional tool to probe Fos homoassociation. Diffusing particle concentration vs. fluorescence intensity curves were generated for EGFP, Fos$^{\Delta\Delta}$-EGFP (a mutant form lacking the DNA-binding and dimerization domains, see Fig. 1), full length Fos-EGFP and Fos-EGFP+Jun-mRFP1 samples (Fig. 4B). The slopes of the fitted straight lines for EGFP and Fos$^{\Delta\Delta}$-EGFP were 30.0 and 30.3

(nM/intensity unit). The similar slopes indicate that equal intensities of EGFP or Fos$^{\Delta\Delta}$-EGFP correspond to equal particle number, suggesting that this mutant contains one fluorophore per particle, i.e., it is monomeric. In contrast, the full length Fos-EGFP protein yielded a slope of 11.5, which is less than half of the previous values. Thus, an equal intensity of Fos-EGFP corresponds to a little less than 1/2× the particle concentration of the monomeric proteins, implying the formation of Fos homodimers. In fact, this curve is not expected to be linear in the low concentration regime because of the monomer-dimer transition. The slope of the Fos-EGFP+Jun-mRFP1 sample (with an average Jun:Fos ratio of 0.7) is between the monomeric and dimeric slopes. Therefore, Fos-EGFP is partially complexed with Jun-mRFP1, where the brightness of EGFP is similar to that of monomers, whereas the rest of Fos-EGFP may form homodimers. We also analyzed the specific particle brightness *F/N*, defined as the ratio of the fluorescence intensity *F* to the number of particles *N*. This parameter characterizes the association state of a labeled protein, and is proportional to the number of fluorophores in a jointly diffusing complex. *F/N* vs. EGFP concentration values are shown in Fig. 4C. Fos-EGFP is brighter than EGFP, Fos$^{\Delta\Delta}$-EGFP or Fos-EGFP+Jun-mRFP1, corroborating the conclusion that Fos-EGFP is homodimerized when there is not enough Jun present. The brightness of Fos-EGFP increases with concentration indicating that dimerization is enhanced at higher concentrations, whereas the brightness of EGFP or Fos$^{\Delta\Delta}$-EGFP does not vary with concentration, just as expected for monomers.

From the autocorrelation functions (ACFs) we also determined molecular diffusion properties (Fig. 4D). ACFs from the EGFP, Fos-EGFP, Fos$^{\Delta\Delta}$-EGFP and Fos-EGFP+Jun-mRFP1 samples were fitted to a model assuming a fast, freely diffusing and a slowly moving component (7). Diffusion coefficients and the fractions of the species are shown in (Fig. 4E). The average fraction of the slow components was 0.35±0.14 for Fos-EGFP expressed alone and 0.38±0.10 for

Fos-EGFP when co-expressed with Jun-mRFP1, whereas it was only 0.19±0.12 for the non-binding $Fos^{\Delta\Delta}$-EGFP mutant, and 0.07±0.05 for EGFP. The similarly increased slow fractions of Fos-EGFP expressed alone or together with Jun-mRFP1 suggest that Fos can bind to chromatin not only as a heterodimer, but also as a homodimer.

SPIM-FCCS confirms stable homoassociation and chromatin binding of Fos proteins

We used fluorescence cross-correlation spectroscopy (FCCS), the two-color version of FCS to characterize the co-mobility of dimer-forming Fos molecules. In FCCS, the auto- and the cross-correlation functions (CCF) from two molecular species tagged with different colors are determined. A non-zero CCF amplitude indicates that the molecules are moving together. The ratio of the CCF and ACF amplitudes from a double-labeled sample is proportional to the fraction of molecules forming a complex. FCCS measurements were performed on a single plane illumination microscope (SPIM) using an EM-CCD camera as sensor, which allows simultaneous measurements at many pixels in a cell. This improves the statistics and provides 2D interaction and mobility maps (40).

Measurements were carried out on cells co-transfected with the following protein combinations: Fos^{215}-EGFP+Fos^{215}-mRFP1, Fos^{215}-EGFP+Jun-mRFP1, $Fos^{\Delta\Delta}$-EGFP+Fos^{215}-mRFP1 (negative control) and EGFP-P30-mRFP1 (positive control, EGFP and mRFP1 connected by a 30-residue long polyproline linker). Cells expressing about equal amounts of green and red fluorophores were selected from the concentration range used in FRET experiments (0.3 – 10 μM). Fig. 7A shows typical correlation curves obtained from the four samples. Amplitudes of the curves were low due to the high concentration required to see Fos homodimerization, but our data showed that quantitative measurements were possible even in this concentration regime. At each pixel we performed a global FCCS fit to the green and red ACFs and the CCF (see (40) and Eqs.

