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"Nuclear FGF Receptor-1 and CREB Binding Protein: An Integrative Signaling Module"

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In this review we summarize the current understanding of a novel integrative function of Fibroblast Growth Factor Receptor-1 (FGFR1) and its partner CREB Binding Protein (CBP) acting as a nuclear regulatory complex. Nuclear FGFR1 and CBP interact with and regulate numerous genes on various chromosomes. FGFR1 dynamic oscillatory interactions with chromatin and with specific genes, underwrites gene regulation mediated by diverse developmental signals. Integrative Nuclear FGFR1 Signaling (INFS) effects the differentiation of stem cells and neural progenitor cells via the gene-controlling Feed-Forward-And-Gate mechanism. Nuclear accumulation of FGFR1 occurs in numerous cell types and disruption of INFS may play an important role in developmental disorders such as schizophrenia, and in metastatic diseases such as cancer. Enhancement of INFS may be used to coordinate the gene regulation needed to activate cell differentiation for regenerative purposes or to provide interruption of cancer stem cell proliferation.

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The founding member of the fibroblast growth factor (FGF) family (Fig. 1A) is basic FGF (FGF-2), purified from bovine brain (Gospodarowicz et al., 1982, 1987). The initial isolated basic peptide represents a truncated protein that is derived from the isolation of a larger 18-kDa protein, discovered later. Early studies tested the actions of the basic FGF peptides as external proteins in fibroblast cell cultures, hence the factors' name. The 18 kDa FGF-2 peptide has become a standard biochemical reagent used in a variety of cell cultures as an effective mitotic reagent. The cloning of FGF-2 cDNA revealed that nontruncated human and rat basic fibroblast growth factor are represented by several polypeptides translated from a single mRNA that lacks a cleavable signal peptide (SP) (Florkiewicz and Sommer, 1989; Powell and Klagsbrun, 1991).

Translation from an AUG codon yields 18 kDa FGF-2, while translation from upstream CUG codons yields additional higher molecular weight isoforms (HMW; 21–24 kDa) (Florkiewicz et al., 1991; Powell and Klagsbrun, 1991; Stachowiak et al., 1994; Joy et al., 1997; Stachowiak et al., 2003b). All HMW isoforms of FGF-2 contain a classical nuclear localization signal (NLS) within extended N-terminal sequences enabling nuclear import (Florkiewicz et al., 1991) (Fig. 1A). The 18 kDa FGF-2 also contains a non-classical C-terminal NLS (Sheng et al., 2004). While some tissues and cells display small amounts of 18 kDa FGF-2 at the cell surface and in the extracellular matrix (Vlodavsky et al., 1991; Schechter, 1992), the nuclear HMW forms are absent from the extracellular environment of the producing cells. These molecules are, thus, intracellular-nuclear signaling factors (Gu and Kay, 1998). In the brain and in many cultured cells both high and low molecular weight FGF-2 are found almost exclusively in the nuclear fraction and thus act as nuclear proteins (Stachowiak et al., 2003b) (Fig. 2A).

There are over twenty members of the mammalian FGF family that share similar sequences and three-dimensional structures. FGFs are not found in single celled organisms, but are common to the diverse multicellular animals (i.e., *C. elegans*, Drosophila, zebra fish, frog, chicken, mouse, and human) suggesting that the generation of tissues and organs with specialized cells requires FGFs (Ornitz and Itoh, 2001; Stachowiak et al., 2011b). Of particular interest during the evolution of the FGF family is the retention or loss of a positively charged nuclear localization signal (NLS), or an acquisition of a cleavable hydrophobic signal peptide (SP) (Fig. 1B). In *C. elegans,* one of two FGF orthologs (LET-756) contains a NLS and acts in the nucleus targeting presumed transcription sites (Popovici et al., 2006). In the mammalian lineage (Fig. 1B), the common primordial FGF gene, FGF-13-like, is thought to contain a NLS and is the source of the various forms of FGFs including NLS containing FGF-13, 14 and 16, and FGF-4, from which nuclear (NLS-containing) FGF-1, 2 have evolved (Ornitz and Itoh, 2001; Stachowiak et al., 2011b). These NLS-containing factors lack the secretion SP sequence, and thus, appear to have an intracellular, nuclear signaling function. FGF-3 retains NLS but also has a SP and thus appears to have evolved to act inside the cell nucleus as well as an intercellular signaling molecule. FGF-5 and FGF-8–23, with SPs and those without a cleavable hydrophobic signal sequence, may also be released from injured cells or via a

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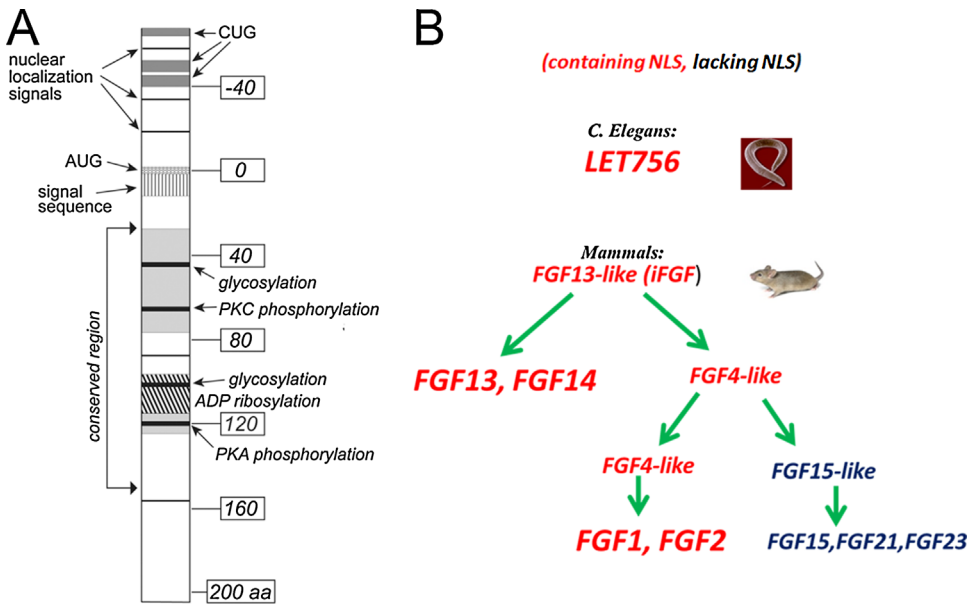

Fig. 1. Structure and evolution of FGFs. (A) General structure of FGF indicating the presence of a signal peptide (SP) and nuclear localization signal (NLS)-like sequences (from Szebeneyi and Fallon [1999]). (B) Evolutionary retention of NLS in FGFs.

phosphorylation-dependent mechanism (Ebert et al., 2010) and interact with plasma membrane FGF receptors (Ornitz and Itoh, 2001).

The nuclear accumulation of FGF-2 is highly regulated and correlates with the transition of cells between major developmental stages (Bryant and Stow, 2005; Stachowiak et al., 2001b, 2003b, 2007). Nuclear translocation of FGF-2 is associated with (i) the growth and differentiation of stem cells; (ii) the growth of glial cells; (iii) the growth, differentiation, and functional activation of neurons; and (iv) the activation of neuro-endocrine adrenal medullary cells. Nuclear accumulation of FGF-2 is induced by various extracellular factors and major intracellular signaling pathways not related to the FGFs (Stachowiak et al., 1994; Joy et al., 1997; Stachowiak et al., 1997b; Peng et al., 2001, 2002) (see also [Grothe et al., 1991; Matsuda et al., 1992; Woodward et al., 1992; Dono and Zeller, 1994; Matsuda et al., 1994; Klimaschewski et al., 1999; Clarke et al., 2001]). The intracellularly expressed nuclear HMW FGF-2 effectively stimulates cellular growth (Arese et al., 1999), differentiation of Schwann cells and neurons and associated gene activities without surface FGF receptor activation (Sherman et al., 1993; Peng et al., 2001, 2002; Stachowiak et al., 2009). Also, FGF-2 stimulates rRNA synthesis (Bouche et al., 1987) and affects gene transcription in vitro (Nakanishi et al., 1992). Thus, natural FGF-2 appears to be a cytoplasmic- nuclear signaling factor rather than an extracellular protein, as initially proposed by the experiments of Gospodarowicz. We have proposed the concept of an "intracrine" growth factor signaling molecule that translocates from the cytosol to the nucleus (without externalization) to induce the cellular responses listed above (Stachowiak et al., 1997a, 2011b).

Nuclear FGF Receptor-1 (FGFR1)

FGFs interact with high (picomolar) affinity tyrosine kinase receptors that mediate FGF cellular responses and low (nanomolar) affinity receptors that are associated with heparan sulfate proteoglycans (HSPGs) (Szebenyi and Fallon, 1999). The high affinity FGFR1–4, encoded by four different genes, share a similar structure consisting of an N-terminal signal peptide (SP) that directs FGFR synthesis to the endoplasmic reticulum (ER), two or three immunoglobulin-like domains that contain the ligand binding site, a single transmembrane domain (TMD), a split tyrosine kinase (TK) domain, and a C-terminal domain (see Fig. 2A).

High affinity FGF-2 binding sites formed by FGFR1 have been found in the nuclei of different cell types (Stachowiak et al., 1996a,b). The nuclear presence of FGFR1 was first reported by the Baird laboratory in 1984, and subsequently demonstrated with an array of different FGFR1 antibodies in different cell types, including non-transformed cells (Maher, 1996; Reilly and Maher, 2001), cancer cell lines (Stachowiak et al., 1996a,b; Peng et al., 2001; Stachowiak et al., 2003b; Bryant and Stow, 2005), cells found in rat and mouse brains (Gonzalez et al., 1995; Clarke et al., 2001; Stachowiak et al., 2003b; Fang et al., 2005; Leadbeater et al., 2006) (Fig. 2A, C), cochleo-vestibular ganglion cells (Bilak et al., 2003) (summarized in [Stachowiak et al., 2011b]), and in human cancer tumors (Chioni and Grose, 2012; Nguyen et al., 2013; Coleman et al., 2014). Importantly, FGFR1 nuclear accumulation has been observed in vivo in both developing neurons and glial cells (Gonzalez et al., 1995; Klimaschewski et al., 1999; Clarke et al., 2001; Horbinski et al., 2002; Fang et al., 2005; Leadbeater et al., 2006).

Nuclear FGFR1 is a full–length, nontruncated protein that can undergo autophosphorylation and phosphorylate other proteins in a ligand stimulated manner (Stachowiak et al., 1996a,b).

