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▶ **To cite this version:**

Adèle Kerjouan, Cyril Boyault, Christiane Oddou, Edwige Hiriart-Bryant, Alexei Grichine, et al.. Control of SRC molecular dynamics encodes distinct cytoskeletal responses by specifying its signaling pathway usage. Journal of Cell Science, 2021, 134 (2), 10.1242/jcs.254599 . hal-03021717

HAL Id: hal-03021717
https://cnrs.hal.science/hal-03021717

Submitted on 24 Nov 2020

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Control of SRC molecular dynamics encodes distinct cytoskeletal responses by specifying its signaling pathway usage.

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Running title: Deciphering SRC pleiotropy.

Keywords: SRC, conformational intermediates, optogenetics, time-resolved phospho-proteomics, pleiotropy, migration, invasion, encoding intracellular signaling, cytoskeletal, adhesions.

Summary

Upon activation by different transmembrane receptors, the same signaling protein can induce distinct cellular responses. A way to decipher the mechanisms of its pleiotropic signaling activity is to directly manipulate its decision-making activity that supports the selection between distinct cellular responses. We developed an optogenetic probe to control SRC signaling, an example of pleiotropic signaling node, and we showed its ability to generate different acto-adhesive structures, i.e. lamellipodia or invadosome, upon distinct spatio-temporal controls of its kinase activity. The occurrence of each acto-adhesive structure was simply dictated by the dynamics of optoSRC nanoclusters in adhesive sites that were dependent on its SH3 and unique domains. These different decision-making events regulated by optoSRC dynamics induced distinct downstream signaling pathways characterized by time-resolved proteomic and network analyses. Collectively, by manipulating differently the molecular mobility of this kinase activity, this study aimed at hijacking and revealing the pleiotropy encoding mechanism of SRC signaling.

Introduction

The numerous extracellular stimuli are integrated by multiple receptors that will activate limited number of intracellular multi-domain signaling molecules that coordinate cell responses. How specific cellular responses are generated through precise control of the spatiotemporal dynamics of activation of a particular signaling molecule has remained a fundamental open question.

To investigate this paradigm, we focused on the first discovered proto-oncogene, c-SRC. Each member of the SRC family kinases (SFKs) is highly pleiotropic since it regulates diverse cellular outputs, such as metabolism, proliferation, gene expression and cell morphology (Parsons and Parsons, 2004). The ability of c-SRC signaling to decide between distinct cellular responses regulating adhesions and cytoskeletal remodeling clearly sustains its role of decision-making in migration and invasion processes (Boateng and Huttenlocher, 2012). C-SRC can even generates antagonistic responses in acto-adhesive structures as invadosomes, since essential for both their assembly and dismantling (Destaing et al., 2008). Consequently, decrypting the numerous potential c-SRC signaling activities in these dynamic acto-adhesive structures is challenging, as these activities occur at micrometer and minute scales.

To investigate the mechanism of SRC encoding activity, we need to integrate dynamically all structural elements that allow c-SRC to sample inputs, to route specific signaling pathways among a large repertoire and to encode specific cellular phenotypes. The pleiotropy of SFK members is supported by their canonical structure in which their kinase domain is targeted to membranes by a SH4 domain and maintained fully closed by two intramolecular interactions: one between the SH2 domain and a C-terminal phosphorylated tyrosine and the second

between the SH3 domain and an internal proline rich region (PRR, Young et al., 2001). Release of these intra-molecular interactions fully activates c-SRC kinase activity, through the opening of its bi-lobal kinase domain and provoking a cascade of events: autophosphorylation of the Y416 (Roskoski Jr., 2015) and solvent exposure of the adaptor SH3, PRR, C-ter phosphorylated-tyrosine and SH2 domains, which become available for extra-molecular interactions. Therefore, the tight coupling between enzymatic activation and adaptor functions modulates the duration and level of SRC kinase activity (Bernadó et al., 2008).

According to c-SRC structural complexity, one can predict that c-SRC signaling is not based on a simple binary activation but instead relies on the relationship between a multitude of possible c-SRC conformational intermediates defined by inter-domain interaction combinations (Fig. 1A, B). Interactions with lipids add an additional layer of complexity by affecting the binding capacities of the poorly structured UD and SH3 domains (Pérez et al., 2013). Finally, this complexity also includes its level of clustering while being able to oligomerize from the poorly knowns c-SRC dimers to 80 nm-nanoclusters of c-SRC molecules (Irtegun et al., 2013; Smith et al., 2016; Spassov et al., 2018). The numerous possible SRC conformational intermediates suggests that the pleiotropy of c-SRC signaling emerges from a heterogeneous population of molecules with different levels of kinase activity targeting dynamically a repertoire of interactors.

Understanding the functional relevance of these multiple c-SRC conformational intermediates in a dynamical manner is impossible with classical genetic methods (Fig.1A). Optogenetics manipulations have emerged as a powerful tool to dynamically regulate cell signaling (Toettcher et al., 2013). Nevertheless, with a temporal resolution ranging from 3 to 300s and a spatial resolution superior to 5 μm (Valon et al., 2015), it remains challenging to access the higher spatial resolution necessary to control acto-adhesive structures. Chemogenetics has been used to directly control the activity of SRC but without achieving both minute-scale and

reversible control of SRC at the subcellular level (Chu et al., 2014; Garske et al., 2011), while optogenetics was only used to obtain reversible and subcellular photo-inhibition of SRC signaling (Dagliyan et al., 2016).

To spatio-temporally activate and control SRC signaling, we engineered a new photoactivable SRC kinase, optoSRC (OS) based on the CRY2 optogenetic module. By only modulating the mobility of OS nanoclusters in adhesive sites, we were able to generate very different SRC-dependent acto-adhesive structures, lamellipodia and invadosomes, showing the possibility to induce different decision-making events optogenetically. Indeed, cyclic light-dependent OS oligomerization sustained over time was sufficient to induce its SH3- and unique site-dependent relocalization to adhesive sites, that could also be modulated by potential membrane association. Modeling of these different dynamics of OS showed that these optogenetic manipulations induced different fluxes of SRC signaling into adhesive sites, that engaged distinct downstream signaling pathways as monitored by time-resolved proteomic of co-purified phosphotyrosine-associated proteins (PY) and network biology. Collectively, with this synthetic approach we succeeded in hijacking the fundamental principles of SRC coding activity, suggesting new aspects of SRC regulation in line with observations on the dynamics of its physiological nanoclustering. We demonstrated the causal link between the dynamics of OS oligomers at adhesive sites and the SRC decision-making activity for triggering different cellular responses.

Results

Development of a light-inducible SRC kinase (optoSRC, OS).

The coupled dynamics of SRC kinase activity and adaptor functions have highlighted that the large repertoire of possible c-SRC conformations supports the complexity of its signaling activity (Fig.1A). According to numerous possible intra- or extra-molecular interactions (Fig.1B), it is not clear how spatio-temporal control of c-SRC activity triggers different cellular responses (Fig.1A,B). Precise spatial control of c-SRC activation is important for acto-adhesive structures, such as lamellipodia or invadosome (Fig.S1A).

Besides trying to reproduce the complexity of endogenous activation of SRC, we developed a CRY2-dependent optogenetic system (Kennedy et al., 2010) to spatio-temporally control its activity. This synthetic tool was based on reported SRC mutations that both affects its membrane localization and the opening of its kinase domain. Our strategy was to create a potentially active and cytosolic SRC mutant displaying only SRC signaling events upon light stimulation while being maintained inactive in the dark. Thus, the CRY2-mCherry module was fused to a SRC mutant deleted of its membrane-anchoring domain (SRCΔmyr-CRY2-mCherry), leading to its absence of localization in cell-cell contacts or adhesive sites of MDCK cells differently from c-SRCWT-mCherry or the active mutant SRCY527F-mCherry (Fig.1C). This mutant was made potentially active by opening its closed conformation through the substitution of phenylalanine at regulatory Y527. Despite not accumulating at cell-cell contacts, the SRCΔmyr,Y527F-CRY2-mCherry mutant was able to localize to adhesive sites in the dark (Fig.1C). An additional point mutation, R175L, inhibited the PY-binding activity of the SH2 domain and abolished the ability of SRCΔmyr,Y527F-CRY2-mCherry to localize in adhesive sites. This final construct named optoSRC (OS; SRCΔmyr,R175L,Y527F-CRY2-mCherry mutant) did not accumulate in any cellular compartment in the dark (Fig.1C).

The functionality of OS was validated by assessing the induction of a typical SRC-driven cellular response by light upon its membrane relocalization. OS relocalization was obtained by CRY2 heterodimerization with a plasma membrane anchored CIBN (CIBN-GFP-Caax) in response to local light stimulation (Fig.1D). As expected for CRY2-CIBN heterodimerization, sustaining cyclic light stimulation (100ms stimulation every 30s over 10 min) induced periodic OS membrane localization with a 5 μm-spatial resolution (Fig.S1B). Local membrane recruitment of OS provoked the rapid formation of large dorsal ruffles (Fig.1D, Suppl. Video 1) and phenocopied the expression of a constitutively active mutant of SRC (SRCY527F, Fig.S1C), the activation of thermosensitive and chemo-activable SRC mutants (Mettlen et al., 2006);(Gentry et al., 2016). The physiological relevance of OS was further confirmed by comparing its ability to activate Erk pathways in the same range as physiological stimuli such as EGF stimulations or cell spreading on different extracellular matrix (Fig.S2). The light-dependent membrane recruitment of OS was associated with self-activation of its kinase domain, as indicated by PP2-sensitive auto-phosphorylation of its Y416 residue (Fig.1E). Furthermore, the specific light-dependent activation of OS and the low leakiness of OS in the dark was confirmed by analyzing paxillin (PXN) phosphorylation, the main cytosolic substrate of c-SRC (Fig.S1D).

