

**Serum regulation of Inhibitor of DNA Binding/Differentiation 1 expression by a
BMP pathway and BMP Responsive Element**

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ABSTRACT

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Immediate Early Genes (IEGs) are expressed upon re-entry of quiescent cells into the cell cycle following serum stimulation. These genes are involved in growth control and differentiation and hence their expression is tightly controlled. Many IEGs are regulated through Serum Response Elements (SREs) in their promoters, which bind Serum Response Factor (SRF). However, many other IEGs do not have SREs in their promoters and their serum regulation is poorly understood. We have identified SRF-independent IEGs in SRF-depleted fibroblasts. One of these, Id1, was examined more closely. We mapped a serum responsive element in the Id1 promoter and find that it is identical to a BMP Responsive Element (BRE). The Id1 BRE is necessary and sufficient for the serum regulation of Id1. Inhibition of the BMP pathway by siRNA depletion of Smad4, treatment with the BMP antagonist noggin, or the BMP receptor inhibitor dorsomorphin blocked serum induction of Id1. Further, BMP2 is sufficient to induce Id1 expression.

Given reports that SRC inhibitors can block Id1 expression, we tested the SRC inhibitor, AZD0530, and found that it inhibits the serum activation of Id1. Surprisingly, this inhibition is independent of SRC or its family members. Rather, we show that AZD0530 directly inhibits the BMP type I receptors. Serum induction of the Id1 related

gene Id3 also required the BMP pathway. Given these and other findings we conclude that the Id family of IEGs is regulated by BMPs in serum through similar BREs. This represents a second pathway for serum regulation of IEGs.

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Chapter 1

Introduction

Immediate Early Genes

In the late 1970's, researchers realized that in response to the insect steroid hormone ecdysone, a group of genes were induced within 5-10 minutes of stimulation [1]. These genes were transiently expressed and their expression led to the expression of a set of secondary response genes that are functionally distinct from the rapidly expressed genes [2]. These findings lead to the study of Immediate Early Genes (IEGs) in fibroblasts [3].

When growth factors are removed from NIH3T3 mouse fibroblasts they become quiescent. These cells enter a reversible, non-growing state; they stop dividing and do not replicate DNA. These cells show no net change in cell number, however, a very small percentage of the quiescent cells transverse the cell cycle [4]. This implies that some cell death may occur. A difference in the genes expressed in growing cells and quiescent cells was observed [5, 6]. Expression of c-fos, one of the earliest known IEGs is very low during the cell cycle but is highly and rapidly expressed upon growth factor stimulation of quiescent cells [7, 8].

Upon re-stimulation with serum or specific growth factors, quiescent cells re-enter the cell cycle [9]. Immediate Early Genes are expressed rapidly-within minutes, and transiently upon treatment with serum or growth factors [10-15]. The expression of these genes is independent of new protein synthesis [16] suggesting that it is a relatively direct response to serum induction.

IEGs are involved in a plethora of processes including growth [17], differentiation [18, 19], lineage determination [20], learning and memory [21]. Many of these genes are potent transcription factors responsible for the regulation of networks of other genes [22]. Others are DNA binding proteins, cytoskeletal protein, receptor subunits or secreted proteins. These genes usually have high affinity TATA boxes in their core promoter, and short primary transcripts with few exons, and over representation of shared transcription factor binding sites in upstream sequences [2].

c-fos, c-jun and c-myc are among the earliest discovered and most well studied IEGs. Some of earliest evidence of c-fos and c-myc being IEGs came in the 1980's when it was observed that their expression can be induced quickly in fibroblast cells which have been stimulated with purified growth factors [8, 23]. c-fos reaches its maximal RNA levels as early as 30 minutes following stimulation, while c-myc reaches its maximal RNA levels 60 minutes following growth factor treatment.

The regulation of these IEGs is tightly controlled and misregulation can lead to many diseases including cancer, neurological disorders [24], bone remodeling disorders [25] and other chronic diseases. As reviewed in [26], IEG expression in many cancers is found to be sustained and abnormally high. Understanding the transcriptional regulation of IEGs is an important step in understanding how their deregulation results in disease and finding better therapies for these diseases.

Serum Response Elements

Hundreds of experimentally validated or hypothesized genes have a Serum Response Element (SRE) or CArG box in their promoter[27-30]. The consensus

sequence of the SRE is a 10-bp cis-element, CC(AT)₆GG. DNA binding assays identified the critical need for an element in the c-fos promoter for binding to a factor [31]. Originally the CArG box was identified as a 23 bp element which showed dyad symmetry within the 5' activating element of the c-fos promoter [31]. Around the same time another group [32] identified a common element in the promoter of chicken, mouse, and human cardiac α -actin genes. They called it the CArG box. This muscle gene element lacked the dyad symmetry of the SRE [33]. Mutagenesis studies established the necessity of the CArG box in the regulation of the promoter activity of IEGs [32, 34]. The CArG box is the core sequence of the SRE, although the two terms are used interchangeably. Over the last couple decades CArG boxes in many IEGs, e.g. c-fos [31], Vinculin (vcl) [35], Cysteine rich protein 61 (cyr61) [36], Connective tissue growth factor (CTGF) [37], Early growth response 2 (egr2) [38] and α -actin [39] have been identified.

Serum Response Factor

The MADS box family member [40], Serum Response Factor (SRF), binds to the SREs of IEGs [24, 41-43]. The MADS box transcription factors were identified based on primary sequence similarity of the founding members MCM1(yeast), *Agamous* (plants), *Deficiens* (plants) and SRF (animals) [44]. SRF has a MADS box, a conserved 56 amino acid region that is made up of a basic DNA-binding domain, a dimerization domain, and an interface for protein-protein interactions [40].

SRF is a 62-67 kDa, evolutionarily conserved [45] protein originally purified from HeLa extracts in complex with the CArG box of the SRE [42, 46]. It is ubiquitously

expressed and binds SRE as a homodimer [45, 47]. The CArG box is important for facilitation of SRF interaction with the SRE [31, 48-50]. SRF is activated by serum and like other IEGs, its induction is not dependent on *de novo* protein synthesis [45].

The use of neutralizing sera showed that SRF is critical in skeletal muscle differentiation *in vivo* [51, 52]. SRF is critical for mesoderm formation during mouse embryogenesis [53]. However, SRF^{-/-} embryonic stem (ES) cells can differentiate into mesoderm marker expressing cells *in vitro* and when introduced into nude mice they can form various cells types [54]. SRF also plays a key role in the differentiation of pro epicardial cells to coronary smooth muscle cells [55]. Although SRF^{-/-} ES cells grow, they are deficient for IEG activation. On the other hand, SRF^{-/-} ES cells display altered cellular morphology, reduced cortical actin expression, and an impaired plating efficiency on gelatin. In spite of these defects, the proliferation rates of SRF^{-/-} ES cells are not substantially altered, signifying that SRF function is not required for ES cell cycle progression [47, 56].

SRF is constitutively present at the promoters of the genes it regulates and its binding does not change upon activation with growth factors [57]. Stimulation of quiescent cells with the growth factors in serum results in the activation of two pathways sufficient for SRE activation, the mitogen activated kinase (MAPK) and the RhoA GTPase pathways. The MAPK pathway, through a cascade of factors, leads to the phosphorylation and activation of SRF co-factors, the ternary complex factors (TCFs). The TCF factors are ETS-like gene 1 (ELK1), Serum response factor accessory protein 1 (SAP1) and ETS related protein NET (NET) [22, 58]. The small GTPase, RhoA, via another group of SRF co-transcriptional activators, also activates SRF. The coactivators

are the myocardin related factors, Megakaryoblastic Leukemia1/2(MKL1/2), also known as MRTF-A, MRTF-B, Mal and BSAC [59-62].

Ternary Complex

TCFs bind an E-twenty six (Ets) motif; CAGGAT, adjacent to the CArG box. TCF binding to the promoter is dependent on SRF [63]. Genetic foot printing studies showed that TCFs are present at the promoter even in unstimulated cells. This indicated that the induction of these factors involved changes in transcriptional activation rather than DNA binding [57]. TCF binding is required for c-fos response to MAP kinase signaling. When the TCF site in its promoter is mutated it is still able to be induced by whole serum but cannot be induced by stimuli such as phorbol myristate acetate (PMA) [64-66].

The three Ets domain proteins which have been identified all have DNA binding properties and contain four regions of sequence homology called domains A, B and C and D [67-69]. DNA binding and cooperative interaction with SRF at the SRE is mediated by domains A and B [70, 71]. The B domain is necessary for the formation of the ternary complex [72] and interaction with SRF [73]. The C domain contains many (S/T) P MAP kinase phosphorylation sites [58, 74]. The D domain serves as the MAP kinase-docking site [75]. Once the kinases have docked they can then phosphorylate residues in the C domain, the transcriptional activation domain [75]. TCFs can be phosphorylated by the three major MAP kinase pathways in mammals, namely: ERK1/2; extracellular signal regulated kinase 1 and 2, JNK; c-Jun N terminal kinase and p38 [76, 77]. Phosphorylation of the transcriptional activation domain of TCFs results in their

increased transcriptional ability and formation of ternary complexes with SRF [74, 76, 77]. SAP1 and Net also contain additional regions that confer the ability to repress transcription [78, 79].

TCFs activate immediate early genes by formation of the ternary complex with SRF at the SREs [80]. Recruitment of TCFs to the promoter of IEGs requires both protein-protein and protein-DNA interactions [80]. In the presence of high affinity ets motifs some autonomous binding of TCF to SREs has been reported [72, 81, 82]. When TCFs are activated they bind to SRF. This interaction increases their affinity to the ets site of the c-fos SRE. The SRF DNA binding domain and the B box are sufficient for this protein-protein interaction [70]. A quaternary complex has also been described on the c-fos promoter that contains an *SRF* duplex and two ELK1s [83, 84].

Ternary complex formation and transcriptional activation is induced upon MAP kinase activation. While the JNK and p38 cascades are activated by cytokines and stress, the ERK cascade responds to growth factors and mitogens. The activation of TCFs results in the recruitment of activating co-factors such as CBP and p300. Other activators and RNA polymerase II are also recruited to the complex and transcription of the IEGs proceeds. In the absence of a signal the c-fos promoter is occupied by a multiprotein complex [57]. Phosphorylation of the TCF C-box results in the exchange of factors and the recruitment of p300 and a mediator complex that recruits RNA polymerase II. c-fos is activated through the histone acetyl transferase activity of the co-activators. De-phosphorylation of the TCF factors [85] or recruitment of co-repressors leads to the subsequent repression of c-fos promoter activity [86]. Phosphorylation may not always be necessary for recruitment of co-activators [87].

RhoA Pathway

The SRE can also be activated without TCF but the presence of SRF is required [64, 66]. Lysophosphatidic acid, a mitogen in serum and heteromeric G-proteins can activate a c-fos promoter, which is unable to bind TCF [88]. This response is mediated by RhoA, a member of the Ras super family of small GTPases [89]. The core SRE can be activated by activated forms of rhoA, rac and cdc42Hs, members of the Rho GTPase family,. In addition, inhibition of RhoA blocks SRE activation in NIH3T3 cells [90].

This pathway involves another group of co-factors, the myocardin related transcription factors (MRTFs) that comprise the cardiac and smooth muscle specific gene, myocardin and the ubiquitously expressed genes megakaryoblastic leukemia-1 (MKL1) and megakaryoblastic leukemia-2 (MKL2) [60]. MKL1/2 bind to the same region of SRF as TCF, therefore, the binding of MKL1/2 and TCF is mutually exclusive. Since the TCF and RhoA pathways are complementary, the activation of the RhoA pathway of SRE induction is often apparent only after mutation of the TCF pathway [91]. RhoA activation leads to changes in the actin cytoskeleton. This results in changes in the nuclear localization and activation of MKL1/2 and consequently activation of *SRF* target gene expression [92-94].

Myocardin is always nuclear and constitutively activates transcription [95]. MKL1/2 have three RPEL domains in their N-terminal region that are required for actin binding [62, 94]. The interaction between MKL1 and SRF is facilitated by the glutamine-rich region [96]. Both the glutamine-rich and basic regions have antagonistic effects on the nuclear import of MKL1. The leucine zipper (LZ) region

can mediate homo- and hetero- dimerization of the MKL1 family members. The function of the SAP region is not well understood but has been involved in nuclear

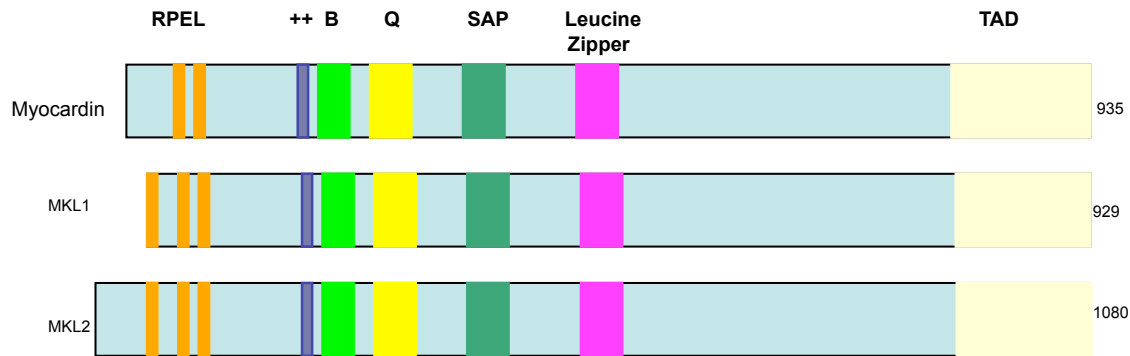


Figure 1.

Sequence similarity in MRTFs. Domain structures of the myocardin protein family. RPEL or actin binding domain, ++ : Basic domain, B: B-box, Q: glutamine rich domain, SAP domain, LZ: Leucine zipper and TAD: trans-activation domain

matrix attachment in other proteins [97]. The C-terminal contains a transcriptional activation domain (TAD) (Figure 1). Deletion of these TAD regions creates a dominant negative mutant [98]. MKL1 phosphorylation upon serum activation results in its nuclear export and inactivation [99].

Activation of RhoA causes stress fiber formation and a reduction in available monomeric G-actin [100]. *MKL1/2* is kept in the cytoplasm by being bound to G-actin. When cells are treated with mitogens, stress fibers form; this causes a reduction in the pool of G actin and MKL1/2 migrates to the nucleus. When in the nucleus these co-factors bind to *SRF* and activate IEG expression [94]. MKL1/2 is needed for the expression of many IEGs [30, 62]. Figure 2 summarizes the two SRF dependent pathways of IEG activation.

Some IEGs do not have clear SREs in their promoters. Their induction may be due to cryptic or distant SREs or entirely different pathways. As described here, some

IEGs do not require SRF for their serum induction. It is important to discover whether there is another common sequence element or pathway through which these SRE-lacking, SRF-independent IEGs are regulated.