S16-S18 in the Supplement). The fit functions were parameterized by the concentrations of three diffusing species (green-only, red-only, green/red-dimers), which were linked over all three curves. We assumed a two-component diffusion model for the ACFs and a one-component model for the CCF. Diffusion coefficients were not linked. Fig. 7B shows exemplary maps and histograms of the relative GR-dimer concentration $c_{GR}/\left(c_{G-only}+c_{R-only}+c_{GR}\right)$ obtained from these fits. Fig. 7C shows the statistics from n>10 cells as average ± standard deviation of the medians extracted from the pixel distributions in each cell. As expected, the negative control had the lowest and the positive control the highest apparent dimer fraction, defining the dynamic range of the measurements (0.06 – 0.32). The upper limit is less than one because of the imperfect overlap of the green and red detection volumes, partial photobleaching of the dyes and imperfect dye maturation/folding producing green-only and red-only species besides doubly labeled molecules. The Fos-Jun heterodimer showed a high apparent dimer fraction (0.22±0.07), whereas the value from the Fos^{215} homodimer was slightly smaller (0.16±0.05), but still significantly larger than that of the negative control.

We also analyzed the protein mobility of the Green/Red dimers that could be extracted from the fits of the CCFs. We used a single component fit; a second component could not be fitted to the CCFs of the Fos-EGFP + Jun-mRFP1, Fos^{215}-EGFP + Fos^{215}-mRFP1 samples suggesting the presence of only a single slow species. The CCFs of the negative control could not be fitted reliably due to their very low amplitude. The diffusion coefficient of the EGFP-P30-mRFP1 fusion protein was D_{cross}~4.3 μm²/s (a mean of two components present for this protein), whereas the average diffusion coefficients of the Fos^{215}-Fos^{215} and Fos-Jun dimers were much lower, ~0.3 and 0.4 μm²/s (Fig. 7D). The presence of a single, slow component for Fos

homodimers and Fos-Jun heterodimers indicated that these complexes could bind to slowly moving nuclear components, supposedly the chromatin.

Fos-Jun and Fos-Fos complexes form stable dimers as revealed by MD modeling

Based on the distance-related data from FRET measurements, we performed MD modeling to testify the stable formation of Fos homodimers. In Fig. 8 the first frames from the production dynamics trajectories are presented. During the 500 ns simulation interval (Supplemental movies 5 and 6) not only the Fos-Jun heterodimer but also the Fos-Fos homodimer remain bound to DNA, and the dimeric structures (coiled coil motifs) stay strongly associated by their leucine zipper regions. It is noteworthy that the Fos-Fos homodimer exists despite the net negative charge of the leucine zipper regions. This indicates that in the dimeric form hydrophobic interactions play a crucial role, and electrostatic forces are largely shielded by counterions. While H-bonds and even salt bridges can contribute to the stabilization of the dimeric structure as well, the H-bond networks between protein chains have variability in their connection patterns, which further supports the importance of hydrophobic interactions in the dimeric structures.

Simulations carried out solely on the leucine zipper region of c-Jun:c-Fos and c-Fos:c-Fos dimers indicated stable structures with coiled coil motif (Figure 8C,D). These results are both in good accordance with former simulations carried out on the c-Jun:c-Fos leucine zipper region (47)and underline again the role of hydrophobic forces even in the stability of the c-Fos:c-Fos homodimer. Whereas, introducing Leu-Asp virtual mutations into these dimeric structures (as a negative control) the contact between the corresponding regions of helices was either weakened (c-Jun:c-Fos) or even destroyed (c-Fos:c-Fos) as demonstrated in Figure 8E,F.