The presence of FGFR1 along with FGF-2 in the nuclei of the developing brain is illustrated in Figure 2A. Cultured human neural progenitor cells (HNPC) that are stimulated to differentiate by cAMP (Fig. 2B, C) or an acetylcholine nicotinic receptor agonist (Fig. 2D, E) also show nuclear accumulation of FGFR1. The ligand that interacts with nuclear FGFR1 is HMW (23 kDa) FGF-2. Co-immunoprecipitation and FRET experiments show that 23 kDa FGF-2 ligand interacts with

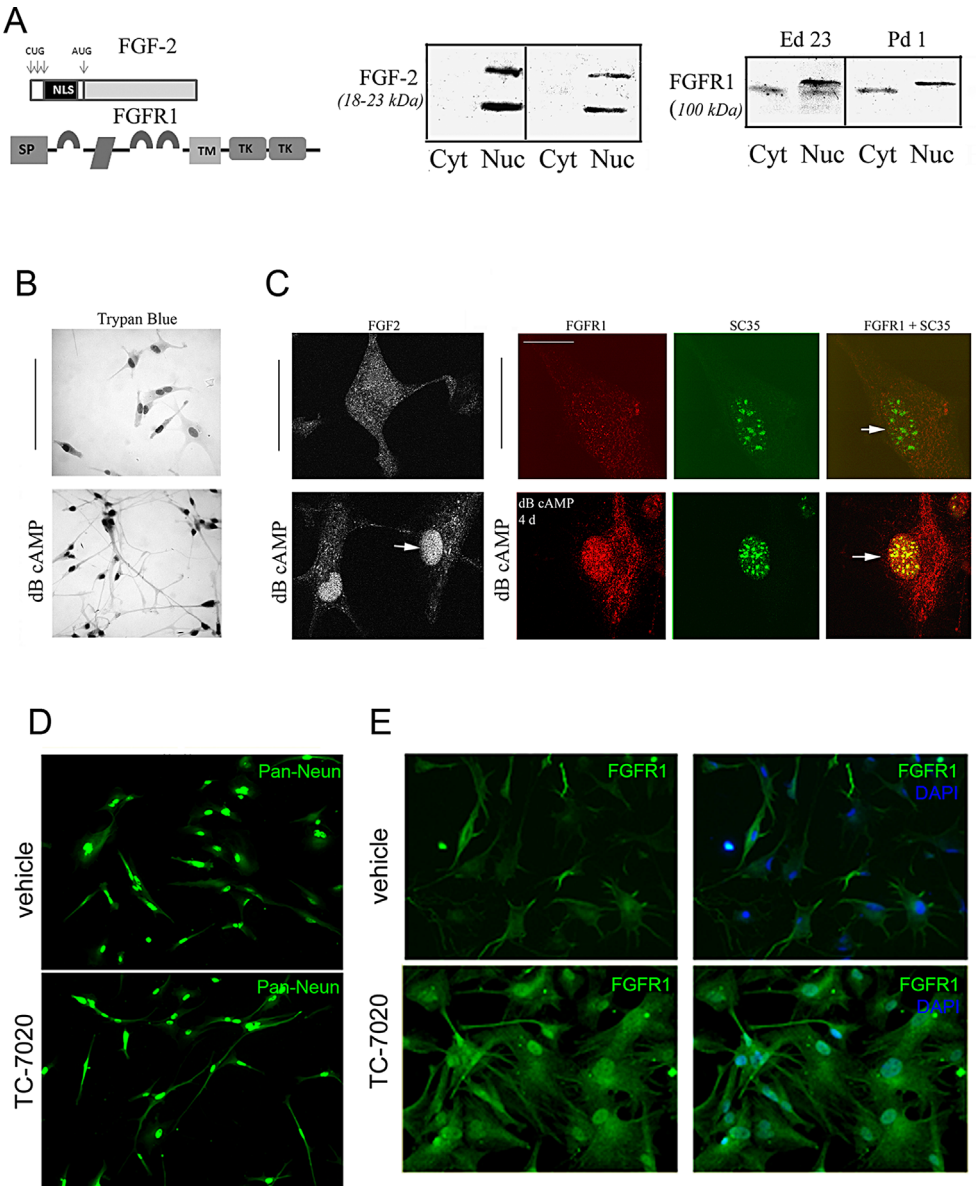

Fig. 2. Structure, subcellular localization and interaction of nuclear FGF-2 and FGFR1. (A) *Left*: schematic structure of FGF-2 and FGFR1 molecules. Low molecular weight (LMW, 18kD) FGF-2 is generated from the typical AUG translational start codon. Higher molecular weight forms (HMW, 21–23 kDa) are generated from additional CUG codons and include a nuclear localization signal (NLS). FGFR1–SP signal peptide, TMD– transmembrane domain, TK – split tyrosine kinase domain. *Middle and right:* Western blot analyses of FGF-2 and FGFR1 in nuclear (Nuc) and cytoplasmic (Cyt) fractions of the whole rat brain at embryonic day 23 and postnatal day 1 (Stachowiak et al., 2003b). (B–C) Non-differentiated proliferating human neural progenitor cells (HNPC) treated with 0.1 mM dB-cAMP to induce neuronal differentiation (Stachowiak et al., 2003b). (B) After 48 h of incubation, cells were fixed and permeabilized with 0.5% Triton X-100 and their morphology revealed at lower magnification by staining with Trypan Blue (Stachowiak et al., 2003b). (C) cAMP-treated cells display nuclear accumulation of FGF-2 and FGFR1 ("B" at 24 h) followed by neuronal differentiation ("C" at 48 h) (Stachowiak et al., 2003b). Cells were fixed with 4% paraformaldehyde and immunostained with FGF-2 Ab or co-immunostained with FGFR1 McAb6 (+ anti-mouse-Alexa 488) and rabbit antibody against splicesome assembly factor SC-35 (+ goat-anti rabbit-CY3). Merged images: FGFR1 and SC35 immunoreactive pixels colocalize (yellow color) within SC35-rich nuclear foci (arrows point to nucleus) (Stachowiak et al., 1996a, 1996b, 2003b; Hu et al., 2004; Peng et al., 2002). Antibody against SC-35 labels nuclear speckle-like domains which correspond to sites of RNA Pol II-mediated transcription and co-transcriptional, pre-mRNA processing (Blencowe et al., 1994). In proliferating HNPC, little or no colocalization between SC-35 speckles and FGFR1 grains was observed. In contrast, colocalization of SC35 and FGFR1 was observed in several larger aggregates of dB-cAMP-treated differentiating cells as shown in this figure. Analyses of consecutive, confocal sections demonstrated FGFR1 sites within the interior of the SC35 speckles (not shown). A–C — were reprinted from J Neurochem 2003; 84:1296–1312, with permission of Wiley-Blackwell Publishing Ltd. (D–E) Neurite outgrowth and nuclear accumulation in HNPC are induced by α7 nicotinic receptor agonist TC-7020. (D) 10x magnification. HNPC were incubated in control medium or with TC-7020 for 48 h. Morphological changes were observed after immunostaining with Pan-Neuronal Marker Ab (Millipore), which labels nuclear NeuN and cytoplasmic cytoskeletal proteins. The average length of neurites extending from cell soma measured on multiple dishes was 79 + 7.8 pixels in control cells, which increased to 136 + 7.8 pixels after TC-70-20 treatment ($P<0.05$). (E) 20x magnification. HNPC were stained with DAPI and immunostained with FGFR1 Ab (Abcam). Images illustrate nuclear accumulation of FGFR1 in TC7020 treated cultures. D–E — were reprinted from Stem Cells –Translational Medicine 2013; 2:776–788, with Journal permission.

FGFR1 in the nucleus and in the cytoplasm whereas, 18 kDa FGF-2 interacts with FGFR1 only in the cytoplasm (Dunham-Ems et al., 2009). Thus, while HMW (23 kDa) FGF-2 serves as a ligand for nuclear FGFR1, LMW (18 kDa) FGF2 does not interact with nuclear FGFR1 (Stachowiak et al., 2011b).

Nuclear Accumulation of Newly Synthesized FGFR1 is Enabled by Unique Transmembrane Domain

In many cells, the binding of extracellular FGF to its surface receptors does not elicit nuclear FGFR1 accumulation; instead, nuclear FGFR1 accumulation is induced by a number of FGF-unrelated factors (reviewed in [Stachowiak et al., 2007, 2011b]). Pulse-chase experiments reveal that both cytosolic and nuclear FGFR1 represent newly synthesized full- length receptor, the notion supported by the fact that much of the nuclear FGFR1 is non- glycosylated. It appears that this receptor enters the cytoplasm/nucleus before Golgi processing and that the ER is one site at which FGFR1 is released into the cytosol (also see below). Also, biotinylation of the cell surface receptors of glioma or TE671 medulloblastoma cells does not lead to the appearance of biotinylated FGF receptor in cell nuclei (Stachowiak et al., 1997b; Peng et al., 2002; Carpenter, 2003), further indicating that nuclear FGFR1 is not derived from the cell surface.

FGFRs are type-1 transmembrane proteins, possessing N-terminal signal peptides, which should predispose these receptors for insertion into cellular membranes. However, biochemical cell fractionation demonstrated that a fraction of cellular receptor is soluble, non membrane- associated protein (Fig. 3B) (Peng et al., 2002; Myers et al., 2003).

Examination of fused FGFR1-EGFP mobility in live cells by Fluorescence Recovery After Photobleaching (FRAP) revealed fast-moving ($t1/2 = 0.2$ sec) cytosolic and nuclear pools of FGFR1 that are not associated with membranes, while the slow-moving population ($t1/2 = 69$ sec) do associate with cellular membranes (Dunham-Ems et al., 2006). The fast moving receptor is generated through FGFR1 transport out of the ER into the cytosol and then into the nucleus.

Important differences among FGFRs reside in the TMD. Typically, the TMD of type-1 transmembrane proteins contains an α-helix of approximately 30 amino acids with more than 11 consecutive hydrophobic amino acid residues. These non-polar amino acid residues are oriented outside the α-helical core and interact with the lipid bilayer, thereby conferring stability to the peptide in the membrane (Creighton, 1984). FGFR4 TMD has a prototypical exclusively α- helical conformation typical to many other type-1 tyrosine kinase receptors (Myers et al., 2003). FGFR2 and FGFR3 TMDs have shorter α-helices as well as β-sheet regions. FGFR1 possesses an atypical TMD consisting of short stretches of hydrophobic amino acids interrupted by hydrophilic, polar amino acids, and a predicted β-sheet conformation (Myers et al., 2003). Such disruption of the hydrophobic region by polar amino acids has been shown in other proteins to make their membrane association less stable allowing proteins to be released from the membrane (Eisenberg et al., 1984).