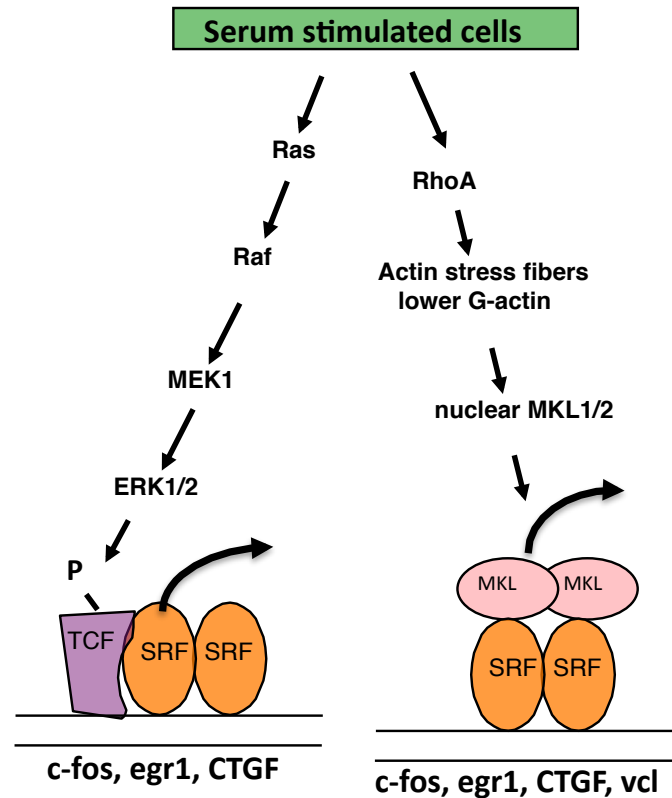


Figure 2.

Model of serum induction.

The MAPK and the RhoA pathways activate iEGs. Both pathways require SRF.

HLH proteins

An important class of gene regulatory proteins contains the basic-helix-loop-helix (bHLH) domain and is involved in proliferation and differentiation pathways [101, 102].

The bHLH domain is a protein motif that is common to a group of transcriptional regulators [103, 104]. More than twenty-seven members of this family have been identified [105]. These DNA binding proteins serve as regulatory factors whose

expression in the appropriate cell type induces the expression of many genes, resulting in

tissue specific phenotypes. These proteins are well conserved and are present in organisms ranging from yeast, nematodes, *Drosophila melanogaster*, and *Xenopus Laevis* to chickens and mammals. bHLH proteins are crucial for the regulation of many cellular processes including, cell growth, differentiation, regulation of lineage commitment, cell fate decisions, and the timing of differentiation [103, 106, 107] .

bHLH proteins are very similar in their structure [103]. These factors have a bHLH domain that is made up of a stretch of about 18 hydrophilic and basic amino acids at the N-terminal end of the domain, followed by two regions of hydrophobic residues which forms an amphipathic α -helix separated by an intervening loop [103]. They have a highly conserved basic region which allow them to bind the major groove of DNA. These proteins are all capable of homodimerization and heterodimerization [101, 108, 109]. The family member bound by a bHLH is crucial in the determination of its role in different cellular processes.

The bHLH proteins bind to a consensus sequence called an E box, CANNTG [110]. bHLH factors are often regulated through protein-protein interactions. Some members of this family lack the basic domain and therefore inhibit DNA binding by the factor with which they heterodimerize. Members of this group include the Inhibitor of DNA binding (Id) family and *Drosophila extramacrochaetae* protein [108, 111]. bHLH genes are also regulated by the availability of binding partners. Some members are repressed in a tissue specific manner, one example is MyoD [112]. *myc* on the other hand is more broadly expressed [113].

There are seven known classes of bHLH families [114, 115]. These classes comprise more than 240 members. Class I proteins, also known as the E proteins, include

E12, E47, HEB, E2-2, and Daughterless. These proteins are expressed in many tissues and capable of forming either homo or heterodimers [108]. Class II includes members such as MyoD, myogenin, Atonal, NeuroD/BETA2, and the achaete-scute complex. These proteins show a tissue-restricted pattern of expression [108]. Class III includes the Myc family of transcription factors, TFE3, SREBP-1, and the microphthalmia-associated transcription factor, Mi. Proteins of this class contain a Leucine Zipper domain adjacent to the HLH motif [116, 117]. Class IV includes Mad, Max, and Mxi; these molecules are capable of dimerizing with the Myc proteins or with one another [118-120]. Class V members are negative regulators of class I and class II HLH proteins. This group includes the Ids and Ems [121-123]. Class VI HLH proteins have as their defining feature a proline in their basic region. This group includes the Drosophila proteins Hairy and Enhancer of split [124, 125]. Class VII HLH proteins are categorized by the presence of the bHLH-PAS domain and include members such as the aromatic hydrocarbon receptor (AHR), the AHR nuclear-translocator (Arnt), hypoxia-inducible factor 1a, and the Drosophila Single-minded and Period proteins [126]. Evolutionary classification of HLH factors has been proposed by Atchley *et al* [105] based on phylogenetic analyses of amino acid sequences. This classification is newer and used less often.

Inhibitor of DNA Binding/ Differentiation 1

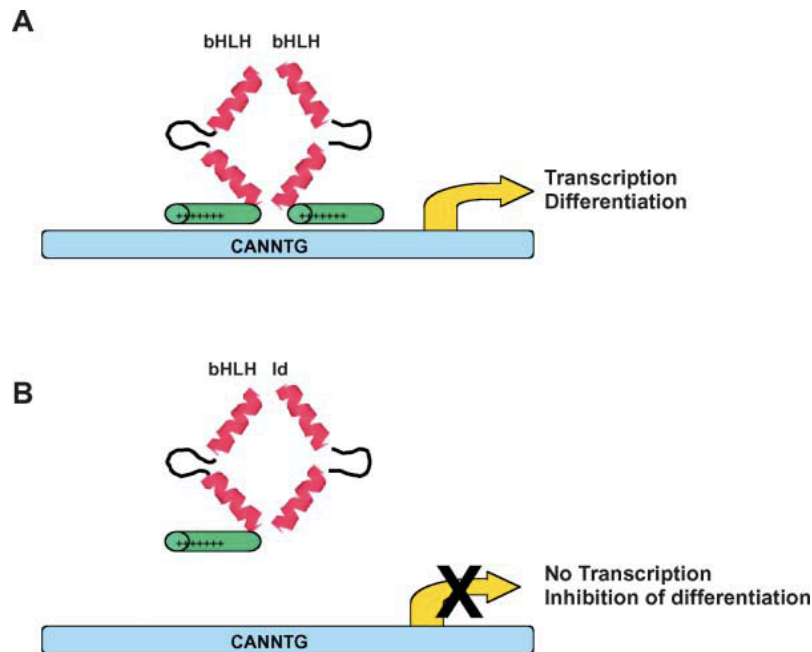
Inhibitor of DNA binding/differentiation 1 (Id1) is an IEG [127]. Inhibitor of DNA binding/ differentiation proteins were isolated as binding partners of the HLH family of transcription factors, namely E12, E47 and MyoD [121]. Id1 is expressed soon

after treatment of quiescent cells with serum and this increased expression does not require *de novo* protein synthesis. Id1 is a member of the HLH family of transcription factors that form heterodimers with other members of the HLH family [121, 128]. To date four members of this family, Id1-4 have been isolated [129-131].

All the Id family members are capable of inhibiting the activity of other members of the HLH family of transcription factors. The Id1 protein lacks a basic DNA-binding domain but it is capable of forming heterodimers with other HLH proteins [132]. These heterodimers are non functional; unable to bind DNA, thereby inhibiting the transcriptional activity of the bHLH proteins (Figure 3). This is an important method of regulation of other HLH factors [133].

Id1 is ubiquitously expressed [134]. Id1 expression appears in many tissues and its expression roughly correlates with the less differentiated phenotype [135-137]. The expression of Id1 is low in differentiation medium and this expression increases when cells are treated with mitogens in serum [127, 138]. The Id1 gene is highly expressed in undifferentiated, cycling cells and tumor cell lines. Id1 expression level decreases dramatically when cells undergo terminal differentiation [121, 139]. Id1 functions as a regulator of cellular differentiation of muscle cells [121]. Id1 binds to muscle differentiation factors such as MyoD and inhibit its activity. This interaction prevents the binding of MyoD to DNA and therefore activation of muscle specific genes regulated MyoD. When Id1 is over expressed, cellular differentiation in many cell culture systems and in transgenic mice is blocked [121, 140-142]. Inhibiting Id protein synthesis by antisense oligonucleotides and microinjection of anti-Id1 antibodies prevents the reentry of arrested cells into the cell cycle [138, 139, 143]. This implies that Id1 is required for

this process. Id1 over expression overcomes the growth inhibitory effect of E2A, suggesting that the balance between E2A and Id1 is critical in regulating cell proliferation [144].



[145] Cancer Cell: June 2003. Vol. 3.

Figure 3. Inhibition of transcription by Id1.

Id1 promoter regulation

The TGF- β family of growth factors regulates Id1. Id1 is upregulated by prolonged exposure to Transforming Growth Factor- β 1 (TGF- β 1) in epithelial cells [146]. Smad3 and ATF binding elements in the Id1 promoter mediate this upregulation. Id1 is also upregulated by TGF- β 1 in the human mammary gland cell line, MCF10A [147] and this regulation is also mediated by Smad3. Upon TGF- β stimulation, activated Smad3 can recruit transcriptional activators such as p300/CBP [148]. Liang *et al.* [147] showed that

TGF- β 1 induces H3 and H4 histone acetylation of the mId1 promoter in MCF10A cells. This is likely because of acetylation activity of p300/CBP [149]. Id1 is also suppressed by TGF- β 1 in some cell lines [146]. For example utilizing RNAi technology, Id1 expression was suppressed in a Smad3-dependant manner in LoVo cells when treated long with TGF- β 1 long-term [150]. Kang *et al.* [146] showed that repression of Id1 is a general feature of the TGF β cytostatic program. Treatment of Mouse Embryonic Fibroblasts (MEFs) with TGF β long-term results in the activation of the TGF β mediator, Smad3. Smad3 activates the expression of ATF3 and recruits it to the Id1 promoter. This is facilitated by Smad Binding Elements (SBEs) located between -1850 to -1467, -1265 to -926 and -255 to +47 of the mId1 promoter [147]. Smad3 interacts with HDAC4 and HDAC5 to repress the Runx2 gene [151]. When this happens Smad3 acts as a transcriptional repressor. Upon TGF- β activation of ATF3, Smad3 may act as a repressor of the Id1 promoter in a similar manner. This repression correlates with decreased levels of acetylated H3. Therefore, Id1 regulation by TGF- β 1 is a complicated event, shifting from early induction to late repression [147]. The early induction of Id1 may be necessary for its inhibition of differentiation while its late repression may allow the right balance to promote cell cycle progression.

Several groups have shown that Id1 is upregulated in response to Bone Morphogenetic Protein (BMP) signaling [152-155]. I will introduce Smads and the BMP pathway before I discuss this regulation.

Smads

Smads are intracellular proteins that transduce extracellular signals from transforming growth factor beta ligands to the nucleus. In the nucleus they activate downstream gene transcription [156]. These genes are homologous to the *Drosophila* protein, mothers against daughters (MAD) and the *Caenorhabditis elegans* protein, small body size (SMA). Eight Smad proteins are encoded in the human and mouse genomes, four in *Drosophila*, and three in *C. elegans* [157]. There are three classes of Smads. The receptor regulated Smads (R-Smads) includes Smad1, Smad2, Smad3, Smad5, and Smad8/9. The common mediator Smad (co-Smad), Smad4 is the only member of the second group. Smad4 interacts with the R-Smads to transduce signals to the nucleus. The inhibitory or antagonistic Smads (I-Smad) make up the final group. This group includes Smad6 and Smad7. These Smads interfere with Smad-Smad or Smad-receptor interactions. Smad2 and Smad3 are the main activators for TGF- β , Nodal and activin signaling. Smad1, Smad5 and Smad8 are the principal factors for BMP and anti-Mullerian receptors [158].

Smad proteins consist of about 500 amino acids which comprise two key domains separated by a globular domain [159]. They have an N-terminal Mad Homology 1 (MH1) and a C-terminal MH2 domain. The MH1 domain is important for DNA binding and is well conserved among the Smads except Smad6 and Smad7. This domain is followed by a linker domain, which contains binding sites for Smad ubiquitination-related factor (Smurf) ubiquitin ligases, phosphorylation sites for several classes of kinases, and in Smad4 a nuclear export signal (NES). The Smad MH2 domain is highly conserved, is one of the most versatile protein-interacting modules in signal transduction and is responsible for Smad interaction with receptors of the TGF- β family [160]. Its

structure contains several α -helices and loops which surround a β -sandwich [161]. It resembles the forkhead-associated domain (FHA), a phosphopeptide-binding domain common in transcription and signaling factors [162]. R Smads have a conserved C-terminal motif, Ser–X–Ser, that is phosphorylated by the activated receptor [158]. Smad4 does not have this motif [158].

Smads are activated when they are phosphorylated in their MH2 domain by activated TGF- β family receptors. This decreases their affinity for their cytoplasmic anchors and increases affinity for their nuclear partners [159, 163]. Smads migrate to the nucleus as a trimer (two R-Smads and one Smad4). In the nucleus the Smad complex directly binds DNA and affect gene transcription. Once in the nucleus the Smad4/R-Smad complex binds to the promoter of target genes and recruits co-activators or repressors. The co-Smad, Smad4 binds to a SBE; GTCT/AGAC of target genes [158]. BMP specific Smads bind a BMP Response Element (BRE). A BRE is a GC rich sequence, i.e. GCCGNC or GRCGNC [164, 165].

R-Smads interact with coactivators such as p300 and CBP. CBP and p300 have histone acetyl transferase (HAT) domains. HATs increase gene transcription by changing the nucleosome and increasing the accessibility to the general transcription machinery [166].

Nuclear R-Smads are later dephosphorylated and returned to the cytoplasm where they can undergo future rounds of activation by phosphorylation. Smad4 constantly shuttles between the nucleus and the cytoplasm [167]. When the R-Smads are phosphorylated they bind Smad4. This masks the NES signal of Smad4 and the pair translocates to the nucleus. This decreases the nuclear export of Smad4. When R-Smads are dyphosphorylated, the NES of Smad4 is exposed and Smad4 is exported from the

nucleus [158].

Bone Morphogenetic Proteins

Bone Morphogenetic Proteins (BMPs) are growth factors which belong to the TGF β super family of receptors [168]. BMPs were discovered in the 1970's as factors necessary for bone formation [169]. It was later understood that a group of proteins, BMPs, were responsible for the formation of bone [170, 171]. BMPs are able to generate bones in extra-skeletal ectopic sites [172]. BMPs are the largest subgroup of the TGF- β super family of growth factors [170, 173]. Over twenty members of the BMP family have been identified [174, 175].

BMPs are critical in many processes including growth, differentiation, apoptosis, embryonic development, mesoderm patterning, bone formation, craniofacial and limb development [176-178]. They are generally made by cells in the region of their physiological requirement and therefore act in an autocrine or paracrine manner [179]. BMPs are divided into four sub-groups BMP2/4, BMP5/6/7/8a/8b, BMP9/ 10, and BMP12/13/14 [180, 181]. This classification was made based on the function of BMPs determined by genetic mutations (see [182] for a detailed review).

BMPs are synthesized as large precursors of about 400-500 amino acids. They consist of an N-terminal signal peptide that directs secretion, a prodomain for proper folding and a C-terminal mature peptide [183, 184]. Carboxy terminal mature proteins are proteolytically cleaved after dimerization of the precursor. They are cleaved in the prodomain at an Arg-X-X-Arg sequence by serine endoproteases, except for BMP4, which is cleaved by Furin, PC6 and PC7 [185]. Active BMPs contain 50-100 amino acids

with seven cysteines. Six of these cysteines form three intramolecular disulphide bonds known as cysteine knots. The seventh cysteine is necessary for dimerization with another BMP monomer by forming a covalent disulphide bond, thus forming a biologically active signaling molecule [186]. With a few exceptions, most BMPs function as homodimers or heterodimers. BMP2 and BMP4 are constituents of serum [187-189].