Altogether, our data show that OS was a fully functional optogenetic SRC system mimicking numerous physiological SRC functions.

Different activation of OS encodes distinct phenotypic acto-adhesive responses.

The ability of CRY2 to oligomerize was used to induce SRC nanoclusters (Fig.1F), whose *in vivo* functions are poorly characterized. Since CRY2 oligomerization and CRY2–CIBN heterodimerization co-exist (Duan et al., 2017), we tested if anchoring OS nanoclusters at the

membrane or not would affect their ability to induce different signaling events. To compare signaling activity of OS nanoclusters associated or not with membranes (depending of CIBN-GFP-Caax expression), and since SRC signaling occurs at cell-matrix adhesions, we used blue TIRF excitation to induce nanoclusters at the vicinity of the plasma membrane. TIRF blue light cyclic stimulation was sustainably applied on MDCK cells stably co-expressing either OS+CIBN-GFP-Caax or OS+GFP-Caax. Recruitment of OS oligomers to membrane (OS+CIBN-GFP-Caax) induced characteristic lamellipodia (Fig.2A; SupplVideo6) that were poorly present in epithelial MDCK cells. Lamellipodia were highly sensitive to OS activation, since rapidly formed after OS activation and disappearing only 5 min after stopping photostimulation (kymograph Fig.2A). These light-induced lamellipodia were not specific of OS nanocluster recruitment to Caax but rather dependent of their recruitment on plasma membrane in general since also observed with the endogenous anchoring domain of SRC (myr-GFP-CIBN, Fig.S3A). Surprisingly, formation of OS nanoclusters in the cytosol (OS+GFP-Caax) induced large, curved, auto-assembling centrifugal actin rings after sustained cyclic photostimulation (33 mHz, Fig.2B; SupplVideo5). These structures were invadosome rings as characterized by their stereotypical auto-assembling behavior, OS localization and accumulation of LifeAct-iRFP (Fig.2B) and cortactin (Fig.2C). Activation of kinase-dead OS mutant (OS-K295M) or PP2 pretreatment before induction of OS oligomerization abolished invadosomes formation demonstrating the need for Src kinase activity for their formation (Fig.S3B-C). Interestingly, both of these acto-adhesives structures implicated in migration or invasion are poorly present in epithelial MDCK cells. Quantitative analysis confirmed that cytosolic OS oligomers essentially induced invadosome formation but poorly induced lamellipodia, whereas recruitment of OS oligomers through membrane essentially induced lamellipodia and dorsal ruffles (Fig.2D). Therefore, the two types of OS oligomers dynamics (membrane or cytosolic diffusion) clearly gave rise to different cellular responses.

Accordingly, with the same photostimulation frequency, lamellipodia were formed immediately after OS activation (1 min$^{+/- 0,5 min}$), while invadosomes appearance needed longer and sustained stimulation (15,6 min$^{+/- 10 min}$). The choice of the frequency of cylic light stimulation have been adapted to the endogenous 5 min reversion-rate of activated CRY2 in order to induce or not a cumulative trend of activated optoSRC (Fig.S1B-S4F). As clearly described (Valon et al., 2015), this strategy allowed to modulated the quantity of activated OS molecules by applying different frequencies of light stimulation over the same duration (around 20 min), and thus test the relationship between the quantity of OS oligomers and the sensitivity of each cellular responses. In marked contrast to lamellipodia induction which was apparent for all frequencies tested (Fig.2F), invadosomes were not able to form if the flux of OS oligomers was not sufficiently important (by photostimulation greater than a 2 mHz, Fig 2E), confirming the importance of sustaining formation of OS oligomers over time to induce this type of structures.

Our data showed that both duration and quantity of activated OS molecules can be controlled optogenetically and can give rise to different and specific SRC-dependent acto-adhesive structures in cells not forming them classically. Surprisingly, without membrane association, OS oligomerization itself is sufficient to induce highly SRC-sensitive structures as invadosome. In conclusion, we were able to directly manipulate SRC pleiotropy and encode different cellular phenotypes by simply modulating SRC oligomers dynamics.

OS nanoclustering drives its relocalization to adhesive sites.

The striking signaling effect of OS oligomers on invadosome formation raised the question of the functionality and localization of these signaling nanoclusters. Firstly, we compared OS oligomerization with observed physiological SRC nanoclusters (2-10 molecules, Smith et al., 2016). To characterize the oligomerization level of light-activated OS in our conditions, we

measured the variations of OS molecular brightness since proportional to the oligomerization status of a fluorophore (Digman et al., 2008), with FCS measurements. Activated OS presented a significant increase of brightness that became comparable to the brightness of two OS that were molecularly fused (double OS) and maintained in the dark (Fig.S4A). This supports that activated OS can form small oligomers (from dimers to low oligomers).

We used cyclic blue light TIRF illuminations (100ms stimulation every 30s over 10 min) to induce mostly OS oligomers at the vicinity of the plasma membrane. Surprisingly, formation of OS oligomers induced their rapid and specific relocalization from the cytosol to adhesive sites, as they colocalized with the focal adhesion markers vinculin, p130CAS and paxillin (Fig.3A-B; Fig.S4D; SupplVideo2) but not with clathrin or caveolin sites, which are other c-SRC-sensitive plasma membrane-targeted loci (Fig.S4B). This relocalization was not dependent on oligomerization with endogenous SFK members as it was still effective in SYF cells (*c-Src*$^{-/-}$*Yes*$^{-/-}$*Fyn* $^{-/-}$cells; Fig.3C). Light-dependent OS relocalization to adhesive sites was typical of the kinetics of CRY2 activation (activation in few sec, reversion in 5min; Fig.S4F) and thus did not present obvious difference with OS relocalization at the membrane in presence of CIBN-GFP-Caax (Fig.S1B). Again, OS expression exhibited poor leakage of its phosphorylation activity under dark conditions, as illustrated by paxillin phosphorylation in MDCK cells or SYF cells (Fig.S5A,B).

Relocalization of OS oligomers to adhesive sites was supported by the SH3 domain functions (Fig.3E, S4G) as evidenced by its strong decrease upon SH3 deletion (OS-ΔSH3), inhibition of SH3 domain binding to PRR-proteins (OS-W118K) or increase of intramolecular SH3-PRR binding (OS-SH3Eng or SRC-K249P/Q252P/T253P). In addition, mutation of the internal PRR of OS (OS-PPR-AAA) had no effect on this process (Fig.S4E). Interestingly, the presence of the phosphorylable Y527 in OS (OS-R175L only) blocked its ability to be relocalized in adhesive sites showing that this tyrosine 527 not only controled the kinase

domains through the opening of the internal SH2-Y527 bridge but was also implicated in OS partners binding when phosphorylated (Fig- S4C). Light-dependent OS relocalization could be either driven by a simple increase of SH3-local concentration induced by the oligomerization or controlled by a specific SRC-SH3 domain conformation which can be dictated by the vicinity of the SH2 domain and/or tyrosine kinase domain. This last hypothesis was confirmed by the reduced ability of activated OS mutants containing a deleted kinase domain or SH2 domain, or perturbation of the kinase domain (OS-K295M) to relocalize in adhesive sites (Fig.S4-D). Finally, reducing clustering properties of CRY2 by using CRY2 low mutant (CRY2-1to488-EED, Duan et al., 2017) slightly reduced OS relocalization in adhesive sites without blocking it (Fig.S4C) showing that lower-size oligomers (probably dimers) can support this relocalization.

In addition to regulating OS adaptor functions, we investigated whether SRC nanoclustering also activated its kinase activity. Light-dependent oligomerization of OS was sufficient to stimulate itself OS kinase activation, as indicated by the increase of PP2-sensitive Y416 phosphorylation (Fig.3F) which also occurred in SYF cells (Fig. S5B). Then, OS kinase activation was increased by a light-dependent trans-phosphorylation process. Finally, light-induced oligomerization of OS in SYF cells activated its kinase domain and phosphorylate paxillin at the same level than c-SRC re-expression, showing the ability of OS to mimic physiological level of c-SRC activation (Fig.S5B).

Therefore, controlling oligomerization formation uncovered the crucial role of the SH3 domain in the functions of SRC nanocluster since regulating the recognition of specific PRR-enriched proteins in adhesive sites and SRC kinase activation. Light-dependent OS oligomers subcellular relocalization in adhesive sites demonstrated therefore marked improvement of the spatial resolution of previous CRY2 optogenetics approach.

Optogenetic control of the different rates of OS recruitment to adhesive sites.

In order to explain, the different signaling events induced by either OS nanocluster freely diffusing from the cytosol to the adhesive sites or either OS nanocluster diffusive at the membrane, we determined the dynamics parameters of OS nanoclusters recruitment in adhesive sites. First, we determined if membrane-associated OS oligomers (OS+CIBN-GFP-Caax) could also relocalize to adhesive sites (Fig.4A; Suppl.Video 4). In the presence of CIBN-GFP-Caax, OS oligomers were recruited with the same rate outside (membrane) and inside of adhesive sites (Fig.4B). By contrast, OS oligomers alone (OS+GFP-Caax) were mainly targeted to adhesive sites (Fig.4C).