DISCUSSION

Homodimer formation of short fragments, mainly the leucine zippers, of Fos proteins has been studied earlier. However, in vitro studies reported low stabilities of the homodimer, and it was assumed that it could not be present in live cells. By combining FRET, FCS and imaging FCCS we demonstrated that Fos proteins formed homodimers in live cells, and presented a method for calculating their dissociation constant. The K_d of Fos homodimers in HeLa cells was 6.7±1.7 μM, which is the same order of magnitude as the value 5.6 μM determined for its isolated leucine zippers in vitro by circular dichroism (14). Values reported for the heterodimers of the isolated leucine zippers (10, 48, 49) or longer polypeptides (50) in vitro varied between 1 and 140 nM. For the Fos-Jun heterodimer we found a K_d range of 10-370 nM in live cells, which depended on the Fos:Jun ratio, and on putting the donor and acceptor tags on one or the other protein. The variation of the K_d with different Fos:Jun ratios may be caused by the formation of Jun homodimers, which could interfere with the heterodimerization process. At lower Fos:Jun ratios, when there is excess Jun present, the relative amount of Jun homodimers is expected to be higher; thus, the amount of available free Jun is less and the heterodimerization process could shift toward higher concentrations (Fig. 6E), resulting in a higher apparent K_d. At higher Fos:Jun ratios, where the Jun homodimer is expected to be less abundant, we got K_d <100 nM for the heterodimer, in agreement with earlier in vitro results. The shift between the Fos-Jun and Jun-Fos curves in Fig. 6E might be due to dark states (51) and incomplete maturation of mRFP1, resulting an error in the acceptor-to-donor ratios. Furthermore, the autofluorescence intensity of HeLa cells in the green channel corresponds to the specific intensity of ~50 nM EGFP; therefore, the signal-to-noise ratio in the concentration range of the K_d is lower than in the case of the homodimer, making the K_d for the heterodimers less accurate.

Several groups used FRET to determine the K_d of isolated proteins (50, 52). Other groups used microscopic FRET to determine K_d in cells, utilizing prior estimates of protein copy number per cell (53), or applying in vitro concentration calibration with purified proteins (54). Here we presented a method to calculate K_d values based on FRET titrations after concentration calibration by FCS, where the whole procedure was carried out in live cells. None of the earlier studies took into account the presence of endogenous, unlabeled proteins. With our method, the absolute concentrations of both overexpressed fluorescent and endogenous non-fluorescent proteins were determined and included in dissociation equilibria. Our concentration calibration method is transferable to measurements performed on different instruments or days by utilizing fluorescent beads as a standard. The procedure can be generally used to determine K_d-s and absolute concentrations of proteins in live cells.

FRET reveals that a certain fraction of molecules are colocalized within Förster distance. We used FCS to assess the co-diffusion of molecules, which is a direct indication of stable interaction. Our molecular brightness analysis of FCS data indicated that Fos-EGFP, when expressed alone, had a higher ($\geq$2×) molecular brightness than its dimerization- and DNA-binding-deficient $Fos^{\Delta\Delta}$ mutant or the free EGFP dye. This corroborated that at a few micromolar concentration Fos was present mainly as a homodimer, which was stable at least for a few tens of milliseconds (the mean dwell time of particles in the focal volume setting the upper limit of observed timescales in our FCS experiments). When fitted with a slow and a fast diffusion component, the slow fraction of Fos was about the same whether expressed alone or together with Jun; in contrast, the slow fraction of the $Fos^{\Delta\Delta}$ mutant was significantly lower, hinting at DNA binding of the wild type Fos either as a homo- or as a heterodimer. The presence of a very small apparent slow fraction in the case of lone EGFP and $Fos^{\Delta\Delta}$ is probably due to molecular crowding in the nucleus leading to anomalous subdiffusion (37). This makes the autocorrelation curves less

steep than for free diffusion, mimicking the presence of a second, slowly moving component with a longer diffusion time.

SPIM-FCCS allowed us to confirm the presence, visualize the distribution and characterize the intranuclear mobility of Fos homodimers. These were stable for at least a few hundred milliseconds, the time window defined by the cross-correlation diffusion time. Their diffusion coefficient derived from the cross-correlation curve was ~0.3 $\mu m^2/s$, similar to that of Fos-Jun heterodimers (0.4 $\mu m^2/s$). The measured diffusion coefficients are similar to those determined by confocal FCCS for the same proteins (7), and to values observed for other chromatin-binding proteins, e.g. nuclear receptors (55, 56) or HP1α (57).

Our molecular dynamic modeling simulations also supported the possibility of homodimerization showing that homodimers stayed together for the duration of the simulation.