As BMPs are so potent, there are several ways to modulate their activity. Extracellular antagonists such as noggin, chordin and follistatin regulate the BMP pathway. Noggin limits the activity of BMP4 by binding to BMP4. This interaction prevents BMP4 from binding to the cognate receptor [190, 191]. The BMP pathway is also inhibited by I-Smads. These Smads bind to the type 1 receptors, thus preventing the phosphorylation of R-Smads by the receptors and preventing the Smad-Smad or Smad-receptor complex formation. Smads are also regulated by protein degradation via the ubiquitin proteasome. Smurf1 and Smurf2 are Smad-specific E3 ubiquitin ligases that selectively interact with BMP R-Smads and mediate their ubiquitination and proteasomal degradation [192, 193]. There are also co-transcriptional repressors which suppress BMP signaling [193]. One repressor, ski can bind to the Smad protein complexes that form in response to BMP. ski represses the ability of BMPs to activate BMP target genes through disruption of a functional Smad complex and through recruitment of transcriptional co-repressors [194].

BMPs bind transmembrane type 1 and type 11 receptors [195, 196]. These receptors encode serine/threonine kinases made up of a short extracellular domain with 10-12 cysteine residues, a single transmembrane domain and the intracellular serine/threonine kinase domain. The BMP type 1 receptors include Activin receptor like

kinases 1-7 (ALK1-7) and the type 2 receptors are BMPR2, ActR11b and ActR11b [195]. BMPR2 is specific for the activation of the BMP pathway. ALKs are classified into three groups based on similarities in their structure and function. The BMPR-1 group includes ALK3 and 6, the ALK1 group includes ALK1 and 2, and the T β RI group includes ALK4, 5 and 7 [175]. The ALK1 group of receptors activates Smad1/5/8 and transduces similar intracellular signals, while those of the T β RI group activate Smad 2/3. Type 2 receptors affect the specificity of BMP binding to type 1 receptors [197]. BMP2 and BMP4 typically activate ALK3 and 6.

Receptors bind with different affinities to individual BMPs. Upon binding of the activated ligands to their receptor there is an increase in oligomerization of the receptors and possible conformational changes. When the activated ligand binds the constitutively active type 2 receptor, the type 2 receptor phosphorylates the type 1 receptor in its glycine and serine-rich domain [198]. This phosphorylation activates the type 1 receptor transforming it to an active conformation.

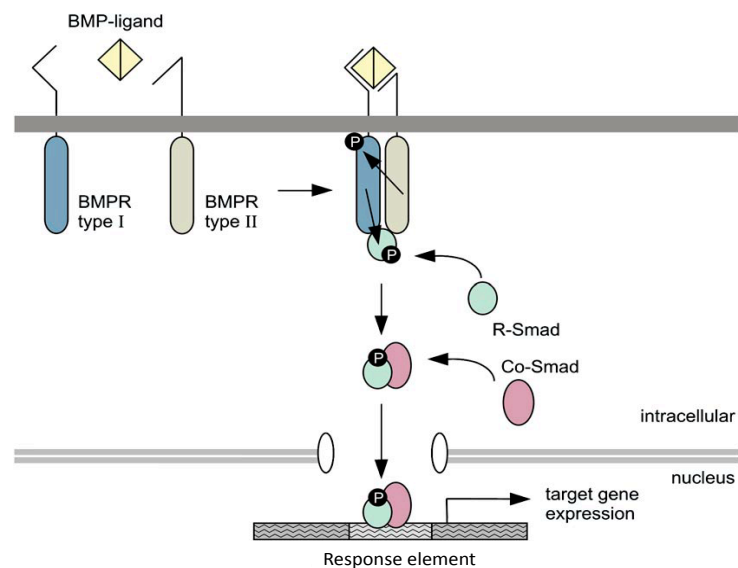


Figure 4. The activation of BMP target genes through the BMP pathway
Balemans and Van Hul, *Developmental Biology*, 2002 [199]

The L45 region in the kinase domain of the activated type 1 receptor phosphorylates the receptor-regulated R-Smads, Smad1, Smad5 or Smad8 in the cytoplasm. The phospho-R-Smad then complexes with the common Smad, co-Smad4. This R-Smad/Smad 4 complex moves to the nucleus where it binds to regulatory regions of target genes [200] (Figure 4). BMPs are capable of activating non-Smad pathways such as the Phosphoinositide-3-Kinase [201, 202], however, for our purpose we will focus on the Smad dependent pathway.

BMP and cancer

BMP plays a key role in the growth of gastrointestinal, epithelial and developmental cancers [203]. Depletion of BMPR2 by RNA interference in mice resulted in gastrointestinal hyperplasia as well as dysmorphogenesis and predisposition to angio-proliferative diseases [204]. Conditional inactivation of BMPR2 leads to colorectal epithelial overgrowth and polyp formation [205]. Mutations in ALK3 as well as Smad4 have been found in some patients with autosomal dominant syndrome juvenile polyposis, an autosomal dominant disorder caused by these mutations [206].

Regulation of the Id1 promoter by the BMP pathway

In 1999 the Id genes were identified as direct targets of BMP [207]. Id1-3 were activated by BMP4 in a number of cell lines. This activation was very rapid and independent of new protein synthesis. In addition, actinomycin D inhibited this response. This indicates that the induction of the Id genes by BMP4 is regulated at the transcriptional level [207]. In the years following many researchers studied the role of

BMP on Id1 activation. In 2002 for example, Valdimarsdottir *et al.* demonstrated that Id1 stimulation by BMP is necessary and sufficient for BMP induced activation of endothelial cells [208]. BMP2 also enhances the expression of Id1 in osteoblastic cells [209].

Katagiri *et al.* demonstrated that BMP2 activates Id1 gene expression within an hour of BMP2 treatment during the differentiation of C2C12 myoblasts into osteoblasts. In 2002 three groups identified a BMP responsive element in the Id1 promoter. Katagiri *et al.*[210] identified a 29 bp GC rich element between -985 and -957 of the human Id1 promoter as a BRE. This was identified in C2C12 myoblastic cells. They found that -985 to -957 of the human Id1 promoter was necessary for Id1 activation by BMP2. This region showed homology to the mouse Id1 promoter in a region identified by Tournay and Benezra [127] as an Id1 expression element, which contains an Egr1 binding site (Figure 5).

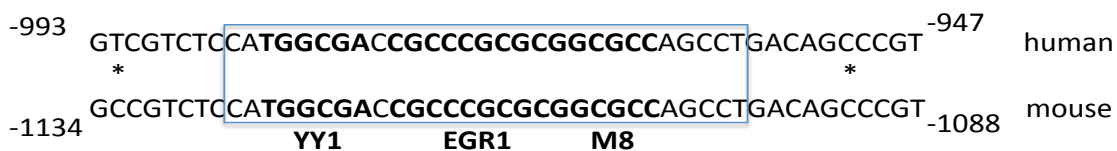


Figure 5. Sequence similarity between the hId1 and mId1 promoter.

Egr1 and M8 are sites identified by Tournay and Benezra. M8 is a GC rich region identified in [127] as being important in Id1 serum expression. * indicates a difference in the human and mouse promoter nucleotide sequence. The blue box shows the 29 bp BRE.

The 29 bp region was both necessary and sufficient for BMP2 induction. Taken together this group identified a BRE in the hId1 promoter. The BRE encompasses the Egr1 binding site [210].

Around the same time another group identified a BRE in the mouse Id1 promoter [29]. Korchynskyi and ten Dijke, 2002 [29] demonstrated that mRNA expression of Id1

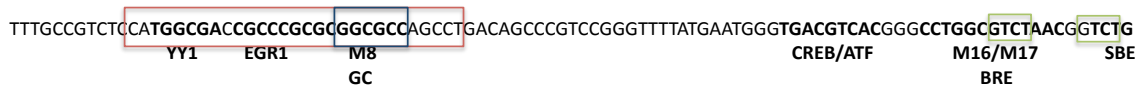


Figure 6. 100 bp conserved region of Id1.

Known consensus sites for binding factors. The blue box is the BRE in [29] while the red box is the element identified by Katagiri *et al* [210]. The green boxes represent the SBEs, which are necessary for Id1 expression. M8 is a GC rich region identified in [127] as being important in Id1 serum expression. M16/M17 was also identified as an important in serum regulation of Id1 [127].

increased in C2C12 muscle cells by treatment with BMP2, BMP6 and BMP7. This increased expression was an IEG response that was transcriptionally regulated. The mouse and human Id1 promoters were activated by BMP2, BMP4, BMP6, and BMP7. This response was mapped to two regions of the Id1 promoter; one contains two SBEs and the other a palandromic sequence, GGCGCC. The BRE was located between -1133 and -1070 of the mouse Id1 promoter. This overlaps the region identified by Katagiri *et al.* [210] (Figure 6). This group also showed that the Smad4 binding motifs CAGC and CGCC are needed for optimal Id1 expression. One of these elements was located in the M16-M17 region that was previously identified as critical for Id1 serum expression [127] (Figure 6). None of the elements was sufficient for Id1 expression suggesting that they work together. Yet another group identified the same region, in addition to downstream SBEs as important for Id1 promoter regulation by BMPs [164]. These groups show that the GC rich/Egr1 binding region and several SBEs are important for Id1 expression. However, it was not clear which is the key element.

The coordinated expression of the Id family by factors was suggested after a common BRE was proposed in the Id family [211]. Two groups later discovered BREs in Id2 [212] and Id3 [213] which are necessary for BMP induction of these genes. An element similar to the element identified in Id3 is in the same region as the M16-M17 and

SBE region identified by Tournay *et al* [127]. This region is well conserved between species and among the Id family members. This indicates that the M16-M17 and SBE regions may be very important for Id1 regulation.

In 2012, Kurooka *et al.* [214] showed that Id2 is inducible by BMP2 through the BRE in its promoter. They showed that BMP2 in serum is responsible for the serum regulation of the Id2 gene. This BRE is identical to the one identified in Id3 that is critical for BMP induction and is in the M16-M17 and SBE region. The element responsible for serum regulation of the Id1 gene was not identified.

Id1 and Cancer

The first evidence of Id1's involvement in cancer came in 1991 when Alani *et al.* [215] demonstrated that overexpression Id1 in human keratinocytes resulted in induction of cell proliferation, inhibition of cellular senescence and differentiation, prolonged life span and eventually immortalization. Telomerase activity in these cells was activated. These are some hallmarks of cancer, therefore, Id1 was implicated in tumorigenesis. Subsequently, Id1 deregulation has been observed in many kinds of cancer including ovarian [216, 217], colon [218], breast [219, 220], thyroid [221, 222] and other cancers. Although Id1 is involved in many areas of tumorigenesis (Figure 8), I briefly will discuss its involvement in two key steps of tumorigenesis, namely metastasis and angiogenesis.

Id1 in tumor metastasis

Tumor invasion involves invasion of the tumor into adjacent or distal sites. This results in metastasis. One important step in metastasis is the dissolution of the Extra Cellular

Matrix (ECM). A family of matrix metalloproteinases is important in the regulation of ECM degradation [223] and cancer progression. Id1 is often upregulated in cancers [224]. Desprez *et al.* found that constitutive expression of Id1 resulted in the upregulation of a MMP protein [225]. It's also been demonstrated that Id1 is one of the key regulators of breast cancer metastasis [226]. Id1 plays a role in epithelial mesenchymal transition (EMT) [227]. EMT is critical for metastasis, which is one of the reasons why people die from cancer.

Id1 in angiogenesis

Angiogenesis provides the blood supply necessary during the growth of the tumor and during metastasis. Many factors such as HIF [228] and VEGF [229] are involved in this process. Studies in knockout mice showed that Id1 expression is essential for angiogenesis during tumor progression [230]. Loss of capillary branching was observed in Id1/Id3 double knockout mice. VEGF was downregulated in the endothelial cells of the knock out mice. VEGF has been identified as a downstream target of Id1 [231]. It has also been observed that Id1 confers angiogenic properties on fully differentiated endothelial cells, contributes to therapeutic angiogenesis [232] and vascularization of tumor xenographs [230]. Id1 expression can be used to mark endothelial progenitor cells that are critical to tumor growth and angiogenesis [230, 233].

Following is a survey of the other areas of Id1 involvement in tumorigenesis:

Id1 promotes lung cancer growth in a BMP2, Smad 1/5 dependent manner [234]. Id1 has been proposed as a molecular target in breast cancer [235] and ovarian cancer [236]. Id1 can localize to centrosomes and induce abnormal centrosome numbers in human

primary cells and tumor cell lines [237]. Id1 protein is required for BCR/ABL-mediated leukemogenesis [238]. Id1 is necessary to confer self-renewal capacity of cancer stem cells [218, 239]. All of these lines of evidence show that Id1 plays varying roles in carcinogenesis. In fact the high expression of Id1 in cancer has been associated with poor prognosis [240] and is considered a prognostic factor in some kinds of cancer.

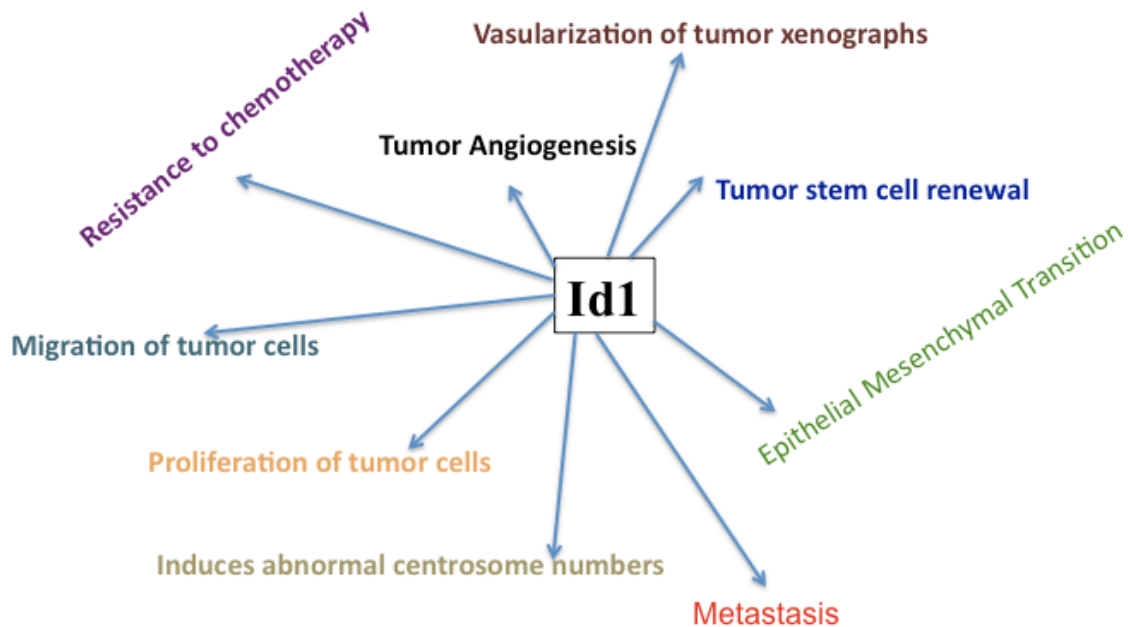


Figure 8.