While these two experimental strategies (direct or membrane-based indirect recruitment) both led to sustained OS recruitment to adhesive sites, we compared the rates of OS passing through these sites in terms of quantity and molecular mobility. At the whole basal cell surface, co-expression of CIBN-GFP-Caax allowed recruitment of more OS oligomers at the plasma membrane than when OS was expressed with GFP-Caax (Fig.4D), probably because of the difference between the large amounts of available membrane anchored CIBN-GFP-Caax and the small number of adhesive sites. However, OS oligomers were more concentrated in adhesive sites than when associated with membranes (OS+CIBN-GFP-Caax), as revealed by their higher OS/vinculin-iRFP ratio after 10 min of cyclic photostimulation (33 mHz, Fig.4E). Therefore, these two strategies of OS recruitment generated different densities of OS in adhesive sites over time. In addition to determining the quantity of activated OS in adhesive sites, it was also essential to analyze the molecular mobility of activated OS in both conditions (GFP-Caax or CIBN-GFP-Caax). Thus, we combined optogenetic manipulation with FRAP analysis to determine the mobility of activated OS molecules in adhesive sites. In comparison to direct recruitment of OS oligomers to adhesive sites, membrane-based indirect recruitment (in presence of CIBN-GFP-Caax) strongly increased the immobilization of

activated OS in adhesive sites (characteristic time of recovery evolving from of 8,4 $s^{+}/-$ 2,6 s to 20,8 $s^{+}/-$ 8,3, associated with a 50% increase of the immobile fraction, Fig.4F).

Therefore, we were able to generate different quantities and molecular mobilities of activated OS in adhesive sites over time by simply controlling its modes of recruitment.

Then, we characterized the two rates of activated OS recruitment to adhesive sites in response to sustained cyclic light activation by modeling. Our optogenetic system can be schematized as a model in which light activates an OS buffer in the cytosol. Following sustained cyclic photostimulation, this activated buffer (OS*) is flushed either by direct adsorption on the membrane (mimicking CIBN-GFP-Caax condition) or direct recruitment to adhesive sites (mimicking GFP-Caax condition, Fig.4G). A first consequence of this model was a 3D-2D reduction of the dimensionality of the mobility for membrane recruitment of OS* compared with direct adsorption of OS* to adhesive sites, explaining FRAP results. Indeed, we expected the rate of recovery of OS oligomers in adhesive sites to be largely smaller for a 2D diffusing membrane-anchored protein ($D \approx 0{,}2\ \mu m^2.s^{-1}$) than for a 3D diffusing cytosolic OS oligomers ($D \approx 10\ \mu m^2.s^{-1}$, Fig.4F).

Based on our experimental results, sustained cyclic light activation of OS induces a transport by diffusion of these activated oligomers (OS*) from the cytosol to adsorption sites (composed of receptors for OS*), generating a local diffusion-limited flux of activated OS controlled by the density of receptors composing the sites. To compare both OS* fluxes, numerical simulation was used to compare the early behavior of OS fluxes that were directly recruited to adhesive sites or after a membrane-binding step. As expected, for the same amount of activated OS, this model demonstrated that the flux for direct recruitment of activated cytosolic proteins, $OS^*_{(r,t)}$, to adsorption sites was higher than that for indirect adsorption through membrane (Fig.4H).

This model describes that both OS dynamics are characterized by both the rate of transport of activated OS and density of receptors for OS in adsorption sites (Fig. 4G) and predict that the probability of phosphorylation for any substrates is dependent of both local concentration of OS and residence time of OS. Therefore, controlling the dimensionality of OS oligomers mobility generates different flux of OS to adhesive sites that encode different OS signaling events.

Specificities of SRC signaling transfer downstream of different OS dynamics.

To understand the causal link between OS dynamics through adhesive sites and decision-making (invadosome versus lamellipodia), we determined how the modulation of OS molecular turn-over affects different downstream signaling pathways. More precisely, we identified and characterized the enrichment dynamics of proteins co-purified by anti-PY affinity after OS activation (referred as "OS-sensitive proteins", Fig.5A). The combination of OS activation with PY co-immunoprecipitation was feasible, as exemplified by light- and PP2-dependent phosphorylation of known SRC substrates, such as paxillin and p130CAS (Fig.5B). We scaled up this set up for both OS fluxes (GFP-Caax or CIBN-GFP-Caax conditions), and OS-sensitive proteins were immunoprecipitated from large MDCK populations photostimulated during 5 or 20 min (33 mHz, Fig.5A). PY-purified proteins were identified by label-free semi-quantitative mass spectrometry, and their fold-changes were averaged from three independent experiments and technical duplicates (Supplemental TableS1). Under non-stimulated conditions (time 0), more than 95% of the identified PY proteins presented the same level of enrichment in non-stimulated MDCK cells co-expressing either OS+GFP-Caax or OS+CIBN-GFP-Caax. OS light activation significantly modulated the enrichment of only a small fraction of PY proteins (90 OS-sensitive proteins out of 506 PY-proteins at time 0, Fig.5C).

Since OS-sensitive proteins are a presumed mixed population of tyrosine phosphorylated proteins and associated proteins, we evaluated the probability of identifying direct c-SRC substrates from these 90 OS-sensitive proteins by analyzing the presence of known SRC partners, known PY-containing proteins, using stringent *in silico* phospho-prediction by c-SRC and sensitivity towards PP2. All of the OS-sensitive proteins were positive for at least one of these conditions, supporting the presumed high enrichment of SRC substrates in our pipeline analysis (Supplemental TableS1). Among OS-sensitive proteins, time-resolved PY-proteome analysis identified many classical SRC substrates (paxillin, p130CAS, Tensins; Sawada et al., 2006; Thomas et al., 1995) or SRC substrates identified by phosphoproteomic approaches (ARHGEF5, p120CAS, SGK269, SGK223, EPHB2; Kuroiwa et al., 2011; Leroy et al., 2009; Luo et al., 2008). In addition, gene ontology (GO) analysis showed a significant enrichment for expected functions, such as motility, kinase signaling and small GTPases (Fig.S6A).

We developed a novel pipeline to compare the enrichment of OS-sensitive proteins over time under two OS flux modes. As already described (Kubiniok et al., 2017; Olsen et al., 2006), we employed soft clustering based on fuzzy c-means partitioning to analyze temporal enrichment patterns and to cluster proteins based on their enrichment dynamics (three clusters presenting a global increase or decrease in enrichment, Fig.5D). Comparison of the cluster compositions showed that modulation of OS dynamics affected PY-proteins enrichment, most likely, the phosphorylation dynamics of OS substrates. First, modulation of light-activated OS flux to adhesive sites did not change the dynamic phosphorylation behavior for most OS-sensitive proteins that are important signaling relays or acto-adhesive regulators (ARHGEF5, ARHGEF7, MAPK1, PPP1CC, GIT2, Tensin3, ITSN2). By contrast, modulation of activated OS flux to adhesive sites was sufficient to significantly change only the amplitude of phosphorylation of key regulators of acto-adhesive structures (SGK223, p130CAS, ASAP2,

Tensin1; Supplemental TableS1). Finally, only 37 proteins out of the 90 OS-sensitive proteins showed cluster changes between both OS dynamics conditions.

To integrate dynamic behavior with signaling specificity downstream of each OS flux, we integrated OS-sensitive protein dynamics with a network analyses based on protein-protein interaction public databases. More than 60% of the 90 OS-sensitive proteins were physically linked and defined three main ensembles of signaling nodes implicated in adhesion signaling (Fig.S6B). The first ensemble was composed of many acto-adhesive elements (paxillin, Tensin1-3, p130CAS) or regulators (ASAP2, p120CAS, EPHB2, SGK269, SGK223, PPP1CC, GIT2). The second ensemble was built over the aminoacyl-tRNA synthetase complex (AIMP1/2, KARS, RARS) and actin-binding protein PTK9. The third ensemble was a mix of DNA stability regulators (CDC47, CDC21, SSPB1), trafficking regulators (COPB, COPG1) and general signaling nodes (SIPA1L1 and PPP2R3A).

Comparison of the OS-sensitive protein networks revealed that a node could harbor different phosphorylation dynamics according to different OS dynamics triggering different cellular responses. These data highlight the existence of correlations between dynamic changes at the network level and decision-making, such as lamellipodia or invadosome (Fig.5E). Intriguingly, induction of lamellipodia correlated with increased phosphorylation amplitude of key regulators of this structure (p130CAS, ASAP2 and PP2AA) and late or sustained increased phosphorylation of EPHB2 and p120CAS. In contrast, invadosome formation correlated with decreased phosphorylation amplitude of key signaling relays (ASAP2, p130CAS), decreased enrichment of several acto-adhesive regulators (EPHB2, p120CAS) and sustained enrichment of key invasion regulators (PXN, SGK269, PTK9) and signaling regulators (PPP2R3A).

Instead of inducing binary phosphorylation, we determined that the modulation of OS dynamics was complex and characterized by its ability to diversely impact the temporal

phosphorylation behavior of OS-sensitive signaling nodes. As our strategy to modulate the flux of OS oligomers at adhesive sites was based on large changes in the dimensionality of their mobility, we wondered if a subtle modulation of OS dynamics was sufficiently sensitive to affect cellular responses.

Importance of the Unique Domain (UD) to modulate OS signaling flux.

Previous structural studies supported the role of the UD domain in the regulation of SH3-binding activity of SRC (Arbesú et al., 2017). Thus, we investigated optogenetically both the poorly known *in vivo* functions of this intrinsically disordered regions on SRC signaling (IDR, Arbesú *et al*, 2018), and the possibility to slightly modulate the flux of OS oligomers to adhesive sites by changing the binding properties of its SH3 domains without changing the dimensionality of OS mobility. Deletion of UD in OS (OSΔUD) led to a significant increase of its recruitment to adhesive sites than OS in response to TIRF photostimulation (33 mHz, Fig.6A-B), suggesting that UD behaved as a negative regulator of SH3 domain binding to PRR proteins in adhesive sites. Deletion of UD did not affect the residence time of OS in adhesive sites (Fig.S6C). Therefore, OSΔUD was used to test the importance of modulating only signaling density and OS concentration in adhesive sites. Phenotypically, even UD deletion affected slightly OS oligomers flux to adhesive sites, this was sufficient to increase by two the number of invadosome in comparison to full-length OS (Fig.6C-D).