The existence of stable Fos homodimers capable of chromatin binding brings up the possibility that they may act as transcriptional regulators, and may explain the importance of Fos overexpression in oncogenesis. Various complexes of different Fos and Jun variants occur in different cell types contributing to cell proliferation or apoptosis (58-60). It is not clear yet whether the Fos homodimer could function as an autonomous transcription factor, or alternatively, it could occupy the binding sites of AP-1 heterodimers preventing their normal function and interfering with their proliferative or antiproliferative effects.

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REFERENCES

1. **Angel P, Karin M.** 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochimica et Biophysica Acta - Reviews on Cancer **1072:**129-157.
2. **Zenz R, Eferl R, Scheinecker C, Redlich K, Smolen J, Schonthaler HB, Kenner L, Tschachler E, Wagner EF.** 2008. Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. Arthritis Research and Therapy **10**.
3. **Shaulian E, Karin M.** 2001. AP-1 in cell proliferation and survival. Oncogene **20:**2390-2400.
4. **Curran T, Franza Jr BR.** 1988. Fos and jun: the AP-1 connection. Cell **55:**395-397.
5. **Hurst HC.** 1994. Transcription factors. 1: bZIP proteins. Protein profile **1:**123-168.
6. **Porte D, Oertel-Buchheit P, John M, Granger-Schnarr M, Schnarr M.** 1997. DNA binding and transactivation properties of Fos variants with homodimerization capacity. Nucleic Acids Research **25:**3026-3033.
7. **Baudendistel M, Müller G, Waldeck W, Angel P, Langowski J.** 2005. Two-hybrid fluorescence cross-correlation spectroscopy detects protein-protein interactions in vivo. ChemPhysChem **6:**984-990.
8. **Nakabeppu Y, Ryder K, Nathans D.** 1988. DNA binding activities of three murine Jun proteins: Stimulation by Fos. Cell **55:**907-915.
9. **Smeal T, Angel P, Meek J, Karin M.** 1989. Different requirements for formation of Jun : Jun and Jun : Fos complexes. Genes and Development **3:**2091-2100.
10. **John M, Leppik R, Busch SJ, Granger-Schnarr M, Schnarr M.** 1996. DNA binding of Jun and Fos bZip domains: Homodimers and heterodimers induce a DNA conformational change in solution. Nucleic Acids Research **24:**4487-4494.
11. **Chinenov Y, Kerppola TK.** 2001. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. Oncogene **20:**2438-2452.
12. **Curran T.** 1992. Fos and Jun: oncogenic transcription factors. Tohoku Journal of Experimental Medicine **168:**169-174.