Involvement of Id1 in cancer.

Id1 is involved many processes important in the biology of cancer formation.

We have found that the serum induction of Id1 is independent of SRF, the MAP kinase and the PI3 Kinase pathways. Therefore, we predicted that Id1's serum regulation is modulated through a novel serum pathway. Here we show that a previously identified element [29, 127] in the Id1 promoter is necessary and sufficient for serum regulation of

the Id1 promoter in NIH3T3 cells. We also demonstrate that the Src kinase inhibitor AZD0530 directly inhibits the BMP type 1 receptors and serum induced Id1 expression. AZD0530 treatment decreases the expression of Id1 in the colon cancer cell line HCT116 in a phospho-Smad1/5/8 dependent manner.

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Chapter 2

Serum regulation of Id1 expression by a BMP pathway and BMP Responsive Element

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Abstract

Background

Immediate Early Genes (IEGs) are expressed upon re-entry of quiescent cells into the cell cycle following serum stimulation. These genes are involved in growth control and differentiation and hence their expression is tightly controlled. Many IEGs are regulated through Serum Response Elements (SREs) in their promoters, which bind Serum Response Factor (SRF). However, many other IEGs do not have SREs in their promoters and their serum regulation is poorly understood.

Results

We have identified SRF independent IEGs in SRF-depleted fibroblasts. One of these, Id1, was examined more closely. We have mapped a serum responsive element in the Id1 promoter and find that it is identical to a BMP Responsive Element (BRE). The Id1 BRE is necessary and sufficient for the serum regulation of Id1. Inhibition of the BMP pathway by siRNA depletion of Smad 4, treatment with the BMP receptor antagonist noggin, or treatment with the BMP receptor inhibitor dorsomorphin all blocked serum induction of Id1. Further, BMP2 is sufficient to induce Id1 expression. Given reports that Src inhibitors can block Id1 expression, we tested the Src inhibitor, AZD0530, and found that it inhibits the serum activation of Id1. Surprisingly, this inhibition is independent of src or its family members and AZD0530 directly inhibited BMP type I receptors.

Conclusion

Serum induction of the Id1 gene required the BMP pathway from BMP receptors to binding of Smads to BREs in the Id1 promoter. The Id family of IEGs is regulated by BMPs in serum through similar BREs. This represents a second pathway for serum regulation of IEGs.

Key words

Immediate Early Gene

BMP Response Element

Id1, Inhibitor of DNA binding/differentiation 1

Serum Response Element

AZD0530, Saracatinib

Background

When growth factors are removed from NIH3T3 mouse fibroblasts they become quiescent. Upon re-stimulation with serum or specific growth factors, these cells re-enter the cell cycle. A class of genes, Immediate Early Genes (IEGs), is expressed rapidly and transiently upon treatment with serum or growth factors [10-13, 241]. The expression of these genes is independent of new protein synthesis, suggesting that it is a relatively direct response to serum induction. IEGs are involved in a plethora of processes including growth [17], differentiation, lineage determination, learning and memory [21]. c-fos, c-jun and c-myc are among the earliest and most well studied IEGs. The regulation of these IEGs is tightly controlled, and misregulation can lead to many diseases including cancer, neurological disorders [24] bone remodeling disorders [25] and other chronic diseases. As reviewed in Dunn et al. [26], IEG expression in many cancers is found to be sustained and abnormally high. Understanding the transcriptional regulation of IEGs is an important step in understanding how their deregulation results in disease and finding better therapies to counter them.

Hundreds of experimentally validated or hypothesized genes have a Serum Response Element (SRE) or CArG box in their promoter region [27-30]. The MADS box family member, Serum Response Factor (SRF), binds to the SREs of these genes [24, 41, 42, 46]. SRF is constitutively present at the promoters of the genes it regulates [57]. When quiescent cells are stimulated with the growth factors in serum, two pathways sufficient for SRE activation are activated, the mitogen activated protein kinase (MAPK; ERK1/2) and the RhoA GTPase pathways. The MAPK pathway, through a cascade of factors, leads to the phosphorylation and activation of SRF co-factors, the ternary

complex factors (TCFs) Elk1, Sap1 and Net [22, 58]. SRF is also activated by the small GTPase RhoA via another group of SRF co-transcriptional activators, the myocardin related factors, Megakaryoblastic Leukemia 1/2 (MKL1/2) [59-62]. RhoA activation leads to changes in the actin cytoskeleton, which directly results in changes in the nuclear localization and activation of MKL1/2 and therefore activation of SRF target gene expression [90, 92-94, 242].

Some IEGs do not have clear SREs in their promoters. Their induction may be due to cryptic or distant SREs or entirely different pathways. As described here, some IEGs do not require SRF for their serum induction. It would be interesting to find out whether there is another common sequence element or pathway through which these SRE-lacking, SRF-independent IEGs are regulated. Inhibitor of DNA binding/differentiation 1 (Id1) is a member of this group.

Id1 is a member of the Helix Loop Helix (HLH) family of transcription factors [121, 128], which form heterodimers with other members of the HLH family. The Id1 protein lacks a basic DNA-binding domain but is still able to form heterodimers with other HLH proteins that contain basic domains (bHLH proteins) [132]. These heterodimers are unable to bind DNA, thereby inhibiting the transcriptional activity of the bHLH proteins.

Id1 is ubiquitously expressed [134] and is regulated by the TGF- β super-family of transcription factors. Id1 expression is increased by prolonged exposure to TGF- β 1 in human epithelial cells [146]. Smad3 and ATF binding elements in the Id1 promoter mediate this regulation. Id1 is also activated by TGF- β 1 in the human mammary gland cell line, MCF10A [147]. Smad3 also mediates this regulation. Several groups have

shown that Id1 expression is also increased in response to BMP signaling [152-155]. Sequences in the Id1 promoter responsible for BMP activation were mapped to two close but distinct regions [29, 172, 210]. Subsequently, common BMP responsive sequences were found for the Id family of genes in *Xenopus* (TGGCGCCAG-N₃-GTCTG) that were conserved in mammals [169]. The element mutated by Korchynskyi et al. partially matches this consensus [29]. We refer to this sequence at -1067 to -1050 in the mouse Id1 promoter as the BMP responsive element (BRE). Overall expression of Id1 was shown to be regulated by an Egr-1 binding site upstream of the BRE [127]. Expression of Id1 in cells grown continually in serum-containing media vs. low serum media was reduced by mutations (m16 and m17) in the BRE region, however it was not clear what factors or pathways activated through this element [127]. It was also not clear whether rapid serum induction acted through this region.

BMP is a member of the TGF β family of transcription factors. Upon activation of BMPs, they bind to their transmembrane type I and type II receptors [195]. These receptors encode serine/threonine kinases. The activated type I receptor phosphorylates the receptor-regulated R-Smads, Smad1, Smad5 and Smad8 in the cytoplasm. The phospho-R-Smad then complexes with the common Smad, Smad4. This R-Smad/Smad 4 complex moves to the nucleus where it binds to regulatory regions of target genes [200]. Here we show that a previously identified element [29, 127] in the Id1 promoter is a BMP responsive element and is also necessary and sufficient for serum regulation of the Id1 promoter in NIH3T3 cells.

Id genes are required for G1 progression and regulate cellular senescence [143]. Deregulation of Id1 expression is observed in many kinds of cancer including ovarian

[216, 217], colon [218], breast [219, 220], and thyroid [221, 222] cancers. Id1 promotes migration and proliferation of cancer cells *in vitro* [243, 244], confers angiogenic properties on fully differentiated endothelial cells, contributes to therapeutic angiogenesis [232] and is required for angiogenesis [245], neurogenesis and vascularization of tumor xenographs [230]. Further, it has been demonstrated that Id1 promotes lung cancer growth in a BMP2, Smad1/5 dependent manner [234]. Id1 has been proposed as a molecular target in breast and ovarian cancers [235, 246]. Here we demonstrate that the src kinase inhibitor, saracatinib (AZD0530), directly inhibits the BMP type 1 receptors and is able to reduce the phosphorylation of Smad1/5/8 and the expression of Id1 in the colon cancer cell line HCT116. Therefore, this and other drugs that inhibit the BMP receptors may have therapeutic potential in cancers, which have perturbed BMP signaling, and increased Id1 expression.

Results

Serum induction of Id1 is independent of SRF

In order to identify IEGs whose expression was independent of SRF we made stable cell lines expressing shRNA lentiviral vectors that target SRF in NIH3T3 mouse fibroblasts. The expression of SRF was significantly decreased in two clones (shSRF-1 and shSRF-2), decreasing SRF mRNA expression by at least 70% at all time points of serum induction (Fig. 1B). The decrease in expression was stronger at the protein level (Fig. 1A) with stronger reduction of SRF in shSRF-2. NIH3T3 cells containing shRNA that does not target any known mouse gene was used as the control cell line. We analyzed the serum regulation of many IEGs in NIH3T3 cells induced with serum for 0 to 2 hours following overnight serum starvation. The transcript levels of 19 IEGs that were previously identified in NIH3T3 cells [30] were measured by quantitative RT-PCR (qPCR). Not surprisingly, the serum regulation of many IEGs such as CTGF, *egr-2*, and *nur77* were SRF dependent while others such as *Id1*, *Id3* and *mig6* were SRF independent (Fig. 1B and supplemental figure 1). Surprisingly the expression of *c-fos*, the longest studied SRF target gene, was only slightly affected. This may be because residual SRF was sufficient to facilitate *c-fos* expression or that there is another mechanism by which *c-fos* can be serum regulated (see Discussion). *cyr61* induction also appeared SRF-independent, despite previous mapping of SREs in its promoter [36]. As with *c-fos*, it may only require low levels of SRF or use a different mechanism. Other genes with previously reported SREs, such as *vcl*, *nur77*, and *egr2* were in-fact SRF dependent (Figs. 1 and S1 and supplemental table 1.)

While, the level of SRF expression is significantly reduced by use of shRNAs targeting SRF, the residual SRF mRNA is still serum inducible (Fig. 1B). This is not surprising as SRF itself is an IEG. In SRF depleted cells, however, many of the genes appeared SRF-independent. (Fig. 1, Fig. S1 & supplemental table 1). Of these, the serum induction of Id1 actually increased upon depletion of SRF at the 30 and 60-minute time points (Fig. 1B). This and the lack of putative SREs in the Id1 gene suggest that Id1 induction is SRF-independent. Consequently, we proceeded to identify sequence elements and factors responsible for the serum regulation of the Id1 IEG.

Serum induction of Id1 does not require the PI3K or the MAPK pathway

SRF target genes are regulated through the RhoA or MAPK pathways. We studied whether these or other known pathways are involved in serum regulation of Id1 by utilizing pathway inhibitors. The serum induction of Id1 was assayed in the presence and absence of phosphoinositide-3-kinase (PI3K) inhibitor LY294002 and MAPK pathway MEK1 inhibitor, PD0325901. NIH3T3 cells were pretreated for 1 hour with either LY294002 or PD0325901, followed by serum induction for 0 to 120 minutes. The potency of the drugs was confirmed by immunoblotting for phospho-AKT and phospho-ERK1/2, respectively (Fig. 2). The RNA levels of Id1 were unchanged when treated with the inhibitors (Figs. 2A and B). The need for ERK1/2 in c-fos serum regulation is well documented, at least partially due to the phosphorylation of the ELK1 family members [241]. Not surprising, c-fos expression was dramatically decreased when NIH3T3 cells were treated with PD0325901, while treating the cells with LY294002 had little effect on c-fos expression (Fig. 2). To study the RhoA pathway we utilized the RhoA inhibitor, C3

transferase [247], using the Id1 reporter gene as described below. There was no effect on Id1 promoter activation by serum while c-fos promoter activation was inhibited in the presence of a C3 transferase expression vector (data not shown). These results suggest that Id1 serum regulation does not require the ERK1/2, RhoA nor PI3K pathways.

Mapping of sequence elements responsible for serum induction of Id1

In order to decipher the factor(s) and pathway(s) involved in the serum regulation of Id1, promoter mapping of the mouse Id1 promoter was undertaken in NIH3T3 cells. We found that a construct containing -1577 to +54 of the mouse Id1 gene was sufficient to mediate nearly a 5 fold serum-induced increase in luciferase expression (Fig. 3A, B). A series of deletion constructs were made between -1577 and -1050 of the mId1 promoter (Fig. 3A). This allowed us to map a region required for serum activation to a 100 bp region between -1150 and -1050 (Fig. 3B). This region of the promoter is particularly conserved as shown by the alignment of the mouse, human and chicken Id1 promoters (Fig. 3C). Critical elements have previously been identified in this region for expression in cells grown in serum containing medium compared to cells starved for serum [127]. These and other studies identified binding sites for YY1, Egr1, CREB/ATF and a Smad binding element (SBE). In addition, systematic point mutation identified the M8 and M16/M17 regions as being required for expression [127]. In order to identify the sequence element(s) important for serum regulation of the Id1 promoter, a series of mutations was made in this 100bp region of Id1 (Fig. 4A, B). Mutation of most of the known sites had some effect, but also retained some serum induction (Fig. 4D). The most striking difference was observed with mutation of the M16/M17 region (mBRE; Fig. 4D).

This region was previously identified as a Smad responsive element [29] and is similar to regions in the Id2 and Id3 promoters required for BMP induction [213, 214] (Fig. 4C).

We will therefore refer to it as a BMP Response Element (BRE). We made finer mutations across the BRE and found that each reduced expression, except for those on the downstream part of the site that overlap the spacer region of the consensus sequence (mBRE-R-b; Fig. 4E). Other mutations in the element (BRE-L and BRE-R) showed that the consensus region is needed for optimal Id1 serum induction (Fig. 4E).

The conserved region of -1150 to -1050 was sufficient to mediate serum induction when placed upstream of an SV40 promoter (Fig. 5B). However, a single copy of the BRE was not sufficient, as a -1177 to -1050 construct (that lacks the YY1-Egr1-GC region) did not mediate serum induction. When four copies of the BRE were placed in front of the SV40 promoter, there was basal expression but it was not inducible by serum (Fig. 5). While the SBE region was also not sufficient, the combination of the BRE and SBE was sufficient for serum induction. This is not surprising as the BRE-SBE combination is conserved in the Id1, 2 and 3 genes (Fig. 4C) and the sequence matches the sequence which is thought to be important for the binding of the co-Smad, Smad4 [158]. It is likely that while the R-Smads bind the BRE, the SBE is bound by co-Smad4. Our efforts to show specific Smad binding in this region of DNA were unsuccessful. We and others have seen weak specific binding of a factor in the BRE – SBE region by electrophoretic mobility shift assays (EMSA) (unpublished results and [127]), but we were unable to successfully identify the factor(s) which, bind this region. Nevertheless, given previous analysis of Smad binding to BRE sequences, it is likely that Smad4 binds with Smad1, 5 or 8 [158, 214].