The information on how FGFR1 protein may be extruded from the ER membrane comes from substitution experiments, in which the atypical (β-sheet containing polar amino acids) TMD of FGFR1 is replaced with the typical (α-helical, hydrophobic) TMD of FGFR4 (Fig. 3A). The wild type FGFR1 associates with cytoplasmic membranes but is also detected in the cytosol and in the nucleus (Fig. 3B). The chimera FGFR1/R4 mutant is primarily associated with membranes and absent from the cytosol and from the nucleus (Myers et al., 2003). In contrast, FGFR1 mutants, with hydrophobic TMD amino acids replaced with increasing numbers of hydrophilic amino acids, show reduced association with membranes and increased cytosolic and nuclear accumulation as TMD hydrophilicity is increased. Thus, the distinct TMD of FGFR1 appears to play a critical role in the weak association with cellular membranes and the FGFR1 accumulation in the cytosol and nucleus (Myers et al., 2003; Stachowiak et al., 2011b).

In a fluorescence loss after photobleaching (FLIP) experiment, repeated photo bleaching of FGFR1-EGFP in a small, defined nuclear region results in a loss of FGFR1-EGFP fluorescence within the entire nucleus as well a delayed loss in the non-vesicular cytoplasm (Fig. 3C) (Lee et al., 2013). These experiments demonstrate that (i) "nuclear" and "cytosolic" FGFR1 are indeed present in distinct subcellular compartments; (ii) nuclear FGFR1 remains in equilibrium with the cytosolic receptor; and (iii) cytoplasmic-nuclear transport limits the rate of nuclear FGFR1 accumulation. Importantly, in nerve growth factor (NGF) stimulated cells, depletion of cytosolic FGFR1-EGFP after nuclear bleaching was accelerated, indicating that the cytosol-nucleus exchange of FGFR1 can be increased (Fig. 3C) (Lee et al., 2013).

These studies have shown that newly synthesized FGFR1 can enter either the 'membrane pathway,' in which FGFR1 is processed through the Golgi to the plasma membrane, and shows limited membrane mobility, or the 'nuclear pathway,' in which non-glycosylated receptor is released from the ER producing highly mobile, cytosolic receptor molecules that accumulate in the cell nucleus (Fig. 3D) (Stachowiak et al., 2007, 2011b). The release from the ER and entry into the nuclear pathway is favored by the interaction of FGFR1 with cytoplasmic pp90 Ribosomal S6 kinase-1 (RSK1), which significantly influences the receptor's mobility and diffusion rate (Dunham-Ems et al., 2006). The interaction occurs via association with the TK domain of FGFR1 (Hu et al., 2004; Fang et al., 2005). The kinase activity of RSK1 is responsible for redirecting FGFR1 synthesis from the Golgi glycosylation pathway into the cytosol, consistent with the known association of RSK1 with ER-attached polyribosomes (Angenstein et al., 1998).

Nuclear FGFR1 import (Reilly and Maher, 2001) and the nuclear role of FGFR1 are importantly supported by the demonstration of a functional dependency on importin β, a component of multiple nuclear import pathways. Since importin β is a soluble protein, which traffics between the cytoplasm and nucleus, the interaction with FGFR1 in this model must occur in the cytoplasm, perhaps after RSK1-associated cytoplasmic release (Fig. 3D). The interaction between FGFR1 and importin β appears to be indirect, as FGFR1 does not exhibit a detectable NLS. The fact that the FGF-2 ligands possess NLS domains makes them potentially attractive linker/chaperone molecules (Stachowiak et al., 2011b).

Regulation of Nuclear FGFR1 Accumulation

Nuclear FGFR1 accumulation is induced by a number of other factors that control cell development including NGF, Angiotensin II, bone morphogenetic proteins, retinoic acid, and by direct activation of cAMP, $Ca++$/Protein Kinase C, and cell depolarization (reviewed in (Stachowiak et al., 2007, 2011b) and is accompanied by an increase in receptor synthesis (Peng et al., 2001, 2002; Carpenter, 2003; Myers et al., 2003; Hu et al., 2004). Hence, this signaling is named Integrative Nuclear FGFR1 Signaling (INFS) (Peng et al., 2002; Stachowiak et al., 2003b). In some cells, extracellular FGFs can stimulate the nuclear accumulation of FGFR1 (Maher, 1996). Biophotonic (FLIP and FRAP) experiments completed in live cells indicate that the nuclear accumulation of FGFR1 is effected by accelerated cytoplasmic to nuclear import, as well as reduced nuclear to cytoplasmic export (Dunham-Ems et al., 2009; Stachowiak et al., 2011b; Lee et al., 2013).

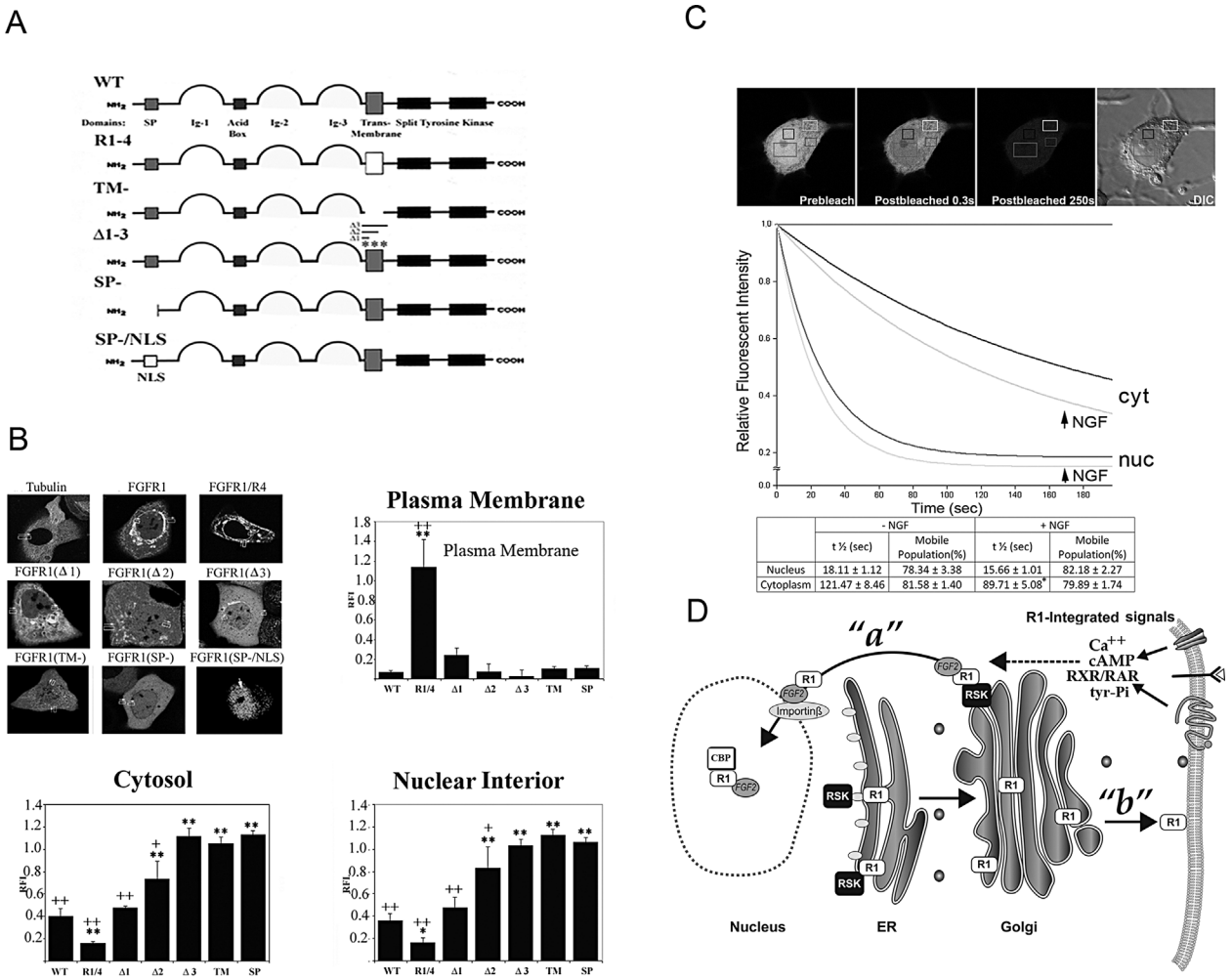

Fig. 3. Cytoplasmic processing and nuclear targeting of FGFR1 (Fang et al., 2005; Stachowiak et al., 2003b). (A) Mutations in transmembrane domain (TMD): TM- deletion of TMD; D1–3 insertion of polar a.a., FGFR1/FGFR4 (R1/R4) replacement of FGFR1 TMD with FGFR4 TMD. Mutations in Signal peptide (SP): SP- deletion; NLS replacement with Nuclear Localization Signal (B) TMD and SP mutations affect subcellular localization of FGFR1–EGFP. Bar graphs show the intensity of FGFR1–EGFP fluorescence associated with the cellular compartments. Cells transfected with control tubulin-EGFP showed fluorescent microtubules, but no EGFP fluorescence was detected in the nucleus. WT FGFR1 associated with cell membranes as well as with the cytosolic and nuclear compartments. Replacement of FGFR1 TM with typical FGFR4 TM prevented cytosolic and nuclear accumulation of chimerical FGFR1/R4 FGFR1. In contrast, insertion of additional polar a.a. (D1–3), TMD or SP deletion facilitated cytosolic and nuclear accumulation (Myers et al., 2003). A–B reprinted from J Cell Biochem 2003; 88:1273–1291. (C) NGF accelerates nuclear trafficking of FGFR1 (from Lee at al., 2013). FGFR1-EGFP was transfected into PC12 cells. Twenty-four hours after transfection, cultures were incubated with or without 50 ng/ml NGF for an additional 48 h and followed by FLIP imaging. Examples of FGFR1-EGFP expressing cells before and after photo-bleaching are shown. DIC image indicates the nuclear and cytoplasmic regions. About 1/3 nuclear area of PC12 cell was bleached by high intensity laser and 2–3 regions of interest (ROI) intensity were measured. Single-exponential analysis of FGFR1-EGFP FLIP regression in cytoplasm and nucleus showed that the diffusion rate of FGFR1-EGFP is affected by NGF treatment in live cells. Individual curves represent means of at least 23 cells. NGF significantly increases the FGFR1-EGFP exchange between nucleus and cytoplasm (half-time decreases) without affecting the FGFR1-EGFP mobile population. Single-exponential analysis of FGFR1-EGFP FLIP regression in the cytoplasm shows that NGF facilitates FGFR1-EGFP trafficking between the cytoplasm and nucleus (half- time decreases from 121.5 sec to 89.7 sec; One-way AVOVA, LSD*$P<0.001$). Figure C was reprinted from PLoS ONE. 2013 8(7):e68931. doi: 10.1371/jo. (D) Model of nuclear trafficking of FGFR1. Cytoplasmic FGFR1 exists in three separate populations: (1) an immobile, newly synthesized ER population (2) highly mobile, non-glycosylated, cytosolic population; and (3) a slowly diffusing, membrane receptor population (Dunham-Ems et al., 2006). FGFR1 can enter either the (a) 'nuclear pathway,' where non-glycosylated receptor is released from the ER into the cytosol and accumulates in cell nuclei or the (b) 'membrane pathway,' in which FGFR1 is processed through the Golgi to the plasma membrane. Integration of diverse signals by the nuclear pathway: activation of diverse classes of surface membrane receptors and their signaling cascades causes association of the adaptor protein RSK1 with the cytoplasmic tail of newly synthesized FGFR1, and subsequent release of FGFR1 into the cytoplasmic pool before trafficking through the Golgi apparatus and to the cell surface. Once in the cytoplasm, FGFR1 indirectly complexes with Importin-β to facilitate nuclear import. In addition, activation of cell surface FGFR1 by FGF-2 induces FGFR1 internalization, which is dependent on the function of the ARF6, Dynamin2 and Rab5 endocytic machinery, and is inhibited by surface E-cadherin adhesion complexes. Once internalized, FGFR1 may potentially be released from endosomes, or trafficked in a retrograde fashion to the ER/Golgi for cytoplasmic release via the RSK1- associated pathway (Stachowiak et al., 2007).