To determine the importance of this subtle change of OS flux of downstream signaling pathways, we analyzed the network architectures and dynamics of OS-sensitive proteins from photostimulated MDCK cells stably expressing OS or OSΔUD. Scaling up materials and improving the enrichment allowed us to identify more OS-sensitive proteins for each time point, consequently improving the depth of analysis (Supplemental TableS2). We found that only one-quarter of PY proteins identified at time 0 were directly modulated by OS

photostimulation (255 OS-sensitive proteins out of 894 PY proteins, Fig.S6D) and had again a high probability of being SRC substrates (Supplemental TableS2). Numerous proteins or GO functions identified after activation of MDCK cells stably expressing OS+GFP-Caax were also found after activation of cell stably expressing OS (Fig.S6E). Mfuzz analysis organized these proteins into 6 clusters of temporal enrichment behaviors (Fig.S6F and Supplemental TableS2).

To reduce the complexity of the networks downstream of both OS fluxes, we focused on the OS-sensitive proteins implicated in acto-adhesive regulation (63 out of 255, Fig 6E). A large number of these proteins were previously identified (Fig.5) and found physically connected. Comparison of the networks of OS-sensitive proteins demonstrated that most acto-adhesives proteins were not affected by the slight change of OS flux induced by UD deletion. However, consistent with a stronger recruitment of OSΔUD to adhesive sites, important invadosome regulators (PTPN6, ARAP3 and ARHGEF5) presented an increased amplitude of enrichment. On the other hand, a subtler response was obtained for other nodes, which either showed decreased phosphorylation (like paxillin) or changes in their phosphorylation behavior, such as components of COP9 signalosome, regulators of membrane trafficking (RAB11-FIP1 and SEC16L), thereby suggesting their involvement in invasion control. Finally, we observed that activation of OSΔUD also stimulated a functional complex composed of the GTPase CDC42, its GEF FGD6 and its effector tyrosine kinase ACK1.

In addition to precisely characterizing the *in vivo* function of an IDR as the UD domain, the comparison of two activated OS and OSΔUD dynamics in the same subcellular compartment identified a set of highly sensitive responsive signaling nodes to subtle modulation of OS flux over time.

Discussion

To investigate the SRC coding activity, we engineered and specify uses of photoactivable SRC kinase to get spatio-temporal control of SRC in adhesive sites. We demonstrated that the modulations of molecular fluxes of SRC activity were sufficient to hijack SRC pleiotropy since generating different downstream signaling pathways and ultimately trigger different cellular responses.

Engineering synthetic SRC to understand its complex structure-function relationship

Controlling directly SRC signaling in space and time is challenging. Previous protein engineering-based approaches targeted directly the kinase domain of SRC by regulating its selectivity, folding or allostery (Chu et al., 2014; Garske et al., 2011; Karginov et al., 2010; Karginov et al., 2014; Shah et al., 1997). Chemogenetic approaches are highly specific but are not suitable to achieve min-scale and reversible control of this kinase at the subcellular level. Coupling allosteric regulation of SRC kinase and the light sensitive domain, Lov2, allowed to generate a photo-inhibitable SRC that is reversible and can present subcellular precision, but without activating specific and exogenous SRC signaling events (Dagliyan et al., 2016). In order to activate SRC in space and time, we engineered a photoactivable SRC based on non-transforming v-Src mutant deleted of its membrane anchoring domain (Cross et al., 1984). In addition to improving the spatial resolution of CRY2-system (Valon et al., 2015), engineering OS also allowed to control the activation/desactivation cycle of SRC with high temporal range (min-scale), revealed new functions of SRC oligomers and the unique domain (UD), a simple IDR sufficient to regulate phenotypic specificity of SFKs (Summy et al., 2003). Despite being mostly described as a monomer, controlling oligomerization with light is physiologically relevant since SRC and other SFK were showed to both dimerize, oligomerize and form larger nanoclusters after activation but without knowing the functional relevance of

these organizations *in cellulo* (Irtegun et al., 2013; Rossy et al., 2012; Smith et al., 2016; Spassov et al., 2018). Thus, our synthetic OS strongly support that SRC oligomerization could be a new stabilizing process in adhesive sites mediated by SH3 domains and a late stage of its activation. The apparent contradiction on the essential role of SH2(Yeo et al., 2006) or SH3 (Fincham et al., 2002; Wu et al., 2016) domains in adhesive sites relocalization can now be explained if one assumes that the level of SRC oligomerization is considered since probably controlling specific partnership with a large repertoire of PRR-containing proteins present in adhesive sites. Since a minimal level of multivalency of SH3 domains drives phase separation (Li et al., 2012), we hypothesized that OS oligomerization favorizes SH3 domains cooperativity that support an increase of the apparent affinity of SH3 domains leading to new binding when SRC molecules are grouped in nanoclusters. Moreover, only the use of OS allowed to confirm the new and essential roles of UD in the stabilization of SRC oligomers in adhesive sites, explaining its ability to modulate the SRC-substrate repertoire *in vivo*. In addition to modulate c-SRC adaptor functions, formation of oligomers controls also kinase activation. CRY2 light-dimerization of cytosolic OS is sufficient to strongly increase Y416 phosphorylation (Fig.3), and confirmed the existence of a transphosphorylation process(Vojtěchová et al., 2006).

By integrating intermediates of a signaling node and getting closer to its characteristic spatio-temporal activation patterns, the synthetic approach of optogenetics is complementary to long-term genetic manipulations for exploring new aspects of molecular dynamics *in vivo* in the decision-making activity of signaling nodes.

Biological relevance of synthetic OS

Our synthetic approach was based on deconstructing some regulatory elements of SRC and this naturally raises the question of its biological relevance. The fact that SRC mutants

containing SH2 or SH3 domain deletions can restore invadosome functions in *c-Src*$^{-/-}$ osteoclasts(Destaing et al., 2008) supported our assumption that other domains of SRC compensate for the loss of function of point-mutated SH2 domain. Moreover, we found that OS activation rapidly induced multiple physiological and classical c-SRC-dependent cellular structures such as adhesive sites and actin cytoskeleton structures (Playford and Schaller, 2004). Indeed, OS activation induces dorsal ruffles as seen for activation of thermo-sensitive SRC mutant(Mettlen et al., 2006) and the activation of chemo-controllable synthetic SRC, containing both functional SH2 and SH3 domains(Gentry et al., 2016; Karginov et al., 2010). OS activation on membrane induced lamellipodia in epithelial cells, suggesting activation a partial EMT process that is in line with previous report showing that only this mode of activation induced mechanical remodeling of epithelia layer (Moitrier et al., 2019). In MDCK cells, another mode of OS activation even induced invadosome rings, which are typical c-SRC-dependent structures in physiological and pathological conditions as revealed by the study of *c-Src*$^{-/-}$ models (Boateng and Huttenlocher, 2012; Luxenburg et al., 2006). Preliminary results on photoactivated optoYes, another member of the SFK poorly present in adhesive sites, revealed its inability to be recruited in adhesive sites in response to light (OD personal communication). This clearly confirmed the specificity of OS to be relocalize in adhesive sites and that our optogenetic approach cannot force SFK molecules to do something that they cannot achieved physiologically. Moreover, activation of optoSRC in SYF cells induces the activation of its kinase domain and the phosphorylation of paxillin in the same range as SRC-WT expressed in SYF cells showing that its level of activation is in a physiological range since comparable to wild-type c-SRC (Fig.S5B). In addition, activation of OS can activate ERK pathways in the same range than physiological stimuli of SRC such EGF treatment or adhesion on ECM (Fig.S2). Finally, mass-spectrometry analysis clearly shows that known physiological substrates of SRC are phosphorylated at min-scale in

response to OS photostimulation (such as its main substrates paxillin, p130Cas, tensins, ASAP2, ARHGEFs, Ephrins…). It is important to notice that most of these substrates are already known to be phosphorylated downstream of physiological stimulations such as PDGF, EGF treatments or adhesion on ECM.

In conclusion, it is striking to observe that our synthetic approach of SRC activation is sufficient to activate numerous and characteristic physiological feature of c-SRC signaling pathways. Thus, this synthetic optogenetic probe provides new direction to explore the causal link between SRC molecular dynamics *in vivo* and its associated signaling.

Control of OS molecular flux supports different decision-making in SRC signaling.

Besides providing a simple qualitative activation approach and decreasing the repertoire of potential SRC conformational intermediates, we showed that specific cyclic and sustain optogenetic activation allowed to control both duration and quantity of activated OS molecules relocalization in adhesive sites over time. Thus, this relocalization-based strategy of our optogenetic design allowed to integrate both quantity and duration of the transport of active OS molecules in an adhesive surface, that can be only described by the notion of flux of signaling OS molecules. The notion of flux of signaling has been proposed to explain the importance of changes of CDC42 activation during cell polarization of budding yeast or to model *in silico* enhanced activation or choice in alternative signaling pathway (Rapali et al., 2017; Selvarajoo et al., 2008). By only regulating either the local density of CRY2-dependent OS oligomers (+/- UD deletion) or the dimensionality of their mobility (+/- membrane-based recruitment) over time, we could experimentally show that this is sufficient to induce different sustained fluxes of the same CRY2-dependent OS oligomers in adhesive sites. As a consequence, and independently of different frequencies of stimulation, our data showed that modulation of the flux of OS molecules in adhesive sites was sufficient to encode different decision-making events leading to the formation of different acto-adhesive responses: invadosomes vs lamellipodia (Fig.2E). Interestingly, we were not able to induce both invadosome and lamellipodia in the same time, even if both structures are not antagonistic.