It has been reported that bovine serum contains a BMP-like factor [248]. This and the requirement of the BRE sequence suggest that the BMP-Smad pathway is mediating serum induction of Id1 expression. To further test this model, we utilized purified BMP2 and known inhibitors of the BMP pathway. BMP2 induced Id1 expression in NIH3T3 cells about twice as strongly as serum (Fig. 6A), confirming that the BMP pathway is sufficient for induction of Id1 and that there are receptors for BMP2 on NIH3T3 cells. BMP2 was also able to activate the Id1 promoter similar to serum in a reporter assay (Fig. 6B). Not surprisingly, serum and BMP2 both induced the phosphorylation of Smad1/5/8 in starved NIH3T3 cells (Fig 6C). We also used the BMP receptor antagonist noggin to study its effect on Id1 serum induction. Noggin-treatment of NIH3T3 cells abolished serum induction of Id1 (Fig. 6A). Noggin also abolished serum activation of the Id1 promoter in a reporter assay (Fig. 6B). This was confirmed by the use of the small molecule inhibitor, dorsomorphin, which is a known inhibitor of BMP receptors [249]. Dorsomorphin strongly inhibited induction of Id1 mRNA in response to serum treatment (Fig. 6D). Finally, we depleted NIH3T3 cells of the co-Smad regulator, Smad4, with siRNAs. Smad4 mRNA was reduced at least 80% by the siRNAs. This strongly impaired serum induction of Id1 mRNA (Fig. 6E). Therefore, these experiments show that the BMP pathway is indispensable for serum induction of Id1.

The src inhibitor AZD0530 inhibits Id1 expression

We were prompted to examine the possibility of the tyrosine kinase Src's involvement in Id1 regulation as the Src inhibitor AZD0530 (sarcatinib) was found to inhibit Id1 expression in the A549 lung carcinoma cell line, while the Src inhibitor PP2

inhibited Id1 expression in the MDA-MB-231 breast carcinoma line [154, 250]. AZD0530 inhibited Id1 expression while preventing activation of Smad1/5 [154]. We investigated whether AZD0530 inhibits the serum induction of Id1. NIH3T3 cells pretreated with AZD0530 showed a complete loss of Id1 serum induction both in mRNA expression and promoter activation (Fig. 7A and B). Induction of both the longer -1150 Id1 reporter and the shorter 4X BRE-SBE reporter were blocked by AZD0530, while as a control, there was little effect on induction of the c-fos promoter (Fig. 7B). We tested for AZD0530 sensitivity on other IEG expression. Id3 was strongly reduced (Fig. 7A), consistent with it containing a BRE-like sequence [213]. There was no effect on c-fos induction (Fig. 7A). The IEG, PAI-1, on the other hand, showed a decrease in serum activation, suggesting that it is partially regulated by an AZD0530 sensitive pathway, similar to Id1.

Surprisingly, the serum-induced autophosphorylation of Src at Tyrosine 416 was unaffected at the concentration of AZD0530 that inhibited Id1 expression (Fig. 8A). We sought to further examine whether src family members are involved in Id1 serum regulation. Neither another Src inhibitor, PP2, nor the BCR-abl inhibitor, imatinib, had any effect on the response of the Id1 gene to serum (data not shown). In addition, we tested SYF cells, which are fibroblasts deficient in the expression of src family members src, yes, and fyn [251]. Serum induction of Id1 was normal in the SYF null cells and there was actually higher serum induction of Id3 in these cells (Figure 8B). src, yes and fyn are the three predominant Src family members expressed in fibroblasts, though it is possible that low expression of other family members could be involved in Id1 regulation. However, since Src phosphorylation was not affected by AZD0530, since loss of the

three Src family members had no effect, and since other src inhibitors did not block Id1 expression, we considered the possibility that AZD0530 was inhibiting Id1 expression through another mechanism.

Since earlier studies have shown that Smad1/5/8 are involved in Id1 expression and that these Smads are necessary for BMP signaling [152, 154], we studied whether treating NIH3T3 cells with AZD0530 affects the phosphorylation status of these Smads. AZD0530 blocked the phosphorylation of phospho-Smad1/5/8 suggesting that it blocks a component upstream of Smad1/5/8 phosphorylation (Fig. 8A). The total level of Smad1/5/8 was not affected by treatment with AZD0530 (data not shown). As a control, there was no effect on phospho-ERK1/2 induction.

One possibility is that AZD0530 directly inhibits the activity of BMP receptors involved in BMP signaling. We tested this hypothesis by testing the inhibition of BMP receptor kinase activity by AZD0530 *in vitro*. The BMP family of receptors consists of two subunits. The type 2 receptor is common, BMPR-2, while there are seven type 1 receptors, termed ALK1 to 7 [182, 184, 195]. There was no inhibition of BMPR-2 kinase activity *in vitro*, however there was strong inhibition of ALK1, 2, and 3 with IC₅₀ values in the 3-30 nM range (Fig. 9). Inhibition of ALK4, 5 and 6 was less sensitive, with IC₅₀s in the 300-800 nM range. For comparison, inhibition by a non-specific protein kinase inhibitor, staurosporine, varied, but was closer to the micromolar range (Fig. 9B). ALK1, 2 and 3 are receptor subunits for the BMP family members BMP2, 4 and 6, which can each activate Id1 [159, 252].

We sought to confirm this inhibition of BMP receptors by AZD0530 *in vivo* using a reporter assay. We transfected constitutively active forms of each of the BMP receptors

[29, 253] with the Id1 reporter gene. We found that the BMP responsive ALKs, ALK1, 2 and 3 activated the Id1 promoter in the absence of serum, while there was no activation by ALK4, 5 and 7 (Fig. 10). The activation by ALK1, 2 and 3 was significantly decreased in the presence of AZD0530. The limited inhibition compared to that with rapid serum induction may have to do with the long-term transfection of the constitutively activated receptors. It is also possible that AZD0530 is less effective at inhibiting the constitutively activated forms compared to the normal forms of these proteins.

Finally we sought to check the efficacy of AZD0530 in blocking Id1 expression in a colon cancer cell line, HCT116, which exhibits high Id1 expression. We found that Id1 expression and Smad1/5/8 phosphorylation were constitutively elevated in these cells (Fig. 11A and B). Upon starvation of HCT116 cells and serum stimulation, there was some further increase in phospho-Smad1/5/8 levels, but there was no further increase in Id1 expression, demonstrating that Id1 expression is misregulated in these cells. Treatment with AZD0530 or dorsomorphin resulted in the inhibition of phospho-Smad1/5/8 levels as well as inhibition of Id1 expression. These results show that AZD0530 can be used to reduce Id1 levels in cancer cells and suggest that altered Id1 expression is dependent upon the BMP pathway.

Discussion

There are many IEGs that do not have clear SREs in their promoters. Whether there is a common pathway/DNA element by which some of these genes are regulated by serum is yet unknown. We sought to identify genes which do not contain SREs and whose serum regulation is independent of SRF. Many SRE containing genes were found to be SRF dependent. Unexpectedly c-fos, the canonical SRF regulated gene, was not SRF dependent. This may indicate that the residual SRF is sufficient to allow c-fos induction. SRF binding to the c-fos SRE in the SRF depleted cells was reduced by 50-70% in chromatin immunoprecipitation assays such that this residual binding may be sufficient for induction (unpublished results). It is possible that due to the different affinities of SREs for SRF that certain IEGs, such as c-fos, require less SRF. Alternatively, there may be another pathway for c-fos induction in NIH3T3 cells. Loss of the SRF alleles in ES cells resulted in a strong reduction of serum-induced c-fos expression [56]. However, there was low residual serum induction in these cells, supporting the existence of an alternative pathway. Understanding whether there is a lower SRF threshold or an alternative pathway for c-fos regulation will be important.

We found that the serum regulation of Id1 and Id3, members of the HLH group of transcription factors, was independent of SRF. The serum induction of Id1 and Id3 both increased when SRF was depleted by shRNAs and was independent of the ERK1/2, RhoA and PI3K pathways. This suggests that there is a novel pathway through which these genes are regulated by serum as SRF. Many IEGs are regulated by one or more of these pathways [60, 241]. In addition, there are no SRE sequences found in the Id1

promoter and no SRF binding was observed in genomic chromatin immunoprecipitation experiments [254, 255].

We subsequently identified a BRE in the promoter of Id1 as being responsible for its serum activation. Although the element in Id1 was identified previously [29, 210], its link to serum activation of Id1 was unknown. A BRE is a GC rich sequence, i.e. GCCGNC or GRCGNC to which BMP responsive Smads bind [164, 165, 256]. It also pairs with a Smad4 binding element (SBE)(GTCT) with a spacer of five bases between the sites (Fig. 4C; [257]). These BMP responsive elements are found in BMP target genes such as Id1 [29], Id2 [214] and Id3 [213]. BREs have been identified across the phylogenetic spectrum, found for example in *Drosophila*, *Xenopus* and mammals [258].

We were able to show that the BRE-SBE element is necessary and sufficient for the serum activation of Id1. This BRE located at -1073 to -1056 in the mId1 promoter, is located within a highly conserved 100 bp region, -1150 to -1050. Although many other transcription factors bind to the Id1 promoter within this 100 bp region, mutation of the BRE had the greatest effect on serum activation of the Id1 promoter. It is quite conceivable that the BRE works with other sequences to obtain optimal serum induction. There were partial effects on Id1 expression when most of these sequences were mutated. Considerable evidence supports the serum activation of Id1 through the BMP pathway and the BRE. The mId1 promoter was activated by BMP2, inhibited by the BMP receptor antagonist noggin, and blocked by the BMP receptor inhibitor dorsomorphin. We show that the BMP receptor inhibitor dorsomorphin inhibits the phosphorylation of Smad1/5/8 in NIH3T3 cells while the total level of Smad1/5/8 was unchanged. Depleting the co-Smad, Smad4, also resulted in decreased activation of Id1. These results

confirmed the involvement of Smads and BMP in serum induced Id1 expression. Recently, it was shown that BMP signaling is also required for serum induction of the Id2 gene [214]. It will be interesting to determine whether the BMP pathway also activates other IEGs. We found strong dependence with the Id3 gene and partial dependence with the PAI-1 gene, but no requirement with the other SRF-independent genes tested.

AZD0530 is a well-documented inhibitor of the Src family. As reported previously [154], the treatment of NIH3T3 cells with AZD0530 caused a dramatic decrease in serum-induced Id1 expression. However, we found that AZD0530 under our conditions (a lower concentration and briefer incubation) did not block Src phosphorylation at a site thought to be due to autophosphorylation. In addition, serum induction of *Id1* was unaffected in SYF cells, which lack the three most abundant Src family members, *src*, *yes* and *fyn*. On the other hand, we found that AZD0530 specifically blocked serum induced Id1 expression, Id1 reporter gene expression and Smad1/5/8 phosphorylation. *In vitro* kinase assays showed that AZD0530 directly inhibits the BMP type I receptors, ALK1, 2 and 3. We confirmed inhibition of these ALKs *in vivo* by AZD0530 using constitutively activated forms of the receptors with the Id1 reporter genes. Together these experiments suggest that AZD0530 blocks activation of the Id1 promoter by direct inhibition of the BMP receptor subunit rather than by inhibition of the Src family.

Increased Id family expression is a hallmark of many cancers [259-261] and Id1 overexpression and BMP constitutive activation [246] is associated with tumor angiogenesis in human pancreatic [262] and other cancers [217, 263, 264]. As we have shown that the small molecule AZD0530 inhibits the serum induction of Id1 at low

concentrations through inhibition of the BMP receptors, AZD0530 may have therapeutic potential in targeting this pathway in addition to its effects on Src.

To check this hypothesis we looked in HCT116 colon cancer cells, which are known to have elevated levels of Id1 [265]. We found constitutive, serum-independent, expression of Id1 in these cells and high basal phospho-Smad1/5/8 levels. Treatment of these cells with AZD0530 or dorsomorphin blocked phosphorylation of Smad1/5/8 and expression of Id1. These results suggest that AZD0530 may be a promising drug for treatment of cancers in which Id1 is constitutively active due to deregulation of the BMP pathway.

Finally, we propose a model of IEG activation in which members of the HLH family of transcription factors are activated by serum through the presence of BREs in their promoters. When quiescent cells are treated with serum, BMPs in serum bind to and activate the BMP receptors. This results in the phosphorylation of the BMP responsive R-Smads, Smad1/5/8, by the type 1 BMP receptors. The R-Smads then recruit co-Smad 4; they migrate to the nucleus, bind BREs in the Id promoter regions and activate transcription of these HLH family members

Conclusion

A novel pathway of serum induction involving BMP receptors and their downstream effectors, in particular Smads regulates the Id family of transcription factors. This identifies a novel way by which SRF-independent IEGs are regulated. In addition, we found that AZD0530 is an inhibitor of BMP type 1 receptors. This drug, currently used as a Src inhibitor, may also be useful as a BMP pathway inhibitor, suggesting additional therapeutic uses.

Methods

Cell culture. SRF shRNA and control cell lines were generated by stable infection of NIH3T3 with lentiviral vectors (pLKO.1) containing short hairpin RNA (shRNA) directed to SRF; shSRF-1, 5' -GCCAGCAUUCACAGUCACCAAC- 3' and shSRF-2, 5' -GAUGGAGUUCAUCGACAACAA -3' ; a non-targeting shRNA was used to generate the control cell line. The lentiviruses were made in Phoenix-ECO cells (ATCC CRL3214). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% New Born Calf serum (NCS) and 10 µg/ml puromycin (Invivogen). NIH3T3 cells were grown in 5% CO₂ in DMEM supplemented with 10% NCS. HCT116 and SYF cells were grown in 5% CO₂ in DMEM supplemented with 10% Fetal Bovine Serum (FBS).

Luciferase assays. NIH3T3 (4×10^4 cells/well) cells were plated on 24 well plates. The following day the media was changed to fresh 10% NCS in DMEM. The cells were transfected with Polyjet reagent (Signagen), 100 ng of pRLSV40P (SV40 promoter driving the Renilla luciferase gene) and 100 ng of pGL3 Id1 or c-fos reporter genes. For transfection with constitutively active BMP receptor genes, caALKs, 25 ng of each ALK was also transfected. The next day the media was changed to DMEM containing 0.2 % NCS (starvation media). For serum/BMP2 induction, the next day the cells were induced with 20% NCS or 20 ng/ml recombinant human BMP2 (Sino Biological Inc.) for 4 hours. The cells were harvested and luciferase activity assayed utilizing the Dual-Luciferase Reporter System (Promega). The Renilla luciferase activity was measured as an internal control and firefly luciferase values were normalized to the corresponding Renilla

luciferase levels. For AZD0530 treatment, the cells were treated with 100 nM AZD0530 (Selleck Chemicals) for 1 hour before the addition of serum. For noggin, 10 ng/ml noggin (STEMGENT) was added overnight while the cells were being starved in 0.2% NCS.

siRNA treatment. Integrated DNA Technologies synthesized siRNA duplexes for mSmad4. Two duplexes were used to deplete Smad4. Smad4-1 contains forward, 5' - GCAAUUGAGAGUUUGGUAAGAAGC-3' and reverse 3' - CUCGUU AACUCUCAACCAUUUCUUCG - 5'. Smad4-2 forward 5' - CAAAUACACCAACAAGUAACGAUGC and reverse 3' - UUGUUUAUGUGGUUGUCCAUUGCUACG - 3'. NIH3T3 cells (2×10^4 cells/well) were plated on 6 well plates. The next day, fresh media was added to each well. The cells were transfected with 50 pmoles siRNA using 3 μ l Powerfect (Signagen) as per the company's protocol. The next day the media was changed to 0.2% NCS in DMEM. The cells were harvested in Tri Reagent (Molecular Research Center, Inc.) and mRNA levels determined as described below.