13. **Porte D, Oertel-Buchheit P, Granger-Schnarr M, Schnarr M.** 1995. Fos leucine zipper variants with increased association capacity. Journal of Biological Chemistry **270:**22721-22730.
14. **O'Shea EK, Rutkowski R, Stafford Iii WF, Kim PS.** 1989. Preferential heterodimer formation by isolated leucine zippers from Fos and Jun. Science **245:**646-648.
15. **Nicklin MJH, Casari G.** 1991. A single site mutation in a truncated Fos protein allows it to interact with the TRE in vitro. Oncogene **6:**173-179.
16. **Mason JM, Schmitz MA, Müller KM, Arndt KM.** 2006. Semirational design of Jun-Fos coiled coils with increased affinity: Universal implications for leucine zipper prediction and design. Proceedings of the National Academy of Sciences of the United States of America **103:**8989-8994.
17. **Greenberg ME, Ziff EB.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature **311:**433-438.
18. **Piechaczyk M, Blanchard JM.** 1994. c-fos proto-oncogene regulation and function. Critical Reviews in Oncology/Hematology **17:**93-131.
19. **Sheng M, Thompson MA, Greenberg ME.** 1991. CREB: A Ca2+-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science **252:**1427-1430.
20. **Johnston SRD, Lu B, Scott GK, Kushner PJ, Smith IE, Dowsett M, Benz CC.** 1999. Increased activator protein-1 DNA binding and c-Jun NH2-terminal kinase activity in human breast tumors with acquired tamoxifen resistance. Clinical Cancer Research **5:**251-256.
21. **Mishra A, Bharti AC, Saluja D, Das BC.** 2010. Transactivation and expression patterns of Jun and Fos/AP-1 super-family proteins in human oral cancer. International Journal of Cancer **126:**819-829.
22. **Güller M, Toualbi-Abed K, Legrand A, Michel L, Mauviel A, Bernuau D, Daniel F.** 2008. C-Fos overexpression increases the proliferation of human hepatocytes by stabilizing nuclear Cyclin D1. World Journal of Gastroenterology **14:**6339-6346.
23. **Saez E, Rutberg SE, Mueller E, Oppenheim H, Smoluk J, Yuspa SH, Spiegelman BM.** 1995. c-fos is required for malignant progression of skin tumors. Cell **82:**721-732.
24. **Kress E, Skah S, Sirakov M, Nadjar J, Gadot N, Scoazec J, Samarut J, Plateroti M.** 2010. Cooperation Between the Thyroid Hormone Receptor TRα1 and the WNT Pathway in the Induction of Intestinal Tumorigenesis. Gastroenterology **138:**1863-1874.e1861.
25. **Mikula M, Gotzmann J, Fischer AN, Wolschek MF, Thallinger C, Schulte-Hermann R, Beug H, Mikulits W.** 2003. The proto-oncoprotein c-Fos negatively regulates hepatocellular tumorigenesis. Oncogene **22:**6725-6738.
26. **Preston GA, Lyon TT, Yin Y, Lang JE, Solomon G, Annab L, Srinivasan DG, Alcorta DA, Barrett JC.** 1996. Induction of apoptosis by c-Fos protein. Molecular and Cellular Biology **16:**211-218.
27. **Clegg RM.** 1995. Fluorescence resonance energy transfer. Current Opinion in Biotechnology **6:**103-110.
28. **Jares-Erijman EA, Jovin TM.** 2003. FRET imaging. Nature Biotechnology **21:**1387-1395.
29. **Bacia K, Kim SA, Schwille P.** 2006. Fluorescence cross-correlation spectroscopy in living cells. Nature Methods **3:**83-89.
30. **Liu P, Sudhaharan T, Koh RML, Hwang LC, Ahmed S, Maruyama IN, Wohland T.** 2007. Investigation of the dimerization of proteins from the epidermal growth factor

receptor family by single wavelength fluorescence cross-correlation spectroscopy. Biophysical Journal **93:**684-698.

31. **Tian Y, Martinez MM, Pappas D.** 2011. Fluorescence correlation spectroscopy: A review of biochemical and microfluidic applications. Applied Spectroscopy **65:**115A-124A.
32. **Vámosi G, Baudendistel N, Von Der Lieth CW, Szalóki N, Mocsár G, Müller G, Brázda P, Waldeck W, Damjanovich S, Langowski J, Tóth K.** 2008. Conformation of the c-Fos/c-Jun complex in vivo: A combined FRET, FCCS, and MD-modeling study. Biophysical Journal **94:**2859-2868.
33. **Pernus A, Langowski J.** 2015. Imaging Fos-Jun transcription factor mobility and interaction in live cells by single plane illumination-fluorescence cross correlation spectroscopy. PlosOne.
34. **Szalóki N, Doan-Xuan QM, Szöllosi J, Tóth K, Vámosi G, Bacsó Z.** 2013. High throughput FRET analysis of protein-protein interactions by slide-based imaging laser scanning cytometry. Cytometry Part A **83:**818-829.
35. **Haustein E, Schwille P.** 2007. Fluorescence correlation spectroscopy: Novel variations of an established technique, vol 36, p 151-169.
36. **Krieger JW, Langowski J.** 2015. QuickFit 3.0 (compiled:2015/01/08, SVN: 3697): A data evaluation application for biophysics. http://www.dkfz.de/Macromol/quickfit/. Accessed
37. **Weidemann T, Wachsmuth M, Knoch TA, Müller G, Waldeck W, Langowski J.** 2003. Counting nucleosomes in living cells with a combination of fluorescence correlation spectroscopy and confocal imaging. Journal of Molecular Biology **334:**229-240.
38. **Petrášek Z, Schwille P.** 2008. Precise measurement of diffusion coefficients using scanning fluorescence correlation spectroscopy. Biophysical Journal **94:**1437-1448.
39. **Greger K, Swoger J, Stelzer EHK.** 2007. Basic building units and properties of a fluorescence single plane illumination microscope. Review of Scientific Instruments **78**.
40. **Krieger JW, Singh AP, Garbe CS, Wohland T, Langowski J.** 2014. Dual-color fluorescence cross-correlation spectroscopy on a single plane illumination microscope (SPIM-FCCS). Optics Express **22:**2358-2375.
41. **Glover JNM, Harrison SC.** 1995. Crystal structure of the heterodimeric bZIP transcription factor c-Fos-c Jun bound to DNA. Nature **373:**257-261.
42. **Hornak V, Abel R, Okur A, Strockbine B, Roitberg A, Simmerling C.** 2006. Comparison of multiple amber force fields and development of improved protein backbone parameters. Proteins: Structure, Function and Genetics **65:**712-725.
43. **Darden T, York D, Pedersen L.** 1993. Particle mesh Ewald: An N·log(N) method for Ewald sums in large systems. The Journal of Chemical Physics **98:**10089-10092.
44. **Berendsen HJC, Postma JPM, Van Gunsteren WF, Dinola A, Haak JR.** 1984. Molecular dynamics with coupling to an external bath. The Journal of Chemical Physics **81:**3684-3690.
45. **Hess B, Kutzner C, Van Der Spoel D, Lindahl E.** 2008. GRGMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation. Journal of Chemical Theory and Computation **4:**435-447.
46. **Pronk S, Páll S, Schulz R, Larsson P, Bjelkmar P, Apostolov R, Shirts MR, Smith JC, Kasson PM, Van Der Spoel D, Hess B, Lindahl E.** 2013. GROMACS 4.5: A high-