This rather suggests that the decision-making process induced here is exclusive. Finally, we showed high sensitivity of SRC decision-making events since low amplitude modulation of OS molecular flux was sufficient to finely tune cell signaling since it was sufficient to initiate specific pathways and acto-adhesive phenotypes (Fig.6).

Except predicting that the probability of phosphorylation for any substrates is dependent of both local concentration of OS and OS residence time, we did not provide a strong model to explain how changing the flux of OS oligomers can support these decision-making process. However, our approach to modulate the dynamics of SRC molecule remembered the importance of considering that stochastic reactions occurring in biological processes implies small numbers of molecules and their inevitable randomness of physical transport processes and binding reactions (Kaizu et al., 2014). In the specific case of the dynamics of OS oligomers relocalized in adhesive sites, it appears essential to consider their probability of rebinding their substrates after a dissociation event: either OS oligomers bind a new substrate (after diffusing back into the bulk) or either OS oligomers will rebind the same substrate. Considering both scenarii is essential to determine the probability of kinase to promote mono-phosphorylation or multi-phosphorylation (on adjacent or distant sites) of the same protein. This physic principle of molecular dynamics was used to show that MAPK or integrin signaling rely on the regulation of molecular mobility through cytosol-to-membrane relocalization or receptors clustering (Block et al., 2015; Kholodenko et al., 2000). Thus, comparing flux of OS oligomers associated or not with membrane could represent two extreme cases where the flux of OS oligomers exchanging quickly with the cytosol could rather promote mono-phosphorylation or multiphophorylation on adjacent sites of a substrate. Thus, considering the difference of flux of SRC could explain how this single kinase can control differently multiple possible phosphorylation sites of its main substrate, paxillin, in different conditions of activation or in different structures (lamellipodia vs invadosome; Webb et al., 2005). Future coupled optogenetics and phosphopeptide studies will be necessary to determine how the coexistence of both fluxes of SRC and its potential substrates (Horton et al., 2016) at adhesive sites is integrated in order to affect the selection and multiphosphorylation processes of specific signaling pathways. Thus, our optogenetics

approach is an experimental proof-of-concept of a new direction to better understand the molecular basis of pleiotropic molecule in cell signaling.

Modulation of SRC molecular dynamics curves the phosphorylation dynamics of SRC substrates.

To understand how subtle modulations of the OS flux coordinate different cellular responses, we mapped the downstream signaling pathways associated with each decision-making process. Although time-resolved phosphoproteomics has been used to globally determine the signaling events downstream of EGFR or TGFβ activation (J. D'Souza et al., 2014; Olsen et al., 2006), it was never coupled to optogenetic studies to follow the dynamic emergence of a signaling pathway and its different associated cellular responses. In regard to the associated phenotype downstream of each OS fluxes, we generated biologically relevant data as we identified key regulators of SRC-driven invadosome formation, such as paxillin and SGK269, and key regulators of lamellipodia, such as p130CAS, EPHB2 and p120CAS (Boguslavsky et al., 2007; Zhang et al., 2014; Zisch et al., 2000).

Our analysis tool revealed the existence of distinct class of phosphorylation behavior (clusters) of OS-dependent substrates (Fig.5D). Modulation of OS flux is sufficient to change the OS-substrate membership in each of these class of phosphorylation behavior (Fig.5E). This ability supports the decision-making switch associated with lamellipodia or invadosome formation, such as for EPHB2 or p120CAS (Fig.5E). Modest modulation of OS oligomer fluxes (OS vs OSΔUD) could affect the functional coupling between acto-adhesive and extracellular matrix degradation activities of invadosome, which is supported by trafficking of WASH-associated endosomes (Monteiro et al., 2013). Indeed, this could occur through the regulation of the CDC42-FGD6-ACK1 complex, which is in agreement with the involvement of activated CDC42 in all invadosome models (Di Martino et al., 2014), the role of its GEF, FGD6, in the regulation of WASH-positive endosomes trafficking (Steenblock et al., 2014),

and the phosphorylation of the invadosome marker cortactin by ACK1(Kelley and Weed, 2012).

Signaling networks in systems biology are usually categorized according to their sensitivity, redundancy and decision-making properties (Azeloglu and Iyengar, 2015). We believe that for SRC signaling, these properties are, at least in part, under the direct control of OS flux. Indeed, modulation of OS flux is sensitive enough to induce different phosphorylation dynamics of different members of the same family (SGK269-223, Tensin1-3 and GIT1-2), and generates redundancy represented by all OS-sensitive proteins without phosphorylation behavior changes.

Exploration of the OS activation regimen revealed new insights into the causal link between molecular signaling and decision-making. This approach paves the way to understand the molecular basis of the pleiotropic coding of other signaling nodes and how oncogenic SRC signaling leads to physio-pathological transitions.

Figure legends

Figure 1. Optogenetic control of the non-receptor tyrosine kinase SRC.

A. Schematic of how coupled adaptor and kinase activities of SRC could trigger different cellular outputs.

B. High combinatorial of SRC conformations is sustained by numerous intra- and extra-molecular interactions.

C. Representative images of different optogenetic probes engineered and expressed in MDCK cells maintained in the dark, that led to the optoSrc (OS) which is a potentially active tyrosine kinase fully cytosolic while not localized in ECM-adhesive sites (blue arrows) and cell-cell contacts (yellow arrows).

D. Representative time serie showing that local cyclic activation of OS (blue square, 100ms pulse every 30s over 5 min) induces its recruitment at the plasma membrane of fibroblasts expressing CIBN-GFP-Caax triggers large dorsal ruffles (red arrows).

E. Light-dependent recruitment of OS at the plasma membrane of MDCK cells expressing CIBN-GFP-Caax induces SRC-dependent activation of its kinase domain (measured by P416Y/total SRC ratio).

F. OS photostimulation can lead to either its membrane relocalization (through heterodimerization with membrane-anchored CIBN) or its oligomerization.

Scale bars: 5 μm.

Figure 2. Different rates of OS molecular flux into adhesive sites generate either lamellipodia or invadosomes.

A. Representative TIRF time serie illustrating how membrane-associated activated OS oligomers induces lamellipodia in MDCK cells (blue light 50ms pulse every 30s over 10 min). Kymograph analysis showed high dependency of lamellipodia to blue light (zoom, white square).

B. Representative TIRF time serie illustrating how activated OS oligomers induces dynamic invadosome rings (LifeAct-iRFP) in MDCK cells (blue light 50ms pulse every 30s over 10 min).

C. These OS-dependent F-actin rings (phalloidin) accumulates the invadosome marker cortactin (confocal imaging).

D. Quantification of the different acto-adhesive phenotypes obtained after 20 minutes of cyclic blue light stimulation of MDCK cells stably expressing either CIBN-GFP-Caax or GFP-Caax (N<6; > 90 cells per condition).

E-F. Quantification of the percentage of cells forming invadosomes (E) or lamellipodia (F) depending of cyclic blue photostimulation frequency (50ms pulse over 20 min, N=4; > 30 cells per condition).

Scale bars: 5 μm.

Figure 3: Light-dependent OS oligomers relocalize to adhesive sites through their SH3 domains.

A-B. Representative TIRF images before and 1 min after cyclic blue TIRF photostimulation (50ms pulse every 15s) showed relocalization of OS oligomers to adhesive sites of MDCK cells (vinculin-GFP, B; or p130cas-GFP, C).

C. Representative TIRF image showed that relocalization of OS oligomers in adhesive sites (paxillin-GFP) even occurs in SYF cells and thus in absence of the main endogenous SFKs.

D. Representative TIRF images showed that light-dependent OS oligomers relocalization in adhesive sites of MDCK cells is dependent of its SH3 domains, while not observed for CRY2 alone or OS mutants (OS-ΔSH3, OS-W118K and OS-SH3Eng).

E. Quantification of OS and its SH3-mutants at the plasma membrane after blue TIRF photostimulation (SD; N=3; >30 cells per condition, unpaired t test).
F. Light-dependent oligomerization of cytosolic OS mediates activation of its kinase domain (measured by P416Y/total SRC ratio) through a PP2-sensitive process.
Scale bars: 5 μm.

Figure 4. Modulation of activated OS molecular flux in adhesive sites by controlling its membrane anchoring.
A. Representative TIRF image of light-dependent OS oligomers relocalization in adhesive sites (vinculin-iRFP) after membrane recruitment mediated by co-expression of CIBN-GFP-Caax in MDCK cells.
B-C. Time series and kymograph characterized relocalization dynamics in response to TIRF photostimulation (50ms pulse every 15s) of OS oligomers inside and outside (dashed line) of adhesive sites when co-expressed in MDCK cells (B) with CIBN-GFP-Caax or (C) the control GFP-Caax.
D-E. Quantification of OS relocalization at the whole basal level of the plasma membrane (D) or specifically in adhesive sites (F, OS/vinculin-iRFP ratio) after blue TIRF photostimulation of MDCK cells expressing either CIBN-GFP-Caax or GFP-Caax (SD; N=3; >18 cells per condition, unpaired t test)
F. Representative TIRF time series of OS relocalization to adhesive sites (white dashed line) after blue TIRF photostimulation, followed by FRAP experiments. Quantification of FRAP parameters (SD; N=3; >15 cells per condition; unpaired t test).
G. Physical principles of a cytosolic OS reservoir that can be relocalized to adhesive sites after its photoactivation (OS*, OS oligomers), directly or through a membrane step, mimicking respectively GFP-Caax and CIBN-GFP-Caax conditions.
H. Numerical simulations of the mean flux of OS* per unit length of an adsorbing cluster and over time, directly (red) or through a membrane step (green).
Scale bars: 5 μm (A), 2,5 μm (B, C, F).