Plasmids. The Id1 promoter fragments were cloned into the pGL3-basic plasmid utilizing restriction enzymes. Mouse Id1 -1577 to +54 and -1050 to +54 were amplified from mouse genomic DNA (Bioline). All other deletion mutants were made utilizing Id1 -1577 to +54 as a template. Point mutations were made utilizing the QuikChange Multi Site-Directed Mutagenesis Kit from Agilent in the mId1 -1150 background. The c-fos WT luciferase construct was as previously described [266]. The expression vector for constitutively active ALK1 pcDNA-ALK1 was a gift from Dr. Kristina Bostrom [253].

Expression vectors for constitutively active HA-ALK 2-5, 7 were gifts from Dr. Peter Ten Dijke [29].

Gene expression. mRNA expression levels were assayed by plating NIH3T3 cells on 6 well plates; the next day the media was changed to 0.2% serum. The following day serum/BMP2 was added for the time indicated to a final concentration of 20% and 20 ng/ml, respectively. For LY294002, PD0325901, AZD0530 and Dorsomorphin treatment, the drugs were added one hour before addition of serum. 10 μ m LY294002 (ChemieTek), 5 μ m PD0325901 (ChemieTek), 100 nM AZD0530 (Selleck Chemicals) or 1 μ m Dorsomorphin (Chemdea) were used. RNA was isolated using Tri Reagent (Molecular Research Center, Inc.) following the manufacturer's instructions. cDNA was made from 1 μ g total RNA using ImProm-II reverse transcriptase (Promega) and random hexamer primers. Individual gene expression was quantified with sybr green present in the Q-PCR master reaction mix (Thermo Scientific) and the Step One Plus machine (Applied Biosystems) was used for real time PCR quantification of gene expression. 18s rRNA expression was measured for normalization of all samples. Supplemental table S2 shows the sequences of all the gene primers.

Immunoblotting. Cells grown as described in preceding methods were rinsed twice in ice cold Phosphate Buffered Saline (PBS) and lysed in 1x passive lysis buffer from Promega. Lysates were kept on ice for 20 minutes, then centrifuged at 11,000 X g for 20 minutes at 4°C. The samples were diluted with one half volume 3X protein sample buffer (188 mM Tris-Cl (pH 6.8), 3% SDS, 30% glycerol, 0.01% bromophenol blue, 15%

β -mercaptoethanol) and boiled for 5 minutes. Proteins were separated by SDS-PAGE on 10% or 12% polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane and the membrane was blocked in 6% nonfat dry milk for 1 hour, washed 3X in 1X Tris Buffered Saline (TBS) and incubated with primary antibody (1:1000) in TBS overnight at 4°C while shaking. The next day the membrane was washed 3X with TBS-tween (TBS, 0.1% tween-20). The membrane was then incubated with fluorescently labeled secondary antibody (1:10,000) (IRDye goat anti rabbit 800 or goat anti rabbit 680 or goat anti mouse 680; LI-COR Biosciences) for one hour at room temperature, washed twice with TBS-tween and once with TBS. The proteins were visualized using the Li-Cor Odyssey Infrared imaging system. Primary antibodies against the proteins were as follows: **Phospho-src** Family (Tyr416) Antibody, rabbit, Cell Signaling; **Total ERK** (p44/42(ERK1/2) antibody, rabbit, Cell Signaling), **Actin** (1-19) Antibody, rabbit, Santa Cruz Biotechnology; **SRF**, rabbit, described in [267]; **Phospho-Smad1/5/8** (S463/S465/Smad8(S426/S428)) Antibody, rabbit, Cell Signaling; **Phospho (ERK)** p44/42 MAP Kinase, rabbit, Cell Signaling; **Phospho AKT** (Thr 308) Antibody, rabbit, Cell Signaling.

***In vitro* kinase reactions.** The Reaction Biology Corporation using the “HotSpot” assay platform performed the *in vitro* kinase reactions. Briefly, purified recombinant kinase/substrate pairs were incubated with the indicated concentrations of AZD0530 (Selleck Chemicals) or Staurosporine and with a mixture of ATP (Sigma) and ³³P-ATP to a final concentration of 10 μ M in reaction buffer (20 mM Hepes pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 0.02% Brij35, 0.02 mg/ml BSA, 0.1 mM Na₃VO₄, 2 mM DTT, 1% DMSO) for 120 minutes at 25 °C. The reactions were spotted onto P81 ion exchange

filter paper (Whatman) and free phosphate was removed by washing of filters in 0.75% phosphoric acid. The kinase activity data were expressed as the percent remaining kinase activity in test samples compared to vehicle (dimethyl sulfoxide) reactions. IC50 values and curve fits were obtained using Prism (GraphPad Software).

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Author contributions

RP and TL initiated and designed this study. Both authors participated in the writing of the manuscript. RP and TL approved the final manuscript. TL performed all of the experiments in this manuscript except the *in vitro* kinase assays.

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Figure Legends

Figure 1. Effect of SRF depletion on the serum regulation of immediate early genes.

A) SRF levels in NIH3T3 cells stably expressing control shRNA or shRNA targeting SRF were measured by immunoblotting with anti-SRF sera. Anti-ERK1/2 antibodies were used as a loading control. B) Control and SRF shRNA cells were starved in 0.2% serum overnight and then serum induced with 20% NCS for the indicated times. The levels of the indicated genes were measured using quantitative real time-PCR (Q-PCR) with 18s rRNA levels used to normalize the samples. The values are the means of three experiments +/- standard deviations.

Figure 2. Inhibition of MAPK and PI3K signaling does not affect Id1 induction.

A) NIH3T3 cells were grown overnight in 0.2% NCS, treated with the PI3K inhibitor LY294002 (10 μ M) for 1 hour and then for 1 hour or the indicated times with 20% serum. Gene expression was analyzed by Q-PCR, as in figure 1. Phospho-ERK1/2 and phospho-AKT levels were measured by immunoblotting with phospho-specific antibodies. B) Cells were treated for 1 hour with the MEK1 inhibitor, PD0325901, (5 μ M for the RNA experiments and 1 or 5 μ M, as indicated, for the immunoblots). The cells were then serum-stimulated, as described above, and measured by Q-PCR for mRNA expression and immunoblotting for ERK1/2 phosphorylation. Actin levels were measured by immunoblotting as a loading control.

Figure 3. Mapping the Serum regulation element of the mId1 promoter.

A) Map of mouse Id1 promoter deletion mutants. B) NIH3T3 cells were transfected with variants of the Id1 promoter along with pRLSV40P as an internal control. The next day transfected cells were starved in 0.2% serum overnight and then induced with 20% serum for four hours. Firefly luciferase levels were normalized to the Renilla pRLSV40P levels and are the means of three experiments +/- the standard deviation. *** indicates a p-value ≤ 0.05 . C) Conservation of sequence elements in the conserved 100 bp Id1 promoter region -1150 to -1050. m, mouse; h, human; c, chicken. M8 and M16/M17 indicated mutated regulatory elements from reference [127].

Figure 4. Mapping the serum responsive element of the mId1 promoter.

A) Sequence of the conserved regulatory region of mId1 with the indicated putative regulatory elements. B) Left (L), mutations in the BRE region and Right (R), mutations in the BRE region. C) Alignment of the BMP responsive elements (BRE) in the mouse Id1, Id2 and Id3 promoters. D, E) The indicated mutants were transfected into NIH3T3 cells and assayed for luciferase activities as in figure 3.

Figure 5. The BRE-SBE elements are sufficient for serum induction.

A) Four copies of the indicated elements of the mId1 promoter were cloned upstream of the SV40 promoter in the pGL3 luciferase reporter. B) These constructs were co-transfected with pRLS40P into NIH 3T3 cells, serum-induced and assayed as in figure 3.

Figure 6. The BMP pathway is necessary and sufficient for serum induction of Id1.

A) NIH3T3 cells were serum-starved overnight and induced with 20% serum or 20 ng/ml BMP2 as indicated. Where indicated, 100 ng/ml noggin was added during serum-starvation. Gene expression was measured by Q-PCR as in figure 1. B) NIH3T3 cells were transfected with the mId1 -1150 and pRLSV40P luciferase reporter constructs, serum starved with or without noggin, and induced with serum or BMP2 as in A. Luciferase levels were measured as in figure 3. C) NIH3T3 cells were starved in 0.2% serum overnight then induced with 20% serum or 20 ng/ml BMP2 for 1 hour. The lysates were immunoblotted with phospho-Smad1/5/8 specific antibodies or anti-actin as a loading control. D) NIH3T3 cells were serum-starved, treated with Dorsomorphin at the indicated concentrations for 1 hour, and then induced with 20% NCS for 1 hour. Id1 mRNA levels were measured by Q-PCR as in figure 1. E) Cells were transfected with control or Smad4 siRNAs. The next day the media was changed to 0.2% serum overnight and the cells serum-induced for 1 hour. Smad4 and Id1 mRNA levels were measured by Q-PCR as in figure 1.

Figure 7. src inhibitor AZD0530 inhibits serum induction of Id1.

A) NIH3T3 cells were starved in 0.2% serum overnight and AZD0530 (100 nM) was added to the cells for 1 hour. Cells were then induced with 20% serum for the indicated times. Gene expression of indicated genes was analyzed by Q-PCR as in figure 1. B) The indicated luciferase reporters (described in figures 3 and 5) as well as a c-fos promoter reporter were transfected into NIH3T3 cells as in figure 3. The cells were treated with

AZD0530 (100 nM) for 1 hour and then with 20% NCS for 4 hours. Luciferase assays were as in figure 3. *** indicates a p-value ≤ 0.05 .

Figure 8. AZD0530 inhibits Smad phosphorylation *in vivo*.

A) NIH3T3 cells were serum-starved overnight and then treated with DMSO, 100 nM AZD0530 or 1 μ M Dorsomorphin for 1 hour followed by serum for 1 hour. Serum-induced phosphorylation was measured by immunoblotting using phospho-specific antibodies for phospho-Smad1/5/8, phospho ERK1/2, and phospho srcY416. Anti-actin antibodies were used as a loading control. B) SYF^{+/+} (wt fibroblasts) and SYF^{-/-} cells (null for src, yes and fyn) were starved in 0.2% serum overnight and induced with 20% serum for 1 hour. Expression of Id1 and Id3 was measured by Q-PCR as in figure 1.

Figure 9. AZD0530 inhibits BMP responsive ALKs *in vitro*.

A) In vitro kinase assays were performed with the indicated purified ALK protein kinases at 10 different AZD0530 concentrations in triplicate. The relative activities are shown. B) The IC₅₀s of AZD0530 and a non-specific protein kinase inhibitor, staurosporine, are shown for each BMP receptor subunit. ND, not done. Alternative names for ALKs are shown.

Figure 10. AZD0530 inhibits the constitutive activation of the Id1 promoter by type 1 BMP receptors.

Constitutively active type 1 BMP receptors, ALK1 to 5 and ALK7 were transfected with the Id1 -1150 luciferase reporter gene along with pRLSV40P into NIH3T3 cells. The

next day the media was changed to 0.2% serum containing AZD0530 (100 nM) or DMSO as a control and the cells were incubated overnight. Luciferase levels were measured as in figure 3. *** indicates a p-value ≤ 0.05 .

Figure 11. AZD0530 inhibits constitutive Id1 expression in HCT116 colon cancer cells.

HCT116 cells were serum starved in 0.2% serum overnight with DMSO, AZD0530 (100 nM) or Dorsomorphin (1 μ M). These chemicals were added again the next day one hour before addition of 20% serum for one hour. A) Immunoblots for phospho-Smad 1/5/8 and actin control. B) Id1 mRNA levels were measured by Q-PCR as in figure 1. *** indicates a p-value ≤ 0.05 .

Figures

Figure 1.

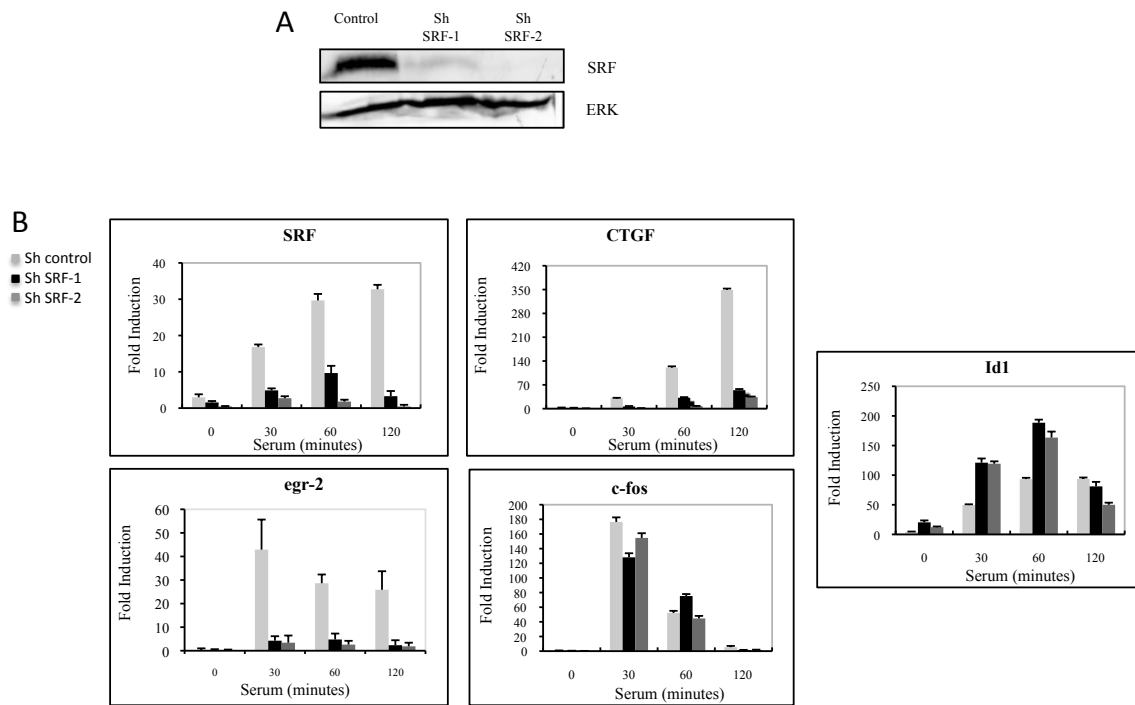


Figure 2.