throughput and highly parallel open source molecular simulation toolkit. Bioinformatics **29:**845-854.

47. **Zuo ZL, Gandhi NS, Mancera RL.** 2010. Calculations of the Free Energy of Interaction of the c-Fos-c-Jun Coiled Coil: Effects of the Solvation Model and the Inclusion of Polarization Effects. Journal of Chemical Information and Modeling **50:**2201-2212.
48. **Heuer KH, Mackay JP, Podzebenko P, Bains NPS, Weiss AS, King GF, Easterbrook-Smith SB.** 1996. Development of a sensitive peptide-based immunoassay: Application to detection of the Jun and Fos oncoproteins. Biochemistry **35:**9069-9075.
49. **Pernelle C.** 1993. An efficient screening assay for the rapid and precise determination of affinities between leucine zipper domains. Biochemistry® **32:**11682-11687.
50. **Patel LR, Curran T, Kerppola TK.** 1994. Energy transfer analysis of fos-jun dimerization and DNA binding. Proceedings of the National Academy of Sciences of the United States of America **91:**7360-7364.
51. **Hendrix J, Flors C, Dedecker P, Hofkens J, Engelborghs Y.** 2008. Dark states in monomeric red fluorescent proteins studied by fluorescence correlation and single molecule spectroscopy. Biophysical Journal **94:**4103-4113.
52. **Song Y, Madahar V, Liao J.** 2011. Development of FRET assay into quantitative and high-throughput screening technology platforms for protein-protein interactions. Annals of Biomedical Engineering **39:**1224-1234.
53. **Mehta K, Hoppe AD, Kainkaryam R, Woolf PJ, Linderman JJ.** 2009. A computational approach to inferring cellular protein-binding affinities from quantitative fluorescence resonance energy transfer imaging. Proteomics **9:**5371-5383.
54. **Erickson MG, Alseikhan BA, Peterson BZ, Yue DT.** 2001. Preassociation of calmodulin with voltage-gated Ca 2+ channels revealed by FRET in single living cells. Neuron **31:**973-985.
55. **Brazda P, Krieger J, Daniel B, Jonas D, Szekeres T, Langowski J, Tóth K, Nagy L, Vámosi G.** 2014. Ligand binding shifts highly mobile retinoid X receptor to the chromatin-bound state in a coactivator-dependent manner, as revealed by single-cell imaging. Molecular and Cellular Biology **34:**1234-1245.
56. **Brazda P, Szekeres T, Bravics B, Tóth K, Vámosi G, Nagy L.** 2011. Live-cell fluorescence correlation spectroscopy dissects the role of coregulator exchange and chromatin binding in retinoic acid receptor mobility. Journal of Cell Science **124:**3631-3642.
57. **Capoulade J, Wachsmuth M, Hufnagel L, Knop M.** 2011. Quantitative fluorescence imaging of protein diffusion and interaction in living cells. Nature Biotechnology **29:**835-839.
58. **Ameyar M, Wisniewska M, Weitzman JB.** 2003. A role for AP-1 in apoptosis: The case for and against. Biochimie **85:**747-752.
59. **Eferl R, Wagner EF.** 2003. AP-1: A double-edged sword in tumorigenesis. Nature Reviews Cancer **3:**859-868.
60. **Shaulian E, Karin M.** 2002. AP-1 as a regulator of cell life and death. Nature Cell Biology **4:**E131-E136.