Figure 5. Modulation of OS molecular flux in adhesive sites regulates different downstream signaling pathways.
A. Signaling pathways downstream of each OS flux were identified by time-resolved PY immunoprecipitation in MDCK cells photostimulated (blue light 50ms pulse every min over 5 or 20 min).
B. Specific PY-enriched proteins were immunoprecipitated (silver staining) and showed that OS activation induces PP2-sensitive phosphorylation of classical c-SRC substrates such as p130Cas and paxillin.
C. Venn diagram of PY-proteins showed high homology between the OS+GFP-Caax (red) and OS+CIBN-GFP-Caax (green) conditions in non-stimulated state. OS-sensitive proteins represented only 17% of all PY-proteins identified at time 0.
D. Detailed fuzzy c-means clustering analysis of OS-sensitive proteins observed in MDCK cells expressing OS+GFP-Caax. The dynamic behavior for each OS-sensitive protein is color coded (green), grouped inside a cluster defined by the average trend (blue). Examples of few OS-sensitive proteins are given for each cluster. Comparing averaged tendency curves showed high similarity for each cluster between each OS flux conditions.
E. Potential physical connections between OS-sensitive proteins was revealed by direct protein-protein interaction network. OS-sensitive proteins (or nodes) presenting only a change in the amplitude of phosphorylation between both conditions were framed, while any OS-

sensitive proteins presenting a change of phosphorylation behavior between both conditions were color coded.

Figure 6. Low modulation of OS oligomers flux in adhesive sites by deletion of the unique domain (UD) is sufficient to regulate cellular response and downstream signaling transfer.

A. Representative TIRF time series of OS and OSΔUD relocalizations in adhesive sites (vinculin-GFP) after cyclic blue TIRF photostimulation of MDCK cells (50ms pulse every 30s).

B. Quantification of OS and OSΔUD relocalizations at the whole basal level of MDCK cells photostimulated (50ms TIRF pulse every 30s over 5 min, SEM; N=3; >16 cells per condition, unpaired t test).

C. Representative confocal images of invadosomes rings (phalloidin) in MDCK photostimulated for 15 minutes.

E. Quantification of the percentage of cells forming invadosomes in MDCK photostimulated for 15 minutes (N=3, 300 cells; paired t test).

F. Connectivity between OS-sensitive proteins implicated in acto-adhesion and trafficking was revealed by direct protein-protein interaction network. OS-sensitive proteins (or nodes) presenting only a change in the amplitude of phosphorylation between both conditions were framed, while any OS-sensitive proteins presenting a change of phosphorylation behavior between both conditions were color coded.

Scale bars: 5 μm.

Methods

Detailed methods are provided in the supplementary data of this paper and include the following:

KEY RESOURCES TABLE (plasmids, reagents, antibodies) : supplemental Table S3a and S3b

EXPERIMENTAL CELL MODELS and CULTURE

Experiments were performed on fibroblasts, SYF cells or epithelial MDCK cells transiently transfected and/or stably transduced by viral strategy. All cell lines were cultivated at 37°C and 5% CO2 in DMEM high glucose 4,5g/L, glutamax, PAA) media supplemented with 10% of Fetal bovine serum (GE Healthcare), penicillin and streptomycin 1% (v/v) (PAA). All cells were transfected using lipofectamine2000 and according the profocol of the manufacturer (Invitrogen).

OPTOSRC PLASMIDS CONSTRUCTION

In this study, c-SRC will be referred to as the wild-type endogenous protein, while SRC represents c-SRC-like activity induced by c-SRC mutants. OS is the resultant of the fusion between the indicated SRC mutant-CRY2-mCherry (from N to C-ter). All the expression plasmids are listed in supplemental STAR Methods table. All the OptoSrc and mutants plasmid construction were cloned in a Nhe1-Not1 digested pSico backbone amplified by PHUSION high fidelity DNA polymerase (NEB) using Gibson assembly (NEB) following the supplier instruction.

Lentivirus production, cell infection and sorting

Lentiviruses were produced by co-transfecting pC57GPBEB GagPol MLV, pSUSVSVG and each plasmid of interest using lipofectamine2000 (Invitrogen) in HEK293 FT (precious gift of Dr Nègre from the ANIRA platform) cells plated in 6 well plates at 50% confluency. Media was changed 24hour later. The viral supernatant was collected 72 hours later and filtered with 0.45µm filters. MDCK cells were plated in 6well plates so it achieved 60% confluency the day of infection. The filtered supernatant was directly used to infect cells of interest. The media was changed 24 hours after infection. After 10 days decontamination, cells were FACS sorted (Aria cell sorter 2000, BD) based on the level of expression of mCherry-tagged OS using 561nm LASER.

OPTOGENETICS EXPERIMENTS BASED ON LIVE IMAGING, FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP) AND LARGE CELL POPULATION ACTIVATION.

Live imaging, photostimulation and FRAP were performed with an iMIC inverted microscope (FEI) using time lapse transmission, confocal (spinning disk) and TIRF imaging (63x/1.46 oil Korr M27; camera EMCCD, image acquisition with the LA software). Optogenetic activation of cells was classically achieved by cyclically stimulated them with one 488nm-TIRF excitation (50ms) every 30 seconds over few minutes (33mHz). OS basal membrane and adhesion sites recruitments were followed using 561nm TIRF imaging, while vinculin-iRFP or LifeAct-iRFP were monitored with 640nm TIRF imaging. FRAP analysis was performed on MDCK cell line expressing stably OS +/- CIBN-GFP-caax and OS ΔUD using the iMIC inverted microscope (FEI) and the same 488 nm TIRF protocol (33mHz) to photostimulate OS during 4min before FRAP experiment. Laser photo-bleaching

of activated OS in adhesive sites were performed with a 561nm laser (100%) for 150ms (with a bleach area of 10x10 microns). Recovery of activated OS in adhesive site was recorded every 400ms during 1 minute. FRAP analysis were performed on OA offline analysis software using the offline FRAP tool/option (FEI). After normalization on the total cell intensity and on the camera background, a single exponential model was applied A*(1-exp[-t*tau_frap]) in order to measure both characteristic time of recovery and the immobile fraction.
Besides single cell analysis, large cell population photostimulation was perform using a home-made blue LED plate (constructed by C. Tucker, I. Wang and M. Balland, Lihpy, Grenoble-Alpes University). The LED illumination were programmed by Arduino software at 2 minutes frequency at 50% LED power for 5 or 20 minutes.

PY-IMMUNOPRECIPITATION, PROTEOME IDENTIFICATION

To characterize the protein-protein interaction networks supporting OS-sensitive signaling pathways, we purposely used PY co-immunoprecipitation rather than phosphopeptide purification to identify both OS substrates and their regulators. Large cell populations were photostimulation using a home-made blue LED plate before lysis and PY-associated proteins purifications. 6 millions of MDCK cells stably expressing OS in different conditions (OS +/- CIBN-GFP-caax, and OS+/- UD) were plated and let adhere for 24 hours. Cell were starved for 12 hours in DMEM media before photostimulation. For some control conditions, inhibition of any SFKs activity was achieved by treating MDCK cells with10µM PP2 (Calbiochem, 529576) 30 minutes before photostimulation. Cells were harvested in modified RIPA lysis buffer [150mM NaCl,20mM Tris-HCl (pH7.4), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1X cOmplete EDTA-free protease inhibitor cocktail (SIGMA#11836170001), and an antiphosphatases mix: 1mM orthovanadate, 10mM Sodium fluoride, 10mM glycerophosphate]. 1,5mg of proteins in lysates were pre-cleared with G sepharose beads Fast flow (SIGMA#P3296) then PY were immunoprecipitated using 5µg of antibodies (PY1000 -Cell signaling 8954) and 4G10-Millipore 05-321) or IgG2B for the IgG control (BD557351)] at 4°C for 4 hours in rotation wheel. 50 µL of G protein sepharose were then added for 1 hour before washed 3 times. The pellet was centrifuged at 3000rpm for 2 minutes at 4°C before resuspension in 50µL of 3X Laemmli buffer.

Mass spectrometry-based proteomic analyses

Three replicates per condition were analyzed. Immunoprecipitated eluates were solubilized in Laemmli buffer, separated by SDS-PAGE (4–12% NuPAGE gel, Invitrogen) and stained by R-250 Coomassie Blue (BioRad). After elimination of bands corresponding to heavy and light chains of IgG, the whole content was in-gel digested using trypsin (Promega) as previously described (Casabona et al., 2013). Resulting peptides were analysed by nanoliquid chromatography coupled to tandem mass spectrometry (Ultimate 3000 coupled to LTQ-Orbitrap Velos Pro, Thermo Scientific) using a 120-min gradient. RAW files were processed using MaxQuant version 1.5.8.3. Spectra were searched against the Uniprot database (*Canis lupus familiaris* taxonomy, April 2017 version), the sequence of OptoSrc and the frequently observed contaminants database embedded in MaxQuant. Trypsin was chosen as the enzyme and 2 missed cleavages were allowed. Precursor mass error tolerances were set respectively at 20 ppm and 4.5 ppm for first and main searches. Fragment mass error tolerance was set to 0.5 Da. Peptide modifications allowed during the search were: carbamidomethylation (C, fixed), acetyl (Protein N-ter, variable) and oxidation (M, variable). Minimum peptide length was set to 7 amino acids. Minimum number of peptides, razor + unique peptides and unique peptides were all set to 1. Maximum false discovery rates - calculated by employing a reverse database strategy - were set to 0.01 at peptide and protein levels. Protein intensities were calculated from extracted MS intensities of unique+razor peptides and used for statistical analyses with ProStaR (Wieczorek et al., 2017). Proteins identified in the reverse and contaminant databases and only identified by site were discarded. After log2 transformation, intensity values were condition-wise median normalized, missing data imputation was realized (replacing missing values by the 1-percentile value of each column) and

statistical testing was conducted using limma t-test. Proteins were considered as differentially expressed between immunoprecipitations performed using Ctrl IgG and PY(phospho-tyrosine) IgG if they exhibit a log2 (fold change) superior to 1 and a p-value inferior to the cut-off fixed to reach a FDR less than 1% using the Benjamini-Hochberg method. The list of proteins to be considered for the time-course analyses was built by aggregating the proteins found to be differentially expressed between immunoprecipitations performed using Ctrl IgG and PY IgG at 0min, 5min and 20min. Raw intensities were median normalized and missing value imputation was performed condition by condition using the 1-percentile value of each column if no value was present or k-NN if at least one value was present. Statistical testing was then conducted using limma t-test.
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD008534.