Differentiation of Multipotent Neural Stem Cells and Pluripotent Embryonic Stem Cells by INFS

Distinct roles for cell surface and nuclear FGFR1 were demonstrated in cultured human neuronal progenitor cells (HNPC) isolated from the fetal brain or from umbilical cord blood (Stachowiak et al., 2003a; Hu et al., 2004; Fang et al., 2005; Stachowiak et al., 2011b). In the presence of exogenous FGF-2, HNPC proliferate and display characteristics of undifferentiated cells. Treatment with cAMP, forskolin, or BMP-7 inhibits proliferation and induces neuronal differentiation accompanied by an outgrowth of neurites, and expression of neuron-specific β-III tubulin, MAP2, NF-L, α-internexin, glutamate and acetylcholine receptors. Figure 2B and D show morphology of proliferating non-differentiated and neuronal-differentiated HNPC. In proliferating HNPC treated with exogenous 18 kDa FGF-2 or other mitogens, FGFR1 is associated with the cytoplasm and plasma membrane. In contrast, treatment with cAMP or BMP7 induce an accumulation of FGF-2 and newly synthesized FGFR1 within the nuclear interior (Fig. 2C) (Stachowiak et al., 2003b, 2011b). In support of the role of endogenous nuclear FGFR1 in neuronal differentiation, transfection with tyrosine kinase deleted dominant negative receptor mutants, either cytoplasmic/nuclear FGFR1(TK-) or exclusively nuclear FGFR1 (SP-/NLS)(TK-), block differentiation of HNPC induced by cAMP (Fig. 4A, C). Thus, these studies demonstrate that nuclear accumulation of FGFR1 is necessary for cAMP- or BMP7 induced differentiation of neural stem/progenitor cells. In support of the role of nuclear FGFR1 in neuronal differentiation, FGFR1 transfection in HNPC results in FGFR1 accumulation in the cytoplasm, as well as in the nucleus and activates induction of long process outgrowth (Fig. 4B) (Stachowiak et al., 2003b). Furthermore, HNPC transfected with a mutant FGFR1, FGFR1(SP-/NLS), in which the signal peptide (SP) was replaced with a nuclear localization signal (NLS), so as to direct it specifically to the nucleus (Fig. 4A), also showed the growth of long processes (Fig. 4B). In contrast, the membrane-associated non-nuclear FGFR1/R4 chimera, in which the atypical FGFR1 transmembrane domain (partially hydrophilic/β-sheet) was replaced with the typical (hydrophobic/α-helical) FGFR4 domain, fails to induce differentiation (Fig. 4A, B) (Stachowiak et al., 2003b). The morphological differentiation of HNPC induced by transfected cytoplasmic/nuclear FGFR1 or exclusively nuclear FGFR1(SP-/NLS) is accompanied by the induction of several neuron-specific proteins. Thus, nuclear FGFR1 accumulation is sufficient to induce neuronal differentiation in the absence of other factors (Stachowiak et al., 2003b, 2011b). Similar experiments have shown that the INFS mechanism mediates retinoic acid, as well as NGF-induced cell differentiation (Lee et al., 2013).

Role of INFS/FGFR1 in Neural Development In Vivo (Stachowiak et al., 2011b)

In proliferating neural stem/progenitor cells (NS/PC) of brain subventricular zone SVZ), FGFR1 is associated with the cytoplasm, while in differentiating cells FGFR1 localizes to the nucleus (Fang et al., 2005; Stachowiak et al., 2009). In agreement with this dual distribution, brain-targeted FGFR1 knockout impairs both the cell proliferation and differentiation (Pirvola et al., 2002; Ohkubo et al., 2004) which may reflect the loss of FGFR1 signaling at the cell surface and the INFS, respectively (Stachowiak et al., 2011b). Nuclear accumulation of FGFR1 can also be observed in developing midbrain substantia nigra (SN) neurons as they grow their telencephalic projections (Fang et al., 2005). As this development is completed, FGFR1 localization becomes predominantly cytoplasmic (Fang et al., 2005; Stachowiak et al., 2003b). To examine the role of FGFR1 signaling in the postnatal development of dopamine-producing neurons, a kinase-deleted FGFR1(TK-) model was employed. The expression of FGFR1(TK-) was restricted to dopamine neurons and other catecholaminergic cells by fusion of FGFR1(TK-) to the 4.5 kb rat tyrosine hydroxylase (TH) gene promoter (Klejbor et al., 2006).

In TH-FGFR1(TK-) mice, significant reductions in the size of TH-immunoreactive neurons are observed in substantia nigra compacta (SNc) and VTA at postnatal days 0 and 360 (Klejbor et al., 2006). Newborn mice have a reduced density of DA neurons in both SN and VTA. Reduced density of the DA transporter in the striatum further demonstrates an impaired development of the nigro-striatal DA projection.

Direct evidence for INFS stimulation of brain neurogenesis was obtained in mice by nanoparticle-mediated gene transfer into the neural stem/progenitor cells in the subventricular zone (SVZ) of lateral ventricles. Transfections of the nuclear FGFR1 (SP-/NLS) or HMW FGF-2, which mobilizes endogenous nuclear FGFR1, blocks proliferation and stimulates differentiation and neuronal development of the NS/PCs (Bharali et al., 2005; Stachowiak et al., 2009). Recently, similar effects were observed in mice following activation of INFS using the specific α7 nicotinic receptor agonist TC-7020 (Narla et al., 2013). These studies show that in vivo targeting the INFS mechanism may effectively reinstate neurogenesis in the adult brain.

INFS in Cancer

Studies of various cancer lines have indicated anti-proliferative effects of nuclear FGFR1. In vitro, the neoplastic pheochromocytoma cells, PC12 cells, proliferate continually in the presence of serum, however when treated with nerve growth factor (NGF), PC12 cells exit the cell cycle, grow neurites, and differentiate to catecholamine-producing sympathetic-like neurons expressing neuronal markers such as doublecortin and TH (Lee et al., 2013). It has been demonstrated that NGF increases expression of the *fgfr1* gene and promotes trafficking of FGFR1 protein from the cytoplasm to the nucleus (Fig. 5A), in part by inhibiting FGFR1 nuclear export (Lee et al., 2013). Nuclear-targeted dominant negative FGFR1(SP-/NLS)(TK-) antagonizes NGF-induced neurite outgrowth (Fig. 5B, C) and activation of the *th* gene promoter (Fig. 5D), while active constitutive nuclear FGFR1(SP-/NLS) mimics the effects of NGF (Fig. 5B, C, E).

When INFS is disabled, as it appears to be in the cancer cell line TE671 medulloblastoma, due to the near total absence of FGFR1, the cells proliferate aggressively and display non-differentiated features. When INFS is re-established in TE671 cells by transfection of FGFR1(SP-/NLS), evidence of cell differentiation is observed. FGFR1(SP-/NLS) induced neuronal-like differentiation also in SHSY5Y neuroblastoma cells, further supporting its anti- oncogenic action (Somanathan et al., 2003; Fang et al., 2005). The TE671 and SHSY5Y cells express little endogenous FGFR1 (Peng et al., 2001, 2002). Hence, the reconstitution of nuclear FGFR1 signaling in those cancer cells inhibits proliferation and induces differentiation (Fang et al., 2005).

Recent studies have identified the presence of nuclear FGFR1 in human neoplasms and suggested an additional complexity to the role of nuclear FGFR1 in cancer. Overexpression of FGFR1 is a feature of pancreatic ductal adenocarcinoma (PDAC). Nuclear FGFR1 and FGF-2 are found in activated pancreatic stellate cells (PSC), the main cells responsible for desmoplasia in pancreatic carcinoma (Coleman et al., 2014). Nuclear translocation of FGFR1 is necessary for the invasion of PSC and subsequently, cancer cells. Overexpression and nuclear translocation of FGFR1 is also