FIGURE LEGENDS

Figure 1

Schematic drawing of c-Fos, its mutants and c-Jun

From top: full length Fos with fluorescent protein tag at the C terminus; C-terminally truncated Fos^{215}, $Fos^{\Delta\Delta}$ where the DNA binding and dimerization domains were deleted; and Jun. Pink color denotes the DNA-binding domain, yellow the leucine-zipper, and the dotted line the linker between Fos/Jun and the fluorescent protein tag (ECFP, EYFP, EGFP or mRFP1).

Figure 2

Subcellular, pixel-by-pixel analysis of dimerization by confocal microscopic FRET on HeLa cells

ECFP (donor channel) was excited at 458 nm and detected between 475-525 nm; in the transfer channel the excitation was at 458 nm and detection between 530-600 nm; EYFP (acceptor channel) was excited at 514 nm and detected between 530-600 nm. Full length Fos-ECFP+Fos-EYFP (top row), Fos^{215}-ECFP+Fos^{215}-EYFP (2nd row), Fos-ECFP+Jun-EYFP (3rd row) and Fos^{215}-ECFP+Jun-EYFP (4th row) showed nuclear localization; the negative control, ECFP and EYFP expressed independently, and the positive control, the ECFP-EYFP fusion protein (5th and 6th rows) were evenly distributed in the whole cell. FRET efficiency *E* was calculated in each pixel. Histograms show the statistics of the subcellular distribution of *E*.

Figure 3

Cell-by-cell analysis of dimerization by confocal microscopic FRET

A and B) FRET efficiencies between donor- (ECFP) and acceptor- (EYFP) tagged Fos^{215} or full length Fos molecules as a function of the acceptor-to-donor molecular ratio (N_A/N_D). Data of 300

cells were grouped into three subsets as a function of donor intensity (low: <800, medium: 800-1200 and high: >1200, a.u.). Cellular data were binned in 0.25 wide intervals of the N_A/N_D values to reduce data scatter. FRET efficiencies increased with increasing N_A/N_D. C) Saturation values of FRET efficiencies at high acceptor-to-donor ratios (N_A/N_D>0.95). ECFP-EYFP fusion protein served as a positive, and independently expressed ECFP and EYFP as a negative control. FRET data of the Fos-Jun and Fos^{215}-Jun pairs were published in (34).

Figure 4

FCS-based concentration calibration and brightness analysis

A) EGFP concentration in HeLa cells was determined from the amplitude of the autocorrelation function. The curve was fitted to a two-component free diffusion model with triplet correction. B) Diffusing particle concentration ($1/G_0$) as a function of the fluorescence intensity per pixel of EGFP, Fos$^{\Delta\Delta}$-EGFP, Fos-EGFP and Fos-EGFP coexpressed with Jun-mRFP1. Data were fitted with straight lines using Deming regression. C) Fluorescence per particle or molecular brightness values characterizing the aggregation state, plotted as a function of the concentration of the EGFP tag. Symbols are the same as in panel B. D) Normalized autocorrelation functions fitted to a two-component free diffusion model. E) Diffusion constants and fractions of the second, slow component derived from the fits (n: number of cells).

Figure 5

Possible combinations of fluorescently tagged and endogenous Fos and Jun

A) In the monomer-heterodimer equilibrium fluorescently tagged and endogenous, unlabeled Fos and Jun molecules participate. The three species containing a donor tag contribute to the measured value of the FRET efficiency E_{meas}: the doubly labeled heterodimer having a FRET

efficiency E_0, and the donor-labeled Fos in complex with endogenous Jun or present as a monomer; the latter two species are characterized by zero FRET efficiency. The fraction of the different heterodimers follows a multinomial distribution. E_{meas} is a weighted average of the species-specific E values (given by Eq. S12 in the Supplement). B) In the monomer-homodimer equilibrium donor-tagged, acceptor-tagged and endogenous Fos and endogenous Jun participate. Four heterodimeric species and the donor-tagged monomer contribute to E_{meas} (derived in Eq. S14 in the Supplement).