Post-analysis of protein lists generated by Mass Spectrometry and TIME-RESOLVED ANALYSIS PIPE-LINE

Analysis of temporal enrichment variations of proteins in datasets. Missing protein identifiers from *Canis lupus familiaris* were manually mapped to *Homo sapiens* after blast analysis (uniprot.org/blast/), applying > 85% threshold of homology for successful identification. R environment (R Development core team, 2008) was used together with Fuzzy c-mean analysis to define clusters of temporal variation on the mean intensity of proteins that have a significant p-value between 5 or 20 and 0 minutes (Kumar and Futschik, 2007), separately, and for each dataset (OS + GFP-Caax or OS + CIBN-GFP-Caax; OS or OSΔUD). We iteratively explored combinations of cluster sizes and fuzzification parameters to find optimal partitioning parameters to compare the OS+GFP-Caax and OS+CIBN-GFP-Caax data sets (c = 6, m = 3)
Average trends were plotted to define cluster correspondence, with default behaviour set on OS + GFP-Caax for OS + GFP-Caax vs OS + CIBN-GFP-Caax comparison, or to OS for OS vs OSΔUD comparison. Significance of variation between datasets was further supported by *limma* t-test at 5 and 20 minutes between OS + GFP-Caax/OS + CIBN-GFP-Caax or OS/OSΔUD. When soft clustering and *limma* t-test were in agreement, we concluded for a cluster change or a lack of change in temporal trend according to fuzzy c-mean results. On the contrary, in case of disagreement between soft clustering and *limma* t-test, we analysed the temporal trends one-by-one with an homemade R script to conclude. If a difference was confirmed by *limma* t-test but did not present any cluster change, we concluded that these proteins present the same behaviour but was associated with a significative change of the amplitude of this behaviour (indicated as “increase of amplitude” Fig. 5E and 6E). In addition, out of 24 proteins in this case in OS + GFP-Caax vs OS + CIBN-GFP-Caax analysis, we eventually validated a temporal trend change for CCT3, EEF1A1, MED13, PPP1CC and PPP2R3A. Likewise, out of 124 proteins in OS vs OSΔUD analysis, we validated a temporal trend change for MTA1, NACA, NOP56, SAR1B, SLC25A3, SRPK1, SUCLA2 and ZWILCH. In both analyses, the majority of remaining proteins from this population was considered as temporally unaltered by OS modulation. To assess and compensate for potential proteome variations that might arouse during cell strain establishment, we systematically compared temporal trends of proteins differentially enriched at 0 minute. All proteins enriched at 0 minutes were removed from further analysis if the temporal trends in compared datasets were identical to each other’s.
As such, CDCP1 and TACC1 were removed from OS + GFP-Caax vs OS + CIBN-GFP-Caax network analysis, while PTPN23, ARHGEF5, PTPN6, SEC16A, EIF3E, CLASP2, HSD17B4, COPS7A, TMRT6, N4BP2, CPSF7, ANKRD17, OSBPL3 and ARAP3 were removed from OS vs OSΔUD network analysis. SRC interaction likelihood, Protein-Protein Interaction Network reconstruction and Gene Ontology enrichment were performed on the resulting t0 minutes subtracted proteins lists.

Assessment of SRC substrates likelihood. To determine the biological relevance of identified proteins, we looked for interaction with SRC in BIOGRID database (Chatr-Aryamontri et al., 2017; database gathered on 28112017 via psiquic services). In addition, we crossed our results with a list of human

proteins annotated with phospho-tyrosin reviewed proteins in uniprot database. We also assessed the potentiality of targeting candidates according to GPS tool (Xue et al., 2011). In this case, FASTA sequences of proteins were retrieved from uniprot database, and consensus sequence for SRC was searched by conservatively setting results threshold to high filter (and score >1,5 reported as "all" in supplemental table1 and 2). To further increase the stringency of the prediction, we scored proteins that show either one or two sites with score in the top first decile (High filter and score >10). Finally, a list of proteins sensitive to SRC inhibitor PP2 was determined by limma t-test at 20 minutes after OS activation.

Gene Ontology enrichment. Lists of identified proteins were analysed through enrichment of the 3 classes of ontologies: Biological Processes, Molecular Function and Cellular Components or PANTHER Overrepresentation Test (BP, MF and CC; amigo2.geneontology.org/amigo, released on 20170413), GO ontology database Released of 20171128, with Bonferoni correction applied.

Protein-Protein Interaction Network reconstruction and analysis. We reconstructed the protein-protein interaction network by gathering BIOGRID, INTACT and MINT protein-protein interaction data from *Homo sapiens* with psiquic retrieval (07122017) and cytoscape environment (Shannon et al., 2003). Custom made approach was used to combine data from these bases, and edges information was implemented accordingly. We applied the discrimination procedure described above in the *analysis of temporal enrichment*, for proteins enriched at 0 minutes. In the case of OS + GFP-Caax vs OS + CIBN-GFP-Caax comparison, we defined the simplest model network representing all proteins that have either fuzzy c-mean cluster change and, or, a temporal interdataset p-value. As mass spectrometry analysis identified more proteins in the OS vs OSΔUD comparison, we chose to describe only proteins with both a cluster change and a temporal interdataset pvalue. We used the cluster identifiers with respect to temporal trend changes in layout to underline the differences described among datasets. When applicable, proteins without cluster change or interdataset temporal p-value were graphically minored. Upon request, generated network maps can be updloaded for public access on CyNetShare (cynetshare.ucsd.edu).

QUANTIFICATION AND STATISTIC ANALYSIS

Data are judged to be significant when $p < 0.05$ by unpaired, two-tailed Mann-Whitney test. We denote statistical significance as follows: ns, not significant (i.e., $p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Graphs and statistical analyses were generated using Prism 6 (Graphpad) and the boxplots indicate the data distribution of the second and third quartile (box), median (line), mean (filled squares), and 1.53 interquartile range (whiskers).

Brightness analysis of OS oligomeric state

Fluorescence Correlation Spectroscopy (FCS) acquisition data was performed with the LSM710-Confocor3 confocal microscope (Carl Zeiss). The inverted AxioObserver stand was equipped with the C-Apochromat 40x/1.2 water-immersion objective, the on-stage cell incubator (PeCon) and the overall environmental chamber stabilized at 37°C at least 1h before acquisitions. Cells were transfected and plated on the 4-well chambered coverslips (LabTek) 24h prior to the experiments at 37° in 5% CO_2 throughout acquisition. The mCherry fluorescence was excited with the 561 nm DPSS laser, reflected by the 488/561 double primary dichroic filter. The laser light power of 2.6 µW was measured at the objective output at 0.3% AOTF transmission. The fluorescence was selected by the 580 nm long-pass secondary dichroic filter and its fluctuations were sampled by the APD detector at 20 MHz rate. The laser power, the pinhole alignment, and the detected molecular brightness and mobility were carefully controlled on the day-to-day basis using the calibration solution of 15 nM sulforhodamine B (Sigma-Aldrich) in water. The shape parameter (axial-to-lateral size ratio) of the confocal volume of 6.9 was determined by fitting the calibration ACF curve to the single-component free diffusion model, and was fixed to this value in the following analyses. In cases of photostimulation of the optogenetic tag CRY2, the 488 nm line of the Ar laser (1% AOTF transmission) was activated simultaneously with 561 nm

FCS laser during acquisition. Due to the high mobility of the cytoplasmic pool of proteins, a stationary regimen of photoactivation was then established and maintained during FCS measurements after a short transition period of ~30 s. Single FCS acquisitions were limited to 10 seconds and repeated 10 times in a cytosolic region of the cell. The presence of the second 488 nm laser increased the apparent brightness of mCherry by *ca.* 8% due to additional excitation in the absorption tail of the fluorophore spectrum. Moreover, the AOTF acousto-optic cross-talk was found to slightly modify the transmitted intensity of 561 nm laser in the presence of 488 wavelengths. The obtained brightness values were accordingly corrected for the proper comparison between no-stimulation and stimulation conditions. The molecular brightness estimated from the FCS curve is also biased by the fluorophore photobleaching and the cytosolic depletion during 488 nm photoactivation. In order to minimize these biases and to be less dependent on the fit quality of the FCS model, we estimated the molecular brightness from the raw photon counts over short time intervals according to the formula:

$$B = \left[\frac{\sigma^2}{< k > -1}\right]\frac{1}{\Delta t}$$

where <k> is the mean number of counts per bin time Δt and σ^2 is the variance of photon counts. The bin time was chosen to be 150 µs since it is long enough in order to average out the photoblinking effects and to obtain higher value of counts per molecule (CPM), but is still considerably shorter than the average diffusion time of the studied molecules, in order to validate the "pseudo static" approximation. The 1 s integration intervals produce sufficient photon statistics for a good signal-to-noise ratio, and are short enough in order to reduce the effects of photobleaching, cytosolic depletion and cell movements. The binary raw data were saved during the standard FCS acquisitions and were then rebinned and analyzed by a custom written ImageJ macro. The molecular brightness within each cell is averaged from the 60 points (the firsts 40 points are excluded due to the equilibrated state of the beginning of the photostimulation). Each average brightness is then averaged on the +/- 15 cells studied in each condition.