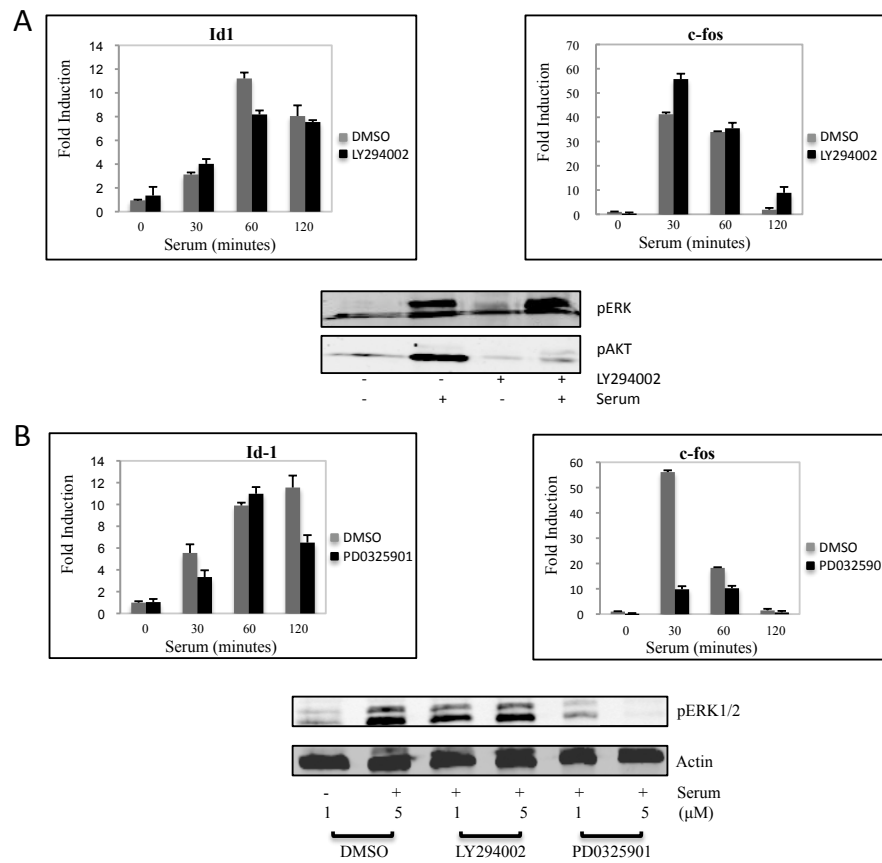


Figure 3.

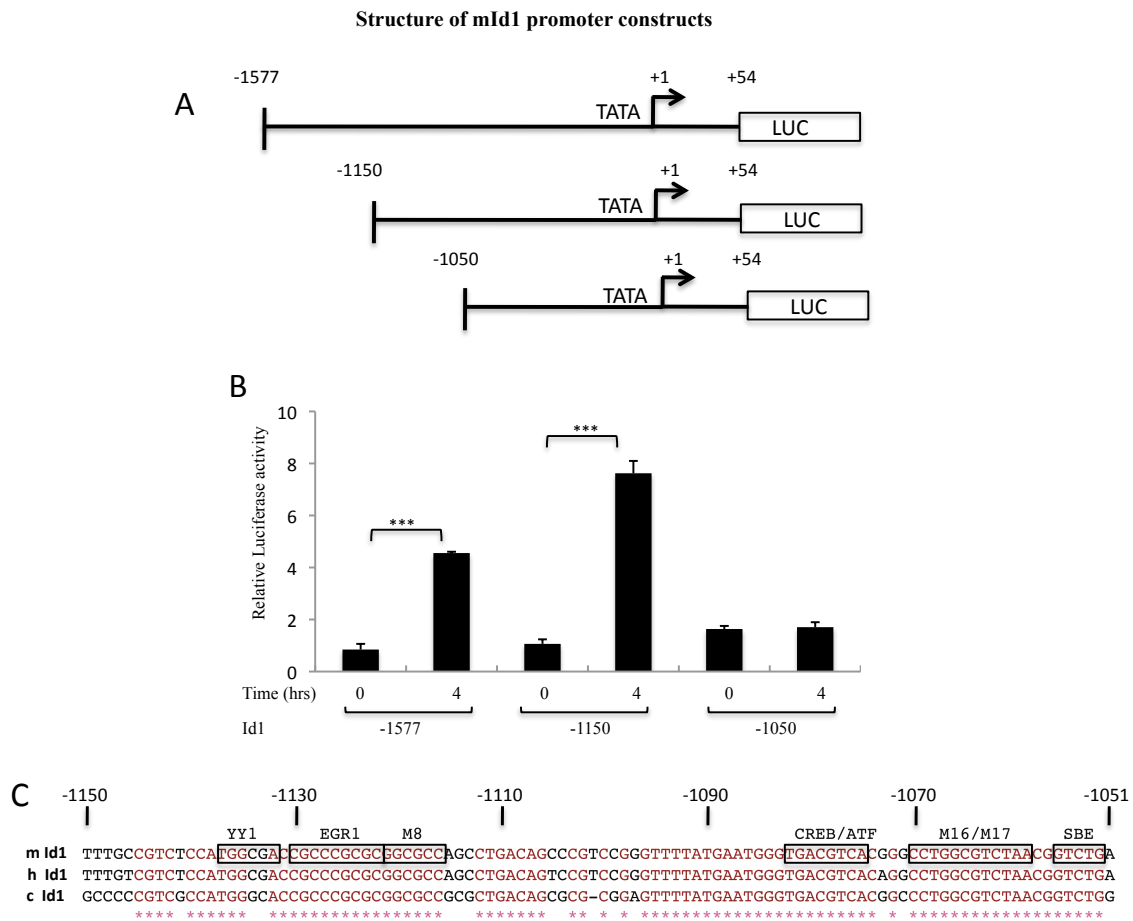


Figure 4.

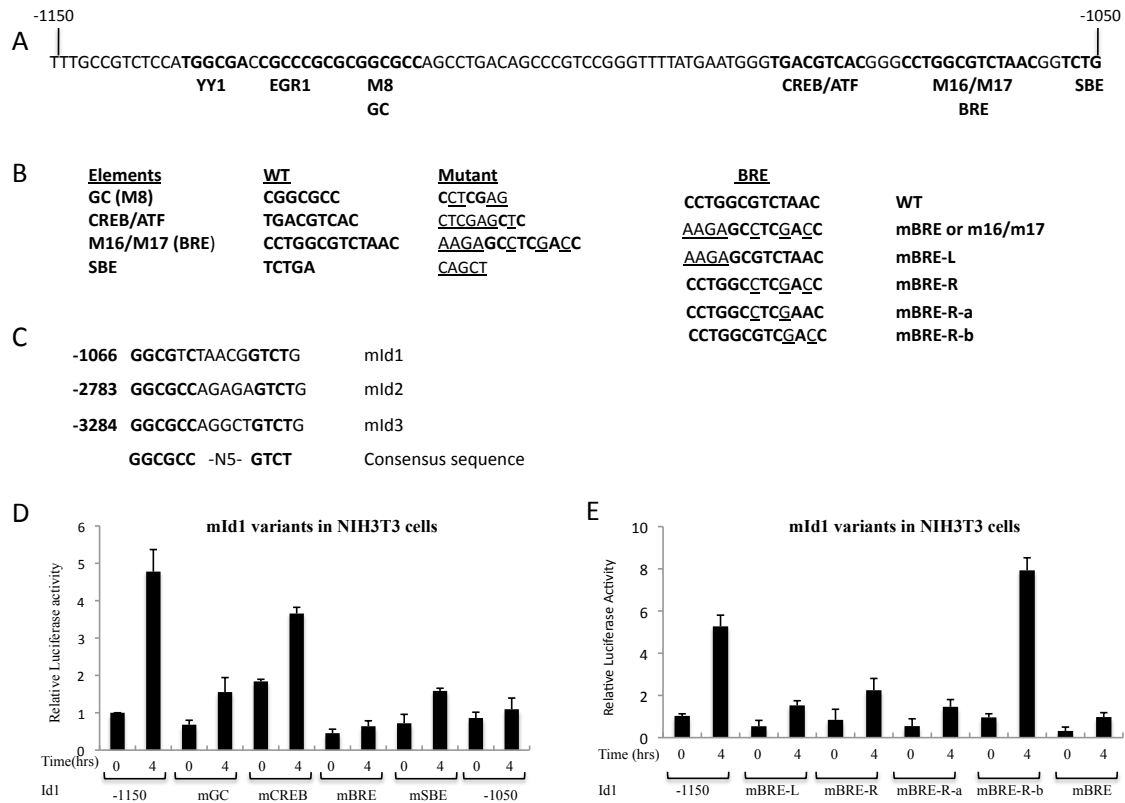


Figure 5.

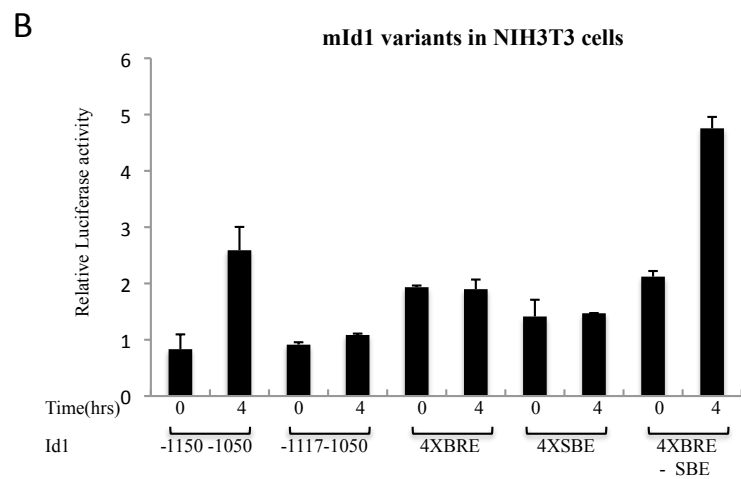
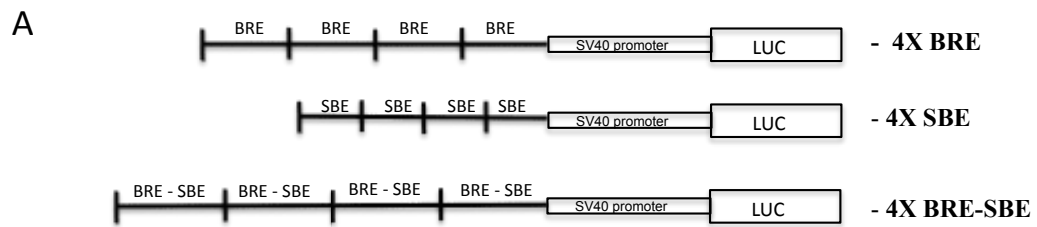


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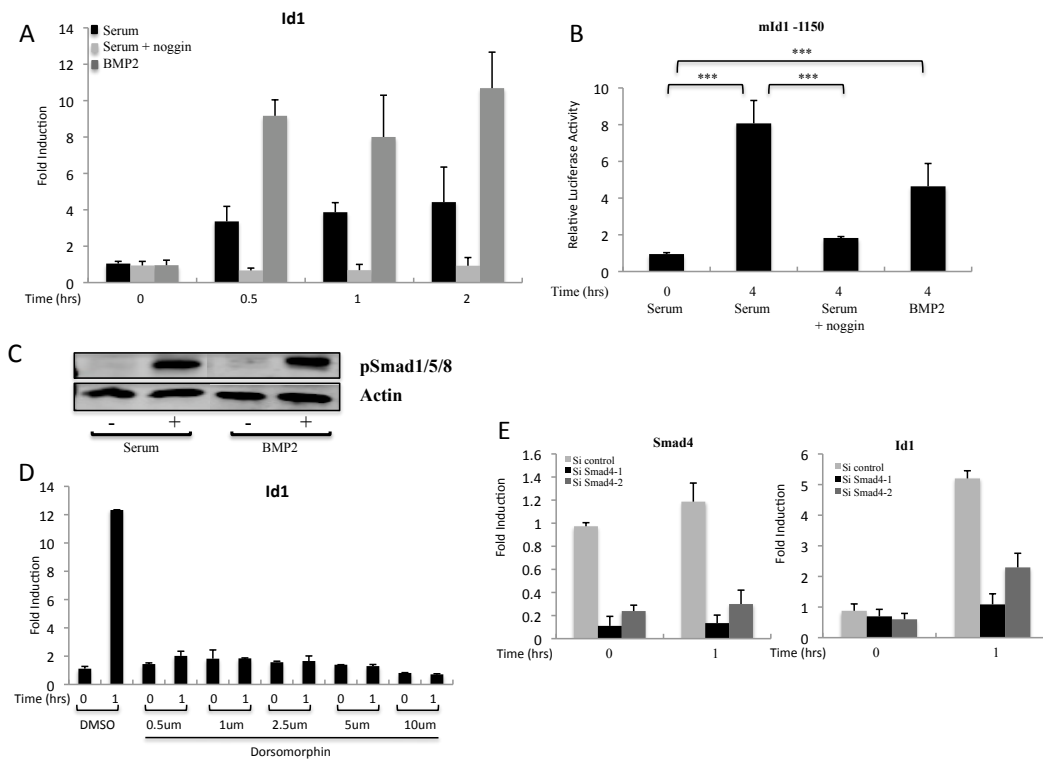


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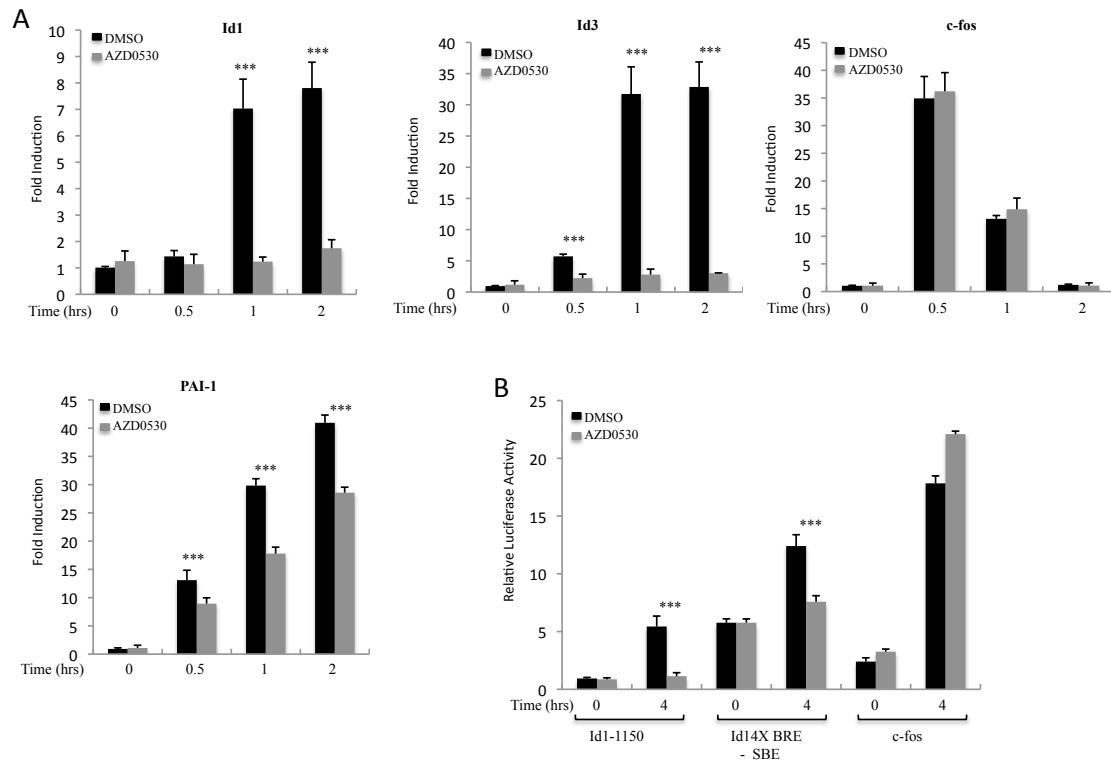


Figure 8.