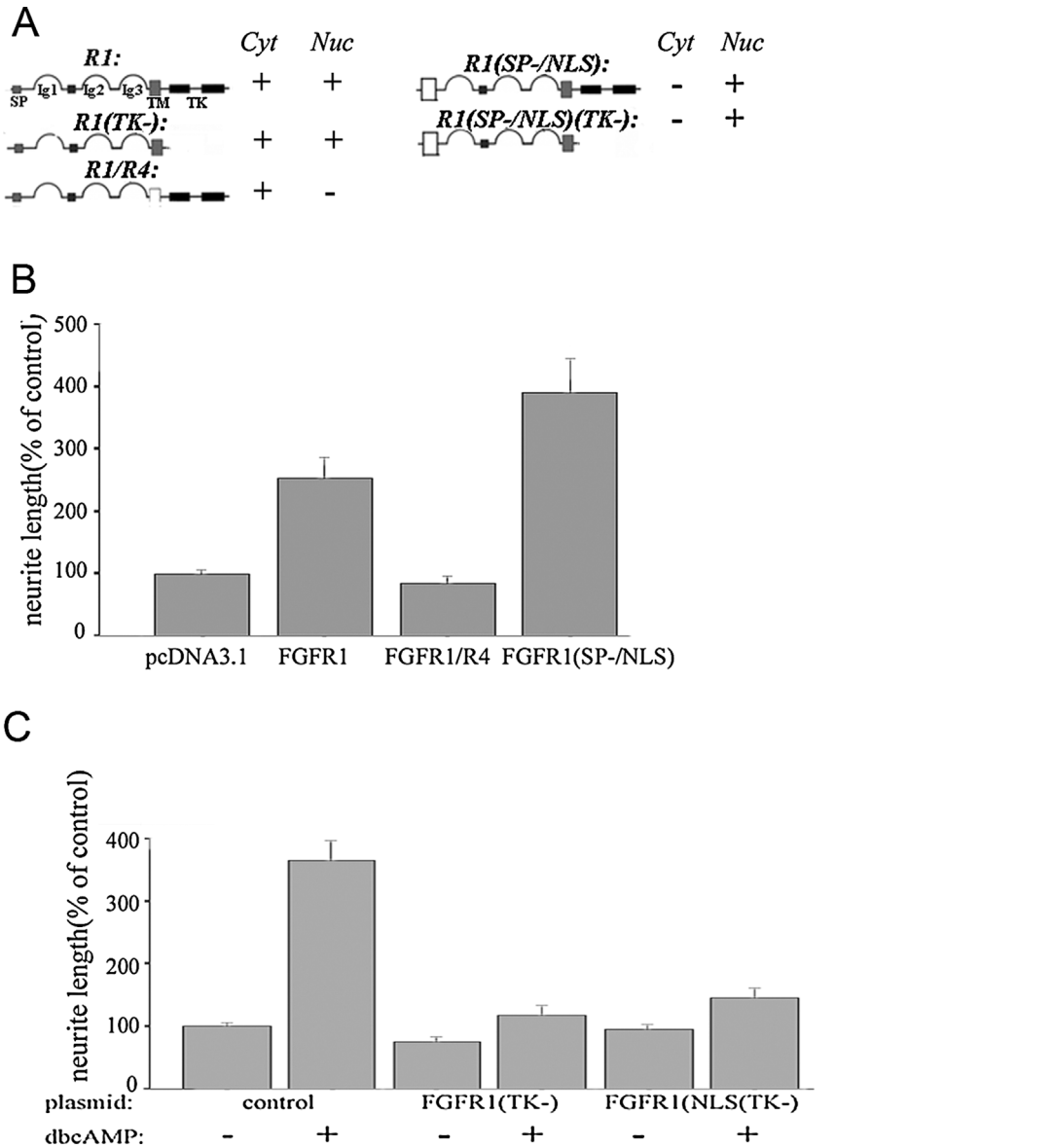

Fig. 4. Nuclear FGFR1 stimulates the differentiation of human neuronal progenitor cells (HNPC) isolated from fetal human brain. (A) FGFR1 (R1) mutants directed to different subcellular locales: Cyt (cytoplasm + membranes); Nuc (nucleus). To direct FGFR1 exclusively into the cell nucleus we replaced the signal peptide (SP) with a Nuclear Localization Signal (NLS). Tyrosine kinase (TK) deletion generates dominant negative FGFR1(TK-) or nuclear FGFR1(SP-/NLS)(TK-) that blocks cAMP, retinoic acid or BMP-7 induced differentiation and gene activities. Replacing the FGFR1 transmembrane (TM) domain with the FGFR4 TM arrests chimerical FGFR1/R4 in cytoplasmic membranes. (B) Effects of transfected cytoplasmic/nuclear FGFR1, exclusively nuclear FGFR1(SP-/NLS), or membrane-associated FGFR1/R4 on neurite outgrowth in HNPC. Bar graph - the length of the single longest process in individual transfected cells was measured (Stachowiak et al., 2003b). The average length of neurite processes in FGFR1 and FGFR1(SP-/NLS) transfected cells was significantly longer than in control pcDNA3.1 transfected HNPC ($P<0.0001$). HNPC transfected with FGFR1/R4 show no additional neurite outgrowth. (C) Dominant negative FGFR1 blocks dB-cAMP induced neurite outgrowth. HNPC were transfected with cytoplasmic/nuclear FGFR1(TK-) or non-membranous, exclusively nuclear FGFR1(SP-/NLS)(TK-) (Stachowiak et al., 2003b). Figures B and C were reprinted from J Neurochem 2003; 84:1296–1312, with permission of Wiley-Blackwell Publishing Ltd.

reported in breast cancer cells (Chioni and Grose, 2012). Nuclear FGFR1 truncation via Granzyme B protease could exert cancer specific transcriptional control. Regulation of FGFR1 levels by means of overexpression or knock down and subsequent ChIP experiments showed that FGFR1 regulates transcription of target cancer genes either directly or in cooperation as a part of larger complex (Chioni and Grose, 2012). Understanding the complex roles of FGF signaling in cancer biology may facilitate treatment strategies.

Nuclear FGFR1 is a Direct Gene Regulator

FGFR1-containing nuclear speckles identified by immunoelectron microscopy and by confocal microscopy correspond to sites of RNA polymerase II-mediated transcription and co-transcriptional pre-mRNA processing (Stachowiak et al., 2003b, 2011b). Consistent with this model, BrUTP labeled sites of transcription were present on the periphery of many FGFR1-rich speckles and nearly 40% of

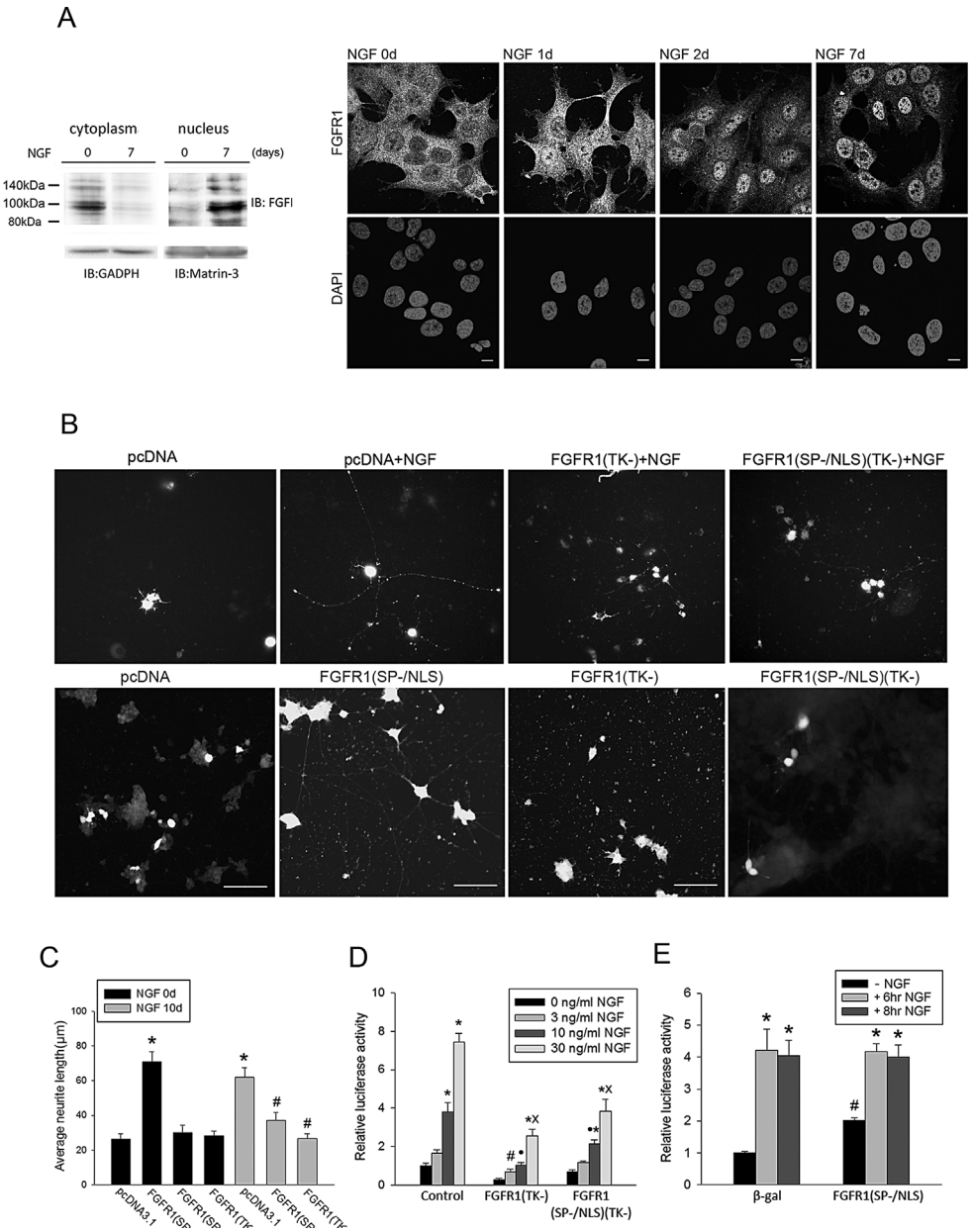

Fig. 5. (A) Nerve Growth Factor (NGF) stimulates nuclear accumulation of FGFR1 in PC12 cells. PC12 cells were maintained in RPMI1640 with 1% horse serum in the presence or absence of NGF (50 ng/ml) for 7 days. A – *left:* The cytoplasmic and nuclear proteins were prepared for electrophoresis (45 μg protein/lane) and immunoblotted with monoclonal N-terminal FGFR1 antibody (Abcam). Cytoplasmic and nuclear immunoreactive FGFR1 protein bands of 140, 110, 100, and 90 kDa correspond to different degrees of FGFR1 glycosylation (Stachowiak et al., 2003b). *Right:* The effect of NGF on FGFR1 expression and nuclear accumulation is illustrated by immunostaining with monoclonal N-terminal FGFR1 antibody plus goat-anti mouse Alexa488. (B) Nuclear FGFR1 mediates NGF induced neurite outgrowth and activation of the *th* gene promoter. PC12 cells were transfected with two plasmids, one expressing recombinant FGFR1 or control pcDNA3.1 and the second expressing EGFP. EGFP diffuses throughout the cell permitting visualization of the entire neuritic network. Twenty-four hours after transfection, cultures were switched to 1% horse serum medium with or without (control) 50 ng/ml NGF for an additional 10 days, after which the cells were imaged using fluorescent microscopy. Bar length – 100 μm. (C) The longest process in an individual transfected cell was measured using ImageJ (Lee et al., 2012). * mark comparison to pCDNA3.1 (-NGF) and # to pCNA3.1 (+NGF). Transfection of constitutive nuclear FGFR1(SP-/NLS) increased neurite outgrowth approximately 3-fold (*$P<0.001$, One-Way ANOVA, LSD). A similar increase was induced by NGF treatment of pcDNA3.1 transfected cells (*$P<0.001$). Cells transfected with dominant negative FGFR1(SP-/NLS)(TK-) or FGFR1(TK-) display no significant changes in average neurite length in the absence or presence of NGF. (D-E) Nuclear FGFR1 mediates NGF induced activation of the *TH* gene promoter. (D) PC12 cells were transfected with a *th-* Luciferase reported plasmid (Kim et al., 1996) and dominant negative FGFR1(TK-), FGFR1(SP-/NLS)(TK-or FGFR1[SP-/NLS][TK-]). (E) – PC12 cells were transfected with th-Luc and control pcDNA3.1(-) or pcDNA3.1 expressing an active constitutive nuclear FGFR1(SP-/NLS). Twenty-four hours after transfection, cells were treated for an additional 6 or 8 h with NGF. FGFR1(SP-/NLS) increases TH promoter activity 2-fold in the absence of NGF but shows no additive stimulation in the presence of NGF. One-Way ANOVA, LSD: * ($P<0.001$) – comparison to (-NGF) within individual plasmid transfection groups; #($P<0.05$) - comparison to pCDNA3.1 (- NGF). Figures A–C were reprinted from PLoS ONE. 2013 8(7):e68931. doi: 10.1371/jo.

all transcription sites colocalized with FGFR1. In contrast, no significant co-localization of DNA replication sites with FGFR1 speckles was found arguing against a direct role in DNA replication (Somanathan et al., 2003; Stachowiak et al., 2003b)

The co-localization of FGFR1 with sites of RNA synthesis indicated that the receptor could act as a direct and global transcriptional regulator that controls the activities of genes located at different genomic locales. Indeed, the increases in the activities of FU-labeled transcription sites accompanying cell stimulation were diminished by transfection of the dominant negative cytoplasmic/nuclear FGFR1(TK-) and exclusively nuclear FGFR1(SP-/NLS)(TK-) (Lee et al., 2013). These tyrosine kinase-deleted recombinant receptors block FGFR1 signaling by heterodimerization with endogenous receptors (Robinson et al., 1995), and by forming an inactive complex with CREB binding protein (CBP), a nuclear target of FGFR1 (see further below) (Fang et al., 2005).