Figure 6

Determination of the dissociation coefficients of Fos-Jun heterodimers and Fos-Fos homodimers from flow cytometric FRET titrations

A) and C) FRET efficiency measured in cells co-transfected with Fos^{215}-EGFP+Jun-mRFP1 and Jun-EGFP+Fos^{215}-mRFP1, plotted as a function of donor-tagged Fos^{215} or Jun concentration. Data were grouped according to acceptor-to-donor molecular ratios (N_A/N_D) and fitted as described (Suppl. Eq. S12, solid lines), yielding the K_d value of the heterodimers and the E_0 FRET efficiency of individual donor-acceptor pairs. Endogenous Fos and Jun were also taken into account. B) and D) The solid lines represent the maximal theoretically attainable E values at different N_A/N_D ratios (assuming E_0=15% and 14.1% based on the fits) when all available Jun-mRFP1 molecules are engaged in heterodimers with Fos; the marked points correspond to the experimental N_A/N_D values (Suppl. Eqs. S13, S15). E) Dependence of the K_d values from the fits on the Fos:Jun ratio. F) FRET efficiency of Fos^{215}-EGFP+Fos^{215}-mRFP1 homodimers as a function of donor-tagged Fos^{215} concentration with K_d and E_0 yielded from a linked fit (see suppl. Eq. S14). G) Maximal attainable FRET efficiencies at different N_A/N_D ratios (assuming E_0=9.47% based on the fit), when all Fos molecules form homodimers.

Figure 7

SPIM-FCCS data analysis show co-diffusion and DNA-binding of Fos homodimers

A) Autocorrelation (ACF) and cross-correlation (CCF) functions from SPIM-FCCS measurements. Green (EGFP ACF), red (mRFP1 ACF) and blue (CCF) solid lines indicate the experimental data, whereas dashed lines are fits assuming two diffusing components (ACFs) or one component (CCFs). The red horizontal line is the cross-talk-corrected red ACF amplitude and the blue line is the level of cross-correlation due to cross-talk. Cross-correlation above this value is due to co-diffusion of green and red molecules. B) The first two columns are fluorescence intensity maps of EGFP (green) or mRFP1 (red) from a selected cell. The third column is a map of the fraction of green-red dimers among all detected molecules, $c_{GR}/\left(c_{G-only}+c_{R-only}+c_{GR}\right)$ determined from the fits, and the histograms show their distributions. C) Average fraction of green-red dimers and D) diffusion coefficients D_{cross} from the cross-correlation fits (mean±s.d., n>20 for each sample). Fits were carried out on a pixel-by-pixel basis, and the median of the respective parameter from each cell was then averaged. ***p<0.0001 for the t-test.

Figure 8

Both Fos-Jun and Fos-Fos complexes form stable dimers and bind to DNA

Molecular dynamics simulations were carried out on small Fos-Jun (**A**) and Fos-Fos (**B**) fragments bound to the DNA fragment. Ribbon representation (colored cyan) was applied for the helical secondary structure of the Fos protein fragment (**A,B**). The atomic details of constituent residues are shown by stick representation coloring the C, H, N, O and S atoms gray, white, blue, red and yellow. For the Jun fragment (**A**) or the second Fos fragment (**B**) solvent excluded surface representation was applied using the above-mentioned color codes. (**C-F**): Visual

representation of trajectories from MD simulations of the Leu zipper region of the Jun-Fos (**C,E**) and Fos-Fos (**D,F**) dimeric structures. Wild type protein fragments (**C,D**) and virtually mutated (Leu280Asp, Leu294Asp in c-Jun- and Leu165Asp, Leu179Asp in c-Fos) fragments (**E,F**) were considered. Mutant residues are shown by stick representation using the same color scheme for the atoms as above. Jun is represented by the orange helix, whereas Fos by green and yellow ones. From each 500 ns dynamics trajectory 100 frames were saved equidistantly and superimposed (after removing rotation and translation). Wild type protein fragments (**C,D**) demonstrate stable coiled coil motifs with relatively low fluctuations. The mutations in the Fos-Jun fragment (**E**) resulted in a somewhat distorted structure and larger fluctuations indicating weakening of the interaction between the monomers. This is even more expressed for the mutant dimeric Fos-Fos fragment (**F**) where hydrophobic interaction between regions affected by the mutations is completely destroyed.