MATHEMICAL MODELING OF DIFFERENT OS FLUXES

In order to specify the flux of OptoSrc (OS) recruitment following light activation, we consider the passive transport mechanisms of activated OS (OS dimers) in the cell volume either with adsorption on the cell membrane or on discrete adhesive sites (Fig.4G). From the mathematical point of view, the model is valid in 2 or 3 spatial dimensions, but it is more tractable in 2D from the numerical point of view. The principal result reported from this modeling is illustrated in Fig. 4H, where it is demonstrated that the OS flux is sustained at a higher level and on a longer time when activated OS is directly recruited to adhesive sites without intermediate membrane adsorption. Explicit solutions for this problem are given to characterize the different time scales.

Call Ω the volume of a spherical cell of radius R_0 in arbitrary dimensions with boundary $\partial\Omega$. $\partial\Omega$ is either a circle in 2D or a sphere in 3D with the same radius. To compute the flux j of activated OS per unit length or surface, consider an initial concentration $u_0(\mathbf{r})$ of light activated OS in the bulk where $\mathbf{r}$ is the radial vector distance to the origin. The model takes into account the diffusion of OS in the bulk with diffusion coefficient D and introduces an effective adsorption rate $k_a^*(\mathbf{r})$ on the boundary $\partial\Omega$. This rate of adsorption is proportional to the density $n_m(\mathbf{r})$ of adsorption sites on $\partial\Omega$. In presence of CIBN-GFP-Caax, $n_m(\mathbf{r})$ is homogeneously distributed on the membrane and k_a^* is constant. In contrast, $n_m(\mathbf{r})$ is exclusively concentrated on the adhesive sites in the GFP-Caax case, see Fig. 1 and 4H, and $k_a^*(\mathbf{r})$ varies on the boundary $\partial\Omega$

$$k_a^*(r) = k_a n_m(r) \quad r \in \partial\Omega$$

(1)

where k_a is the rate per unit surface at full coverage. To compare case 1 where activated OS is recruited to the membrane to case 2, where OS is only recruited to clusters, we assume that $n_m(\theta)$ has a square wave distribution, with $n_m(\theta)$ alternating between 0 and n_m along the boundary. Following illumination, the two geometries give different flux densities. For the same buffer of cytosolic activated proteins, the flux through adhesive clusters in case 2 must be larger than in case 1, since the same amount of material must be adsorbed at infinite time, but on a smaller spatial domain. Using this sink analogy, we also see full adsorption takes a longer time on clusters than on a homogenous boundary.

To summarize, the concentration $u(r,t)$ solves the following problem:

$$\begin{aligned} &\frac{1}{D}\frac{\partial u}{\partial t} = \Delta u \quad r \in \Omega \\ &u(r,t=0) = u_0(r) \quad r \in \Omega \\ &-D\hat{u}_r \cdot \nabla u(r,t) = k_a^*(r)u(r,t) \quad r \in \partial\Omega \end{aligned} \tag{2}$$

where the first two equations are the diffusion equation together with the initial condition at zero time. The last equation equals the flux φ of adsorption normal to the boundary with the probability that the protein is absorbed on a site assuming that they are in contact (Robin boundary condition). We are interested in computing the time dependent solution of **Erreur ! Source du renvoi introuvable.** from which the total amount of OS can be calculated.

For what follows, it is useful first to consider the dimensionless ratio to compare the diffusion-limited rate of Schmoluchowski with the rate of OS association with the membrane assuming close contact.

$$\frac{k_a^* R_0}{D} \tag{3}$$

When this ratio is smaller than 1, we are in the fast diffusion regime where, following illumination, the concentration in the bulk becomes first homogenous and OS proteins subsequently adsorbed. This is the relevant experimental regime which ensures that our results will not depend on the geometry. Following initial bulk homogenization, the buffer of cytosolic proteins is cleared on a much longer time scale. Calculations below show that this occurs on a characteristic time scale t_1 we compare with the diffusion time scale t_0

$$t_1 = \frac{R_0}{2k_a^*} \gg t_0 = \frac{R_0^2}{4D} \tag{4}$$

so that the flux j exhibits a sharp increase at short times, $t < t_0$, and decreases more slowly at longer times, $t \simeq t_1$: See Fig. 2. For a typical cytosolic protein with $R_0 = 10\,nm$ and $D = 10\,nm^2.s^{-1}$, $t_0 = 2.5s$. Since typical association rates of proteins are of the order of a tenth of $nM.s^{-1}$, t_1 is of the order of a few ten of seconds. In problem **Erreur ! Source du renvoi introuvable.**, we have neglected desorption. This approximation is valid if the characteristic time for this process is longer than all other characteristic time and it has the advantage to decouple the adsorption problem from the diffusion problem of adsorbed proteins along the boundary. If diffusion along the boundary occurs, desorption will take place homogeneously along the boundary with small modifications of the bulk diffusion flux contour lines, so that problem is a good approximation at short time.

In the homogenous case 1 (indirect relocalization of activated OS in discrete adhesive sites through a membrane step as in the conditiokn of expression of CIBN-GFP-Caax), our problem is solved in 2D by separation of variables $u(r,t) = \exp[-D\lambda_m^2 t]u(r)$. Sturm-Liouville theory gives the solution as a series

$$u(r,t) = \sum_{m=1} c_m J_0(\beta_m r) e^{-D\beta_m^2 t} \tag{5}$$

The boundary condition in **Erreur ! Source du renvoi introuvable.** gives the set $\{\beta\}_{m=1}$ is the ordered set of positive roots of the equation

$$\beta_m R_0 J_1(\beta_m R_0) = \frac{k_a^* R_0}{D} J_0(\beta_m R_0) \tag{6}$$

where $J_{0,1}(r)$ are standard Bessel functions. The coefficients c_m are obtained by operating on the initial condition

$$c_m = \frac{1}{N(\beta_m)^2} \int_0^{R_0} dr\, r J_0(\beta_m r) u_0(r) \tag{7}$$

with

$$N(\beta_m)^2 = \int_0^{R_0} dr\, r J_0(\beta_m r)^2 \tag{8}$$

The limit of small $k_a^* R_0/D$ is interesting. In this limit,

$$\beta_1 R_0 \simeq \left[\frac{2k_a^* R_0}{D}\right]^{1/2} \ll 1 \qquad (9)$$

Thus, according to **Erreur ! Source du renvoi introuvable.**, the characteristic time scale for the exponential decrease in flux density is

$$D\beta_1^{\,2} \simeq 2k_a^* / R_0 \qquad (10)$$

We conclude that the first term in the series dominates and that the flux of proteins adsorbed on the membrane will decrease with a characteristic time $t_1 = R_0/2k_a^*$ after a sudden increase on time scale t_0. The same result holds for the 3D solution with the same symmetry of revolution. Eq. **Erreur ! Source du renvoi introuvable.** should be adapted using the appropriate basis. Because of dimensional analysis, the same characteristic time scales enters into the problem.

In the non-homogenous case 2 (direct relocalization of activated OS in discrete adhesive sites as in the condition of expression of GFP-Caax), we solve problem **Erreur ! Source du renvoi introuvable.** using finite element methods for a square wave distribution of clusters. For convenience, we take the density of adsorption sites in the square as equal to the density of adsorption sites for the homogenous membrane.

Fig.4H compares the solution for the homogenous problem with the one for the cluster geometry with averaged quantities per unit length. Since the clusters occupy only 50% of the total perimeter, the area under the curves are the same when conveniently renormalized, so that the total amount of proteins absorbed at infinite time is the same at time infinity. The two curves exhibit the same sharp increase at short time t_0, which only depends on diffusion in the bulk but not on cluster geometry. Again, the mean adsorbed flux is higher in the non-homogenous geometry than in the homogenous case and decreases slowly since all material have to be only adsorbed on the discrete adhesive sites and not between. Depending on the cluster geometry, this happens on different time scales as it can be seen from Eq. **Erreur ! Source du renvoi introuvable.** by renormalizing the adsorption rate k_a^* by the fraction of area occupied by the adsorption sites.

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Acknowledgements
We would acknowledge the benevolence and talent Dr S. Wickström for critical reading of this manuscript. We would also thank Dr. C. Gaggioli, Prof. R. Baron, Dr. M. Coppey, Dr E. Miroshnikova, Prof. A. Delon, Dr. I. Wang, prof. M. Digman and prof. E. Graton for experimental help, critical reading and exciting discussions. This work was funded by JCJC ANR-13-JSV2-0003-01 program, by LLNC as "Equipe labellisée Ligue 2014" (EL2014.LNCC/CAR) and by FRM as Equipe labellisée FRM 2017 (FRM grant, DEQ20170336702). The PhD of A.K. was funded by "Ligue Nationale contre le Cancer" (LLNC, GB/MA/VSP 11211) and "la Fondation pour la Recherche Médicale" (FRM, FDT20170437267). We thank the support of the discovery platform and informatics group at EDyP. Proteomic experiments were partly supported by the Proteomics French Infrastructure (ANR-10-INBS-08-01 grant) and Labex GRAL (ANR-10-LABX-49-01).

Most of the data related to this work were posted the **25th of May 2019** in BioRxiv (**doi**: https://doi.org/10.1101/648030).

Authors Contributions

A.K., C.O. and O.D. generated constructs and cell lines, performed and analyzed data in cell biology. A.K. and A.G. developed brightness analysis. C.B., A.Kr. and Y.C analyzed mass-spec data and developed pipe-line analysis. B.F. developed the kinetic model and performed computational simulations and analysis.
A.K. and O.D designed the study, and O.D., E.F., C.A-R., A.K., B.F. and C.B. wrote the paper with significant contributions from all authors.