The *fgf-2* gene, located on human chromosome 4, (Peng et al., 2002) and the tyrosine hydroxylase (*th)* gene, located on human chromosome 11, were the first genes shown to be regulated by nuclear FGFR1 (Peng et al., 2001, 2002). TH, the rate-limiting enzyme in catecholamine biosynthesis, is expressed specifically in catecholamine producing cells. Co- transfection of FGFR(TK-) prevents the activation of transfected *fgf-2* and *th* gene promoters as well as their endogenous genes by angiotensin receptors, acetylcholine receptors, cell depolarization, and by PKC and cAMP signaling pathways, demonstrating that FGFR1 is essential for the transcriptional activation of the *th* and *fgf-2* genes by various stimuli (Peng et al., 2001, 2002). In contrast, the extracellular FGFR1 antagonists do not prevent the same gene activations, suggesting that the promoter activation is mediated by intracellular, nuclear FGFR1 and the INFS mechanism (Peng et al., 2001, 2002). Indeed, *th* and *fgf-2* gene promoters and other endogenous genes are activated by transfected wild type FGFR1, by soluble (non-membrane) cytoplasmic/nuclear FGFR1(SP-), as well as by soluble nuclear FGFR1(SP-/NLS). Thus, an increase in the nuclear FGFR1 content is sufficient to activate the *th* and *fgf-2* genes. Importantly, blocking cell surface FGFR1 and the extracellular ligand-induced FGFR1 internalization does not affect gene activation by transfected FGFR1 or by its nuclear ligand, HMW FGF-2 (Peng et al., 2001, 2002). This supports the conclusion that nuclear FGFR1 mediates gene transactivation via INFS, which is not a consequence of ligand- induced receptor internalization from the cell surface (Stachowiak et al., 2003b, 2011b).

Although the activation of transcription by nuclear FGFR1 is prevented by deletion of the receptor tyrosine kinase domain, the K514A mutation, which eliminates the FGFR1 kinase activity and receptor auto-phosphorylation, has little effect on transcription. Indeed, both FGFR1 (K514A)(SP-/NLS) and FGFR1(SP-/NLS) transactivate the th gene as well as co-transfected TH and FGF-2 promoters and CRE linked to a luciferase reporter (Fang et al., 2005). Thus, activation of transcription by nuclear FGFR1 does not require receptor tyrosine kinase activity and, therefore, is distinct from the tyrosine kinase activity-dependent effects of plasma membrane FGFR1 (Stachowiak et al., 2003b, 2011b).

Other genes thus far shown to be regulated by nuclear FGFR1, include neuron-specific enolase (NSE) (Somanathan et al., 2003), neurofilament-l (NF-l), c-Jun, cyclin D1 (Reilly and Maher, 2001), doublecortin, βIII-tubulin, nurr1, and nur77 (Lee et al., 2013; Narla et al., 2013), Different chromosomal locales of these nuclear FGFR1-regulated genes further supports the role of INFS in global gene regulation.

Nuclear FGFR1 Partnership With Common Transcription Co-Activator, CREB Binding Protein (CBP)

Th gene activation by nuclear FGFR1 is mediated by the classical cAMP response element (CRE). The promoters of other genes regulated by nuclear FGFR1 contain CRE, AP1 and other cis-acting elements. Transfected nuclear FGFR1(SP-/NLS) transactivates CRE, as well as AP1-, retinoic acid receptor-, Nur-, and NF-κB-binding elements (Fang et al., 2005; Stachowiak et al., 2011b) all of which are known to be co-activated by CBP (Stachowiak et al., 2011b). The cooperation between these two nuclear proteins was documented using diverse experimental strategies (Fang et al., 2005; Dunham-Ems et al., 2009). Double immunostaining revealed that the FGFR1- and CBP-labeled pixels were closely co-localized within the nuclear speckles in cAMP- differentiated HNPC (Fig. 6A) and in developing mouse brain neurons (Fang et al., 2005). Evidence for a direct interaction of FGFR1 with CBP comes from co-immunoprecipitation experiments of FGFR1, FGFR1(TK-) or FGFR1(K514) with CBP. CBP interacted predominantly with non-glycosylated FGFR1 (Fig. 6A) (Fang et al., 2005), which is consistent with our previous finding that non-glycosylated FGFR1 is subject to nuclear trafficking. This interaction was also seen in the nuclear extracts of the developing rodent brain and was further confirmed by GST-CBP pull down of FGFR1 (Fang et al., 2005).

Nuclear FGFR1 Regulates Transcription Gating Activity of CBP

Gene activities in developing cells are affected by a variety of extracellular signals transduced by diverse intracellular messengers including cAMP, Ca^{++}, NF-κB, STATs, and nuclear receptors. CBP and its homolog p300, function as signal integrators and coactivators of diverse sequence-specific transcription factors (ssTFs), including CREB, AP1, STATs, NF-κB, and retinoic acid receptors, and modulate the expression of genes involved in cell development and homeostasis (Heasley et al., 1991; Chrivia et al., 1993; Kwok et al., 1994; Lundblad et al., 1995; Janknecht and Hunter, 1996; Glass et al., 1997; Kwok et al., 1998). CBP facilitates the formation of the RNA Pol II holoenzyme (RPIIH) and is involved in histone acetylation, leading to chromatin remodeling, which is essential for initiation and continued transcription (Chrivia et al., 1993; Arany et al., 1994; Kwok et al., 1994; Lundblad et al., 1995; Eckner et al., 1996; Horvai et al., 1997; Boyes et al., 1998; Kwok et al., 1998; Kraus et al., 1999).

In the nucleus, CBP is present in limiting quantities and, in addition, its activity is suppressed by other nuclear proteins including Ribosomal S-6 serine/threonine kinases, RSK2 (Nakajima et al., 1996) and RSK1, which bind to a region of CBP that is also recognized by the repressor E1A oncoprotein (Arany et al., 1994). Cell differentiation triggered by cAMP, PKC and other signaling pathways promotes the disassociation of RSK-CBP complexes allowing CBP and RSK to exert their functions as a RNA Pol II activator, HAT enzyme (CBP) and ssTF/histone kinase (RSK) (Nakajima et al., 1996; Sassone-Corsi et al., 1999; Cheung et al., 2000a). The histone phosphorylation along with acetylation constitute an important step in chromatin remodeling essential for gene transcription (Janknecht and Hunter, 1996; Wolffe and Pruss, 1996; Kwok et al., 1998; Cheung et al., 2000b). The disassociation of CBP-RSK1 complex, was shown to be necessary for CREB-mediated activation of genes such as *th* (Nakajima et al., 1996).

The signaling events that regulate critical CBP-RSK interactions, to date are not clearly elucidated. Dynamic sequestering of CBP by RSK may allow channeling of the cell signals via mitogenic Ras for proliferation or via cAMP for

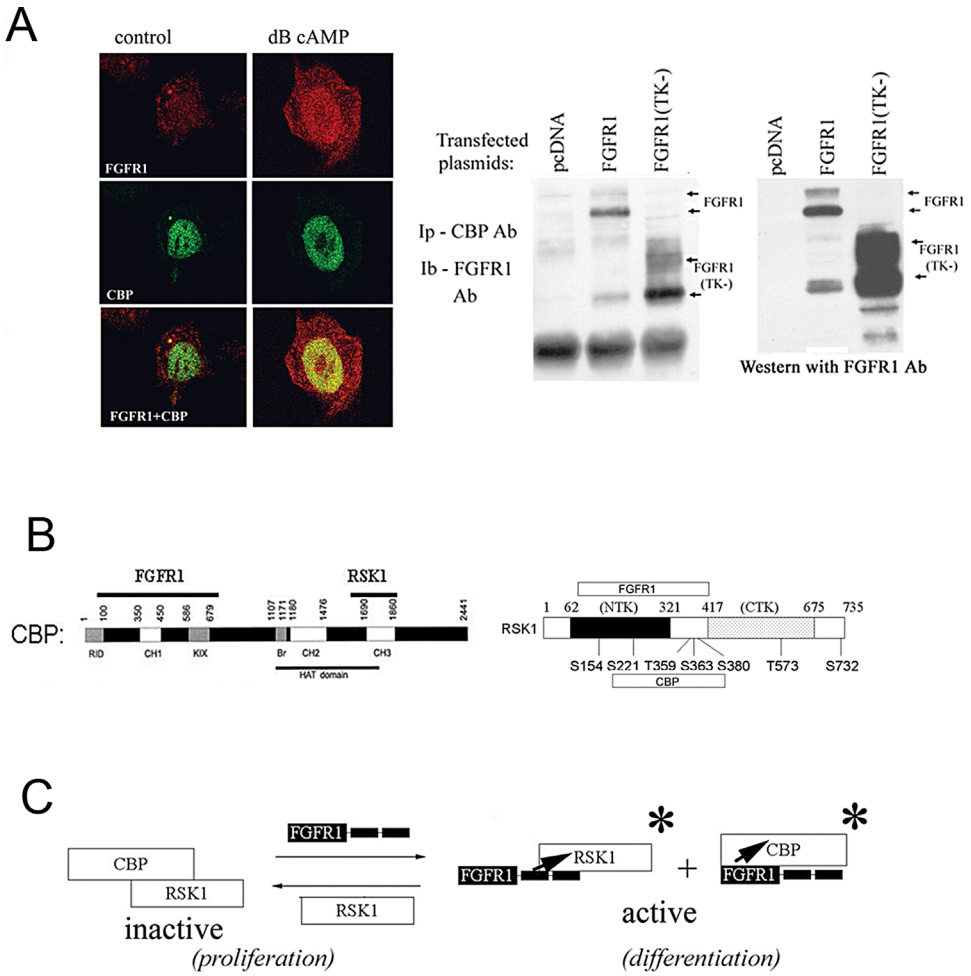

Fig. 6. Nuclear FGFR1 as a transcriptional regulator – partnership with CBP (Peng et al., 2001, 2002) (A) *Left* - FGFR1 colocalizes with CBP in differentiating neural stem cells. Cultures of neural progenitor-like cells isolated from human umbilical cord blood were treated with 0.1 mM dB-cAMP for 48 h and co-immunostained with FGFR1 McAb6 (+ anti-mouse- Alexa 488) and with rabbit CBP (+ goat-anti rabbit-CY3). Confocal sections through the middle of the nuclei are shown. Merged images - FGFR1 and CBP immunoreactive pixels colocalize within CBP-rich nuclear foci (Fang et al., 2005). *Right* - Both FGFR1 and tyrosine kinase deleted FGFR1(TK-) bind to CBP. TE671 medulloblastoma cells were transfected with FGFR1 or FGFR1(TK-). Cell extracts were co-immunoprecipitated with CBP Ab and immunobloted with FGFR1 McAb6 or were directly subjected to western analysis with FGFR1 McAb6. The interaction between CBP and transfected FGFR1 was further confirmed by CBP co- immunoprecipitation with FGFR1 Ab in developing brain and by GST-CBP pull down of FGFR1 (Fang et al., 2005). (B) Interactions among FGFR1, CBP and RSK1 (Fang et al., 2005; Hu et al., 2004). FGFR1 and RSK1 bind to distinct CBP domains (Fang et al., 2005). The binding regions in CBP were established by CBP-GST binding assays (Fang et al., 2005). FGFR1 and CBP bind to the overlapping regions in RSK1 (Hu et al., 2004). (C) CBP, FGFR1 and RSK1 are in a dynamic equilibrium in which nuclear accumulation of FGFR1 disrupts the RSK1-CBP complex while an increase in RSK1 restores the CBP-RSK1 binding. Serum and other mitogens promote RSK1-CBP binding in proliferating cells thereby blocking CBP transactivation of genes involved in cell differentiation. cAMP, PKC and other signaling pathways that differentiate cells, trigger nuclear accumulation of FGFR1. Nuclear FGFR1 binds to RSK1 and disassociates the inactive RSK-CBP complex allowing CBP and RSK1 to exert their functions as a RNA Pol II activator, histone acetyltransferase (CBP) and ssTF/histone kinase (RSK) (Cheung et al., 2000a; Nakajima et al., 1996; Sassone-Corsi et al., 1999). FGFR1 direct binding to CBP and RSK1 augments their transcription-activating functions and activates genes involved in differentiation (Fang et al., 2005). Figure A was reprinted from J. Biol Chem, 2005;280:28454–28456, with permission of American Society for Biochemistry and Molecular Biology Inc. Figures B and C were reprinted from DNA Cell Biol. 2007;26(12):811-26.

differentiation. Our investigation has shown that the CBP-RSK1 complex that forms in mitogen-stimulated proliferating cells is effectively disrupted by increasing the levels of nuclear FGFR1. These findings framed a three-fold mechanism for nuclear FGFR1 activation of transcription (Fig. 6B, C) (Stachowiak et al., 2003b, 2011b). In proliferating cells, CBP is found in a complex with RSK1. As FGFR1 accumulates in the nucleus, the TK domain binds to the N-terminal kinase domain of RSK1 and disrupts the CBP-RSK1 complex (Fig. 6B, C). The N-terminal domain of another FGFR1 molecule interacts with the N-terminal domain of CBP. The FGFR1-CBP complex activates transcription utilizing the N-terminal portion of the CBP in a process that leads to the recruitment of RNA Pol II and histone acetylation (Fang et al., 2005). RSK1-bound FGFR1 augments RSK1 activity potentially resulting in the phosphorylation of ssTF and/or chromatin proteins.

Nuclear FGFR1 and CBP Co-Associate with Targeted Genes: Dynamic Model of Gene Regulation (Stachowiak et al., 2011b)

EMSA showed the FGFR1 ability to associate with the *th* gene promoter CRE, along with CREB and CBP (Fig. 7A).

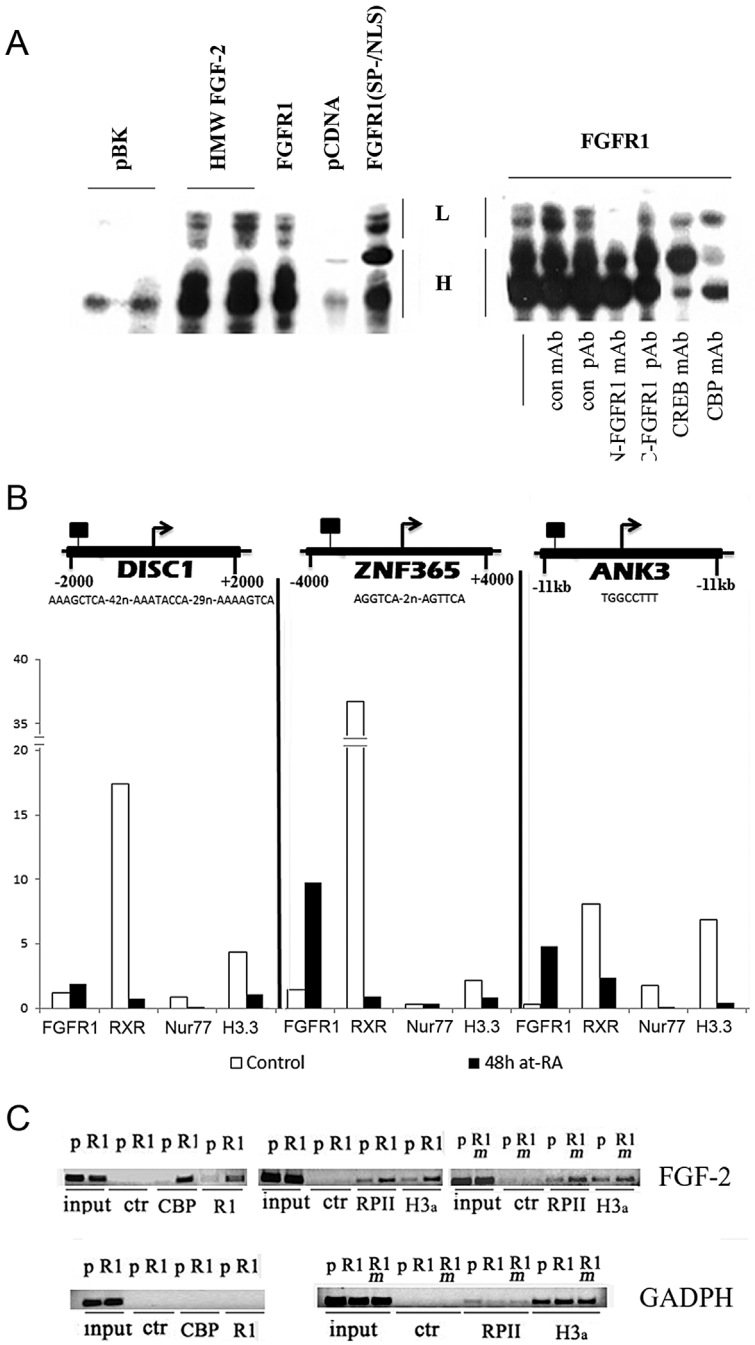

Fig. 7. FGFR1 associates with targeted genes. (A) Electrophoretic Mobility Shift Assay (EMSA) shows FGFR1 binding along with CREB and CBP to the th gene promoter CRE region. Left - TE671 cells were transfected with pBK expressing HMW (23 kDa) FGF-2 or pcDNA3.1 expressing FGFR1, FGFR1(SP-/NLS) or control, empty plasmids. Nuclear extracts were incubated with the 32P-labeled (60/0 bp) TH promoter CRE probe and analyzed by EMSA. Lanes 1–5 and 6–7 represent separate experiments. _L_ and _H_ indicate low and high mobility retarded protein-DNA complexes. Right – nuclear extracts of TE671 cells transfected with pcDNA3.1-FGFR1 were incubated with a 32P-(60/0 bp) CRE probe. Subsequently, the reactions were treated with antibodies for an additional 8 h at 4°C and then analyzed by EMSA. – No antibody; control (con), monoclonal (m) Ab; polyclonal (p) Ab; N-terminal (N); C-terminal (C). Figure was reprinted from J Neurochem 2002;81:506–524, with permission of Wiley-Blackwell Publishing Ltd. (B) Chromatin immunoprecipitation (ChiP) shows FGFR1 binding to neurodevelopmental genes linked to schizophrenia. Immunoprecipitated DNA was analyzed by Real Time PCR. Note the opposite effects of retinoic acid induced neuro-ectodermal programing of human ESC on binding by FGFR1 and its partners RXR and Nur77. Figure was reprinted from Schizophrenia Research 2013, 143(2–3): 367–76. (C) FGFR1 stimulates CBP and RNA polymerase II binding and histone H3 acetylation at the FGF-2 core promoter. TE671 cells were transfected with FGFR1 (R1), TK inactive FGFR1(K514A) (R1m), or pcDNA (p). Chromatin was cross-linked and immunoprecipitated with the indicated antibodies (anti-CBP, anti-FGFR1 [R1], anti-RNA polymerase II [RPII], antihistone H3a [H3a], or control [ctr] antibodies). The immunoprecipitates were analyzed by PCR for the FGF-2 promoter (211 = þ112 bp) and a fragment of the GADPH gene (þ2245 = þ2765 bp) as a control (Fang et al., 2005). Figure was reprinted from J Biol Chem, 2005;280:28454–28456, with permission of American Society for Biochemistry and Molecular Biology Inc.

Cross-linked chromatin immunoprecipitation experiments (Fang et al., 2005) showed that FGFR1 associates with FGFR1-activated gene promoters of neurogenic genes (*nurr1* and *nur77*, *fgfr1*, and *fgf-2*), neurodevelopmental genes, including genes affected in schizophrenia (Fig. 7B), and with NGF-activated neural genes (*dcx*, *th*, and *βIII tubulin*) (Lee et al., 2012, 2013).

Transfected FGFR1 associates with the core *fgf-2* gene promoter and induces the binding of CBP within the promoter (Fig. 7C). Both kinase-active and -inactive FGFR1 increase RNA Pol II binding and acetylation of histone H3 at the promoter. Thus, nuclear FGFR1 may stimulate the recruitment of CBP and RNA Pol II as well as chromatin remodeling in a FGFR1 kinase activity-independent manner. Nuclear accumulation and gene binding by FGFR1 is also accompanied by H3 methylation and incorporation of the H3.3 variant further indicating the roles of FGFR1 in chromatin remodeling (Lee et al., 2012).

An emerging dynamic model of nuclear function proposes that gene regulation is dependent upon probabilistic collisions of fast moving molecules (Misteli, 2001). Fluorescence recovery After Photobleaching (FRAP) has shown a dynamic nature of gene regulation by the nuclear FGFR1 and its partner proteins (Dunham et al., 2004; Dunham-Ems et al., 2009; Baron et al., 2012; Lee et al., 2012). The transcriptional activation by cAMP or other stimuli is accompanied by conversion of immobile, nuclear matrix-associated FGFR1, and hyperkinetic nucleoplasmic FGFR1 into a hypokinetic, chromatin-associated FGFR1 and with a decreased rate of its movement (extra-slow) (Fig. 8A) (Dunham-Ems et al., 2009). Changes in FGFR1 chromatin kinetics during gene activation reflect interactions with CBP and with RSK1. We proposed that by increasing the residence time of FGFR1 and its CBP and RSK1 partners, the transcriptional reaction may be initiated and carried to the completion of RNA transcripts (Stachowiak et al., 2011b). We postulated a model (Stachowiak et al., 2011b) (Fig. 8B) in which nuclear FGFR1, CBP and RSK1 collisions at the gene sites drive formation of the preinitiation complex (PIC) and transcript elongation. The rates of FGFR1 oscillations at the corresponding phases of gene transcription are similar to those of RNA Pol II (Darzacq et al., 2007) (Stachowiak et al, 2011b). Stochastic generation of slower oscillating FGFR1/CBP and FGFR1/RSK1 create efficacious molecular complexes that support transcription reactions (Dunham-Ems et al., 2009) (Stachowiak et al., 2011).

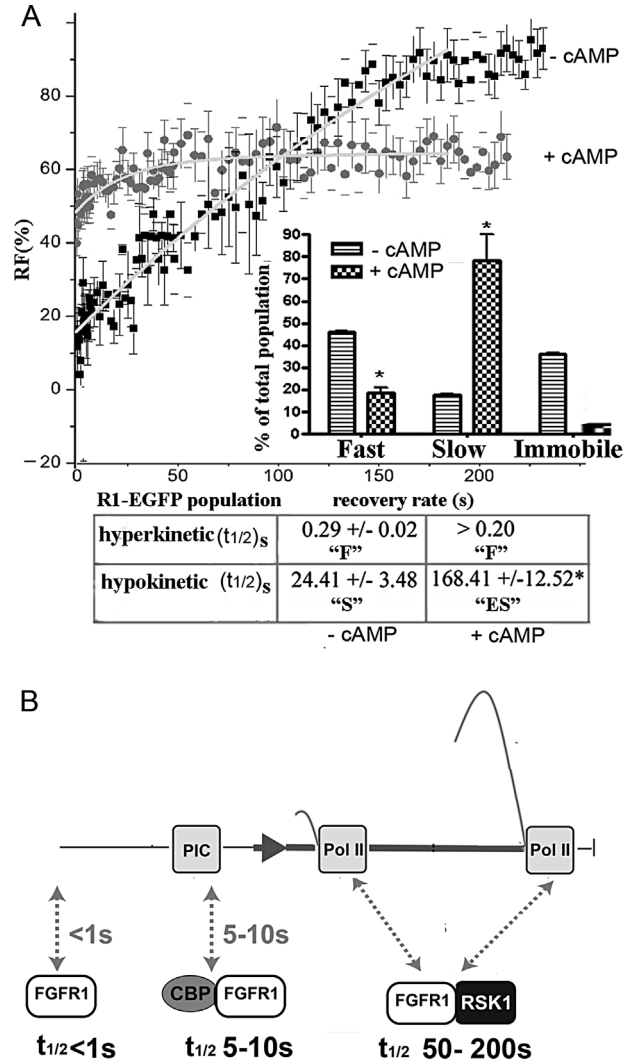

Fig. 8. (A) Transcription activation affects FGFR1 (R1)-EGFP nuclear FRAP mobility. Bimodal analyses of the R1-EGFP recovery demonstrates that nuclear FGFR1 contains a hyperkinetic ("F" Fast recovering t1/2<1 sec; present in nucleoplasm), a hypokinetic ("S" Slow-recovering, t1/2 = 24 sec; chromatin-associated), and nonrecovering (Immobile, nuclear matrix-associated) populations (bar graph). dB-cAMP treatment eliminates the immobile, reduces hyperkinetic and increases hypokinetic populations ($P<0.001$). The recovery halftime of hypokinetic R1-EGFP is increased ($P<0.001$), thereby generating an Extra-Slow ("ES"; t1/2 = 168 sec) R1-EGFP. These effects are reproduced by transfection of R1-binding CBP and reversed by antisense CBP or transcription inhibitors (Dunham-Ems et al., 2009). Transfection of RSK1 converts the "S" R1 into "ES" without increasing the total hypokinetic population (Dunham-Ems et al., 2009). Figure was reprinted from Mol Biol Cell. 2009; 20 (9):2401–12. (B) Kinetic model of INFS: FGFR1 that is not engaged in transcription associates with nuclear matrix and is immobile. Activation of transcription by cAMP releases FGFR1 from matrix via FGFR1 interaction with 23 kDa FGF-2 generating "F" R1, which engages in rapid (t1/2<1 sec) "non-productive" molecular collisions and chromatin scanning. R1-CBP binding converts "F" R1 into hypokinetic R1 (t1/2 5–10 sec). We propose that the "S" FGFR1 represents FGFR1-CBP oscillations that drive the formation of the RNA Pol II (Pol II) Preinitiation Complex (PIC). CBP binding to DNA–associated transcription factors may extend the CBP-FGFR1 chromatin residence thereby promoting initiation during transcription activation, the rate of oscillations is further reduced ("S" R1 converts into "ES," (t/12>50 sec) possibly reflecting FGFR1 and RSK1 binding events and formation of the productive elongating complexes.

Summary

The function of INFS can be described as a "feed-forward-and-gate" module in which developmental signals are transduced by diverse membrane receptors and classical signaling cascades towards ssTFs (Fig. 9A) (Fang et al., 2005; Stachowiak et al., 2007). The same signals are fed forward by nuclear FGFR1 to the common and limiting transcriptional coactivator, CBP. Nuclear FGFR1 releases CBP from inactive complexes and the coupled activation of CBP and RSK1 by INFS and ssTFs enables the coordinated regulation of multi-gene program involved in differentiation. The direct activation of the INFS via overexpression of nuclear FGFR1 or its HMW FGF-2 ligand suffices to convert the proliferating stem/progenitor-like cells into their differentiated progeny, thus bypassing the action of the many external and internal factors needed for such a transition. This has been demonstrated in diverse cultured cells, as well as in vivo in the mouse brain (Bharali et al., 2005; Stachowiak et al., 2011a). Hence, INFS, which directs cells toward postmitotic development, that is, neuronal differentiation, complements the pluripotency module responsible for self-renewal and the cell cycle module for mitotic expansion (Fig. 9B) (Stachowiak et'al., 2011a).

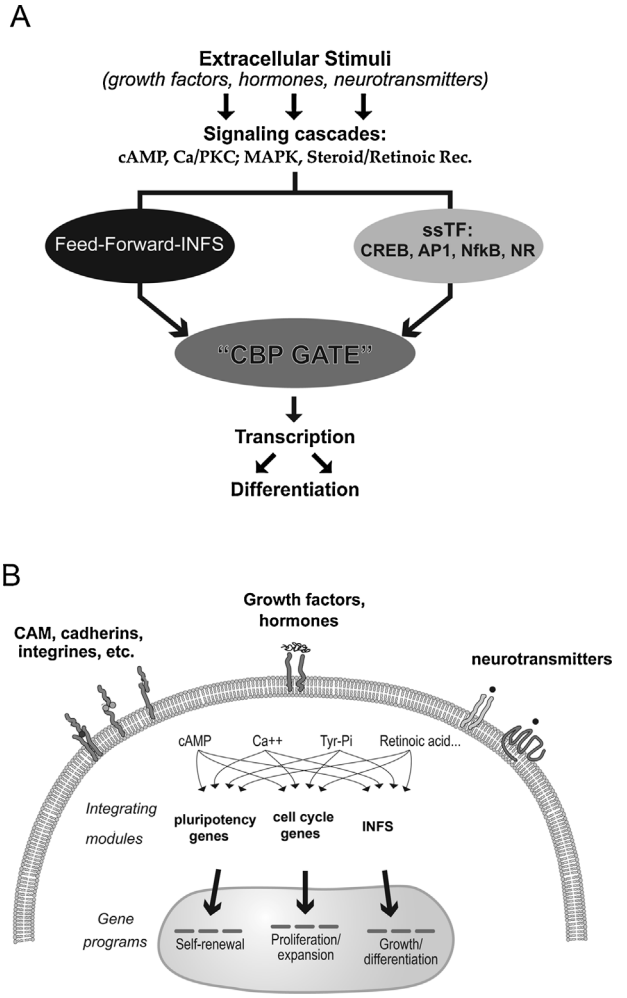

Fig. 9. (A) "Feed-Forward-And-Gate" signaling module of INFS is initiated by diverse stimuli that activate various signaling cascades and sequence specific transcription factors (ssTFs) (Fang et al., 2005). The stimuli trigger nuclear translocation of FGFR1 (R1-INFS), a parallel signal that "feeds forward" the stimulation to CREB Binding Protein (CBP), the functional equivalent of the "End Gate." CBP is present in limited quantities and, when released from inactive complexes by nuclear FGFR1 (R1) enables gene activation and neuronal differentiation. Transfection of an engineered nuclear FGFR1(SP-/NLS) is sufficient to open the CBP "gate" and trigger neuronal differentiation. (B) Integrative signaling modules in stem cell development. Self-organizing pluripotency network for cell renewal involves positive feedback-interactions among transcriptional factors Oct3/4, Sox2 and Nanog. Cell cycle proteins (cyclins and cyclindependent kinases) integrate mitogenic signals. Cell differentiation mediated by Integrative Nuclear FGFR1 Signaling (INFS).

Recent studies have reported a rapidly increasing number of identified INFS regulated genes, nevertheless, at the present time, the full extent of the INFS gene regulation remains undetermined. However, given that mutation and knockout of FGFR1 prevents normal gastrulation, development of mesodermal somites, neural plate and neural crest, by affecting diverse development-controlling genes (Partanen et al., 1998; Dequeant and Pourquie, 2008) (Ciruna et al., 1997; Partanen et al., 1998; Ciruna and Rossant, 2001) and microRNAs (Bobbs et al., 2012; Stuhlmiller and Garcia-Castro, 2012), we have hypothesized that nuclear FGFR1 may control many key developmental genes (Stachowiak et al., 2011b). Whether nuclear FGFR1 targets these genes directly or only a subset of genes that initiate a cascade of downstream gene programs is under investigation.

Acknowledgements

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