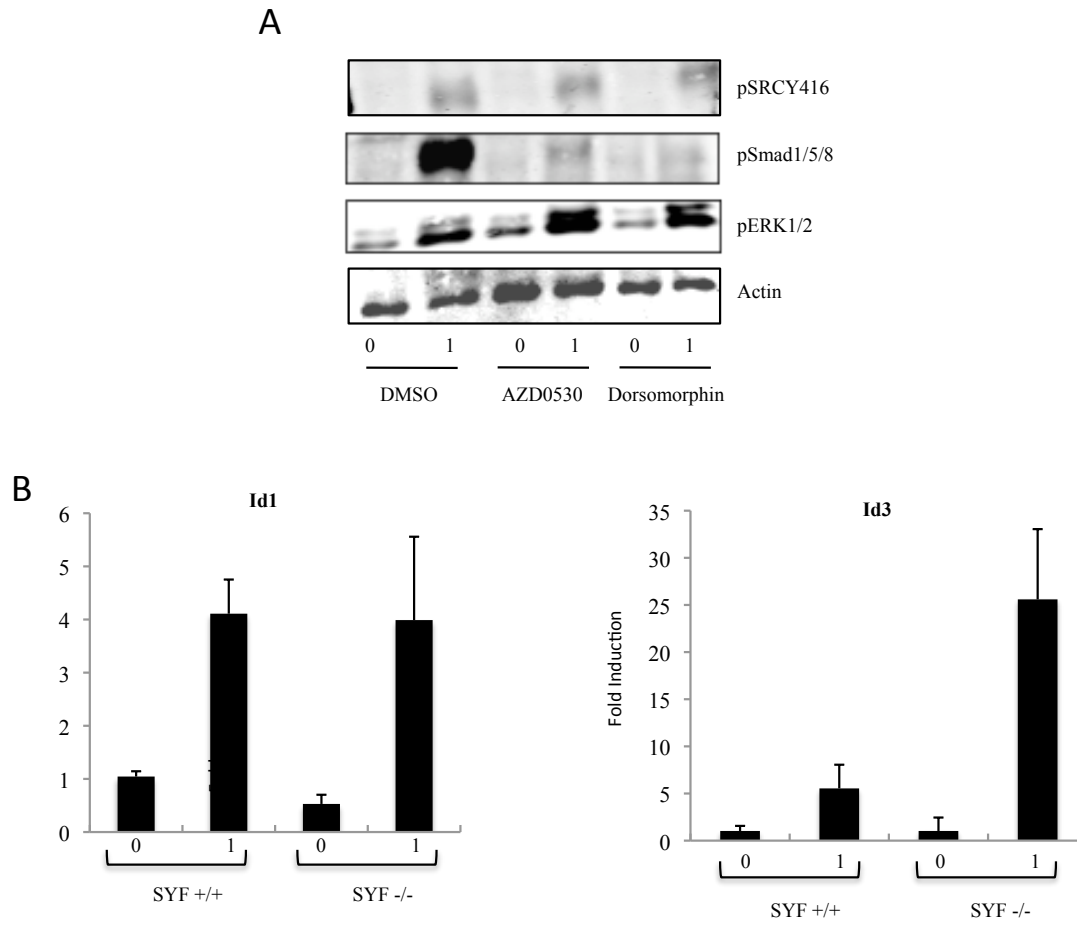
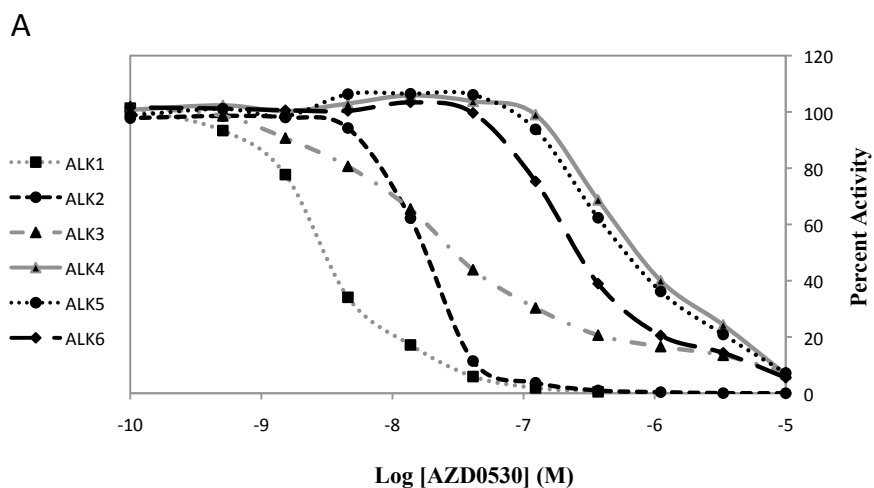


Figure 9.



B

Kinases	Compound IC50 (M)	
	AZD0530	Staurosporine
ALK1/ACVRL1	3.20×10^{-9}	3.55×10^{-7}
ALK2/ACVR1	1.74×10^{-8}	6.50×10^{-6}
ALK3/BMPR1A	3.07×10^{-8}	4.61×10^{-7}
ALK4/ACVR1B	8.16×10^{-7}	5.14×10^{-6}
ALK5/TGFBR1	6.65×10^{-7}	1.61×10^{-5}
ALK6/BMPR1B	2.95×10^{-7}	ND
BMPR2	No Inhibition	1.23×10^{-7}

Figure 10.

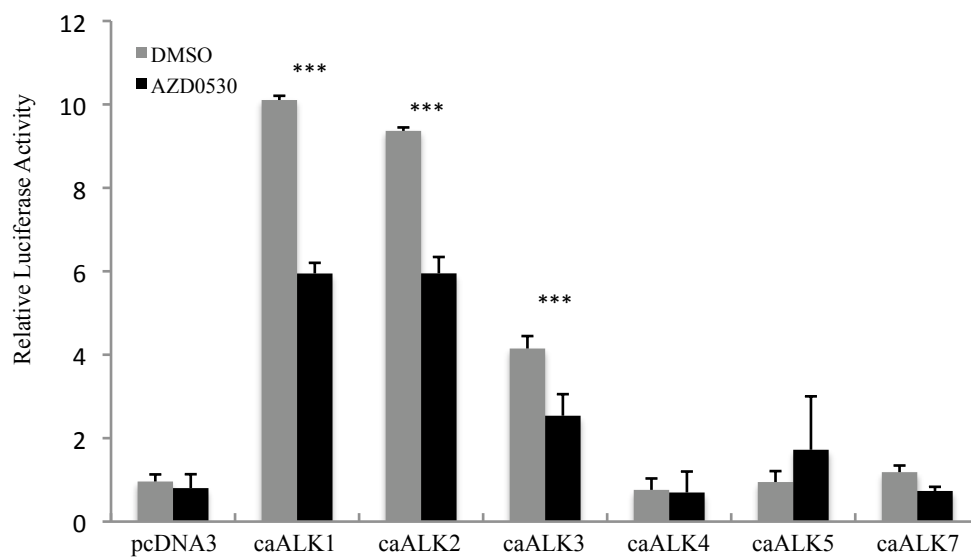
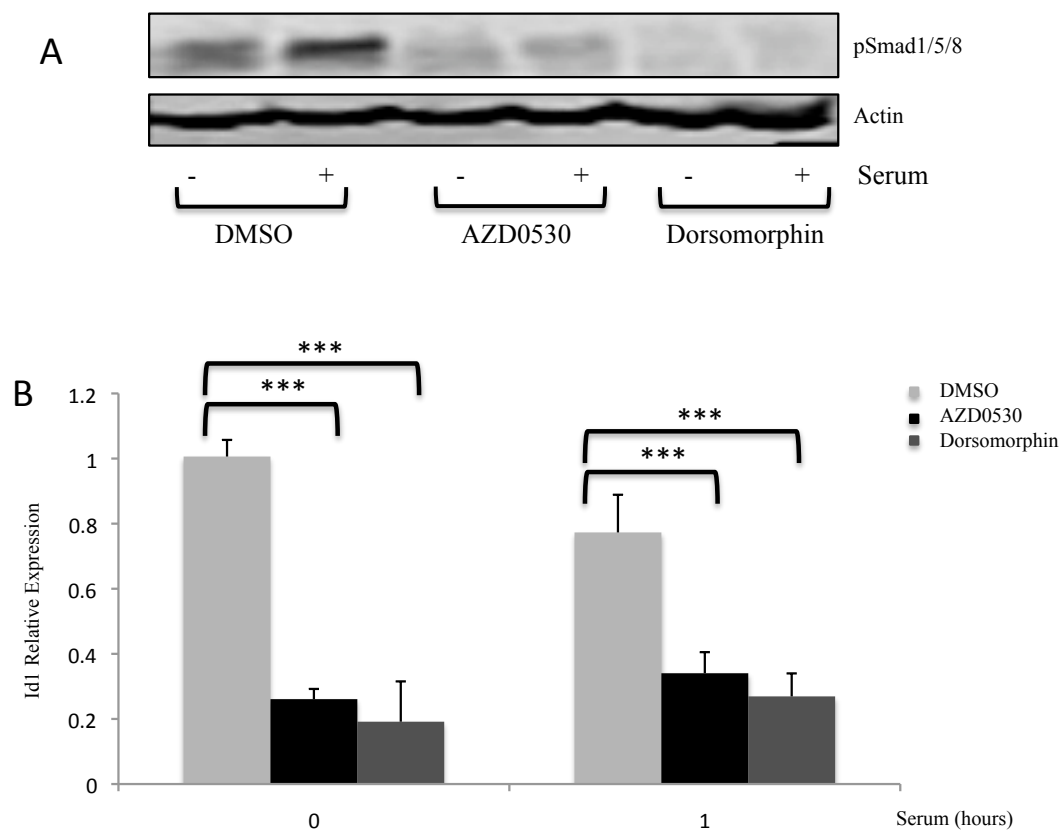


Figure 11.



Supplemental

Methods

Cell line.

SRF shRNA 2-2 cell line was generated by stable transfection of NIH3T3 with pLKO.1 containing short hairpin RNA (shRNA) directed to SRF; shSRF-2, 5' – GAUGGAGUUCAUCGACAACAA -3' ; NIH3T3 cells were used as the control cell line. The lentiviruses were made in Phoenix-ECO cells (ATCC CRL3214). Cells were grown in 5% CO₂ Dulbecco's modified Eagle's medium (DMEM) containing 10% New Born Calf serum (NCS) and 10 µg/ml puromycin (Invivogen). NIH3T3 cells were grown in 5% CO₂ in DMEM supplemented with 10% NCS.

Figure legends

Figure S1. Effect of SRF depletion on IEG expression.

Control and SRF knockdown cells were starved in 0.2% NCS overnight and then serum induced with 20% NCS for 0, 30, 60 or 120 minutes. Total RNA was isolated from the cells. The levels of the indicated genes were measured using quantitative real time-PCR (Q-PCR). 18s rRNA levels were used to normalize for overall RNA levels.

Table S1. Effect of SRF depletion on serum induction of immediate early genes.

The indicated IEGs were assayed as in figures 1 and S1. The average percent decreases at the peak time of induction +/- the standard deviation is shown. A decrease of greater than 50% was considered significant. Genes whose induction was changed less than 50%

or increased in the SRF depleted cells were labeled as SRF independent. The final column indicates the SRF dependence.

Table S2. Nucleotide sequence of primers utilized for Q-PCR.

Figures

Figure S1

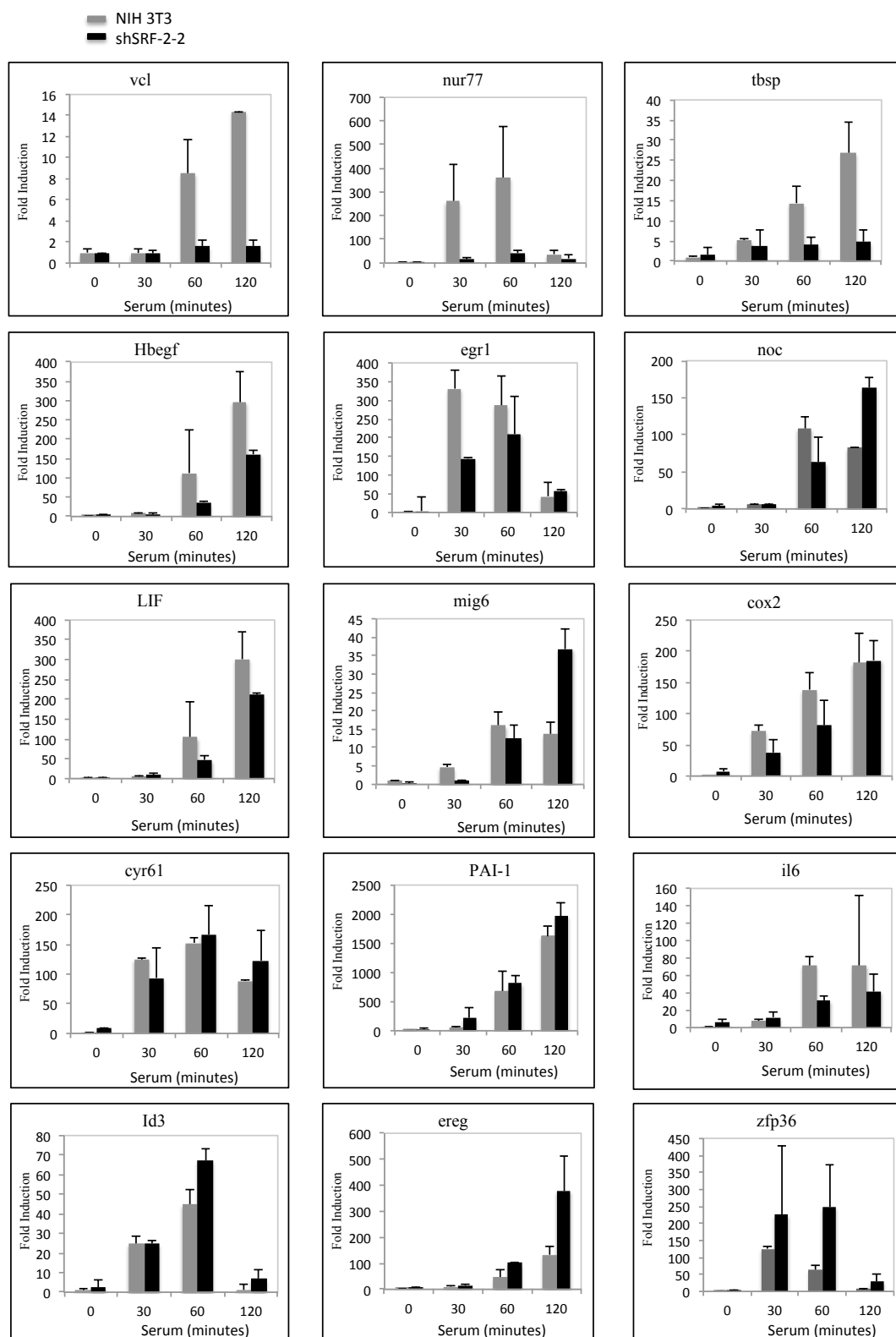


Table S1

Gene	Gene Symbol	Percent decrease at peak +/- standard deviation	SRF Dependant
Early Growth Response 2	egr2	91.5 +/- 1.8	Yes
Connective Tissue Growth Factor	CTGF	90.2 +/- 2.0	Yes
Vinculin	vcl	89.1 +/- 4.3	Yes
Nuclear hormone receptor 77	nur77	87 +/- 4.0	Yes
Thrombospondin	tbsp	80.1 +/- 17	Yes
Heparin binding EGF like growth factor	Hbegf	51.3 +/- 8.3	Partial
Early Growth Response-1	egr1	58.2 +/- 23.8	Partial
Nocturnin	noc	39 +/- 40.3	No
Leukemia inhibitory factor	LIF	28.0 +/- 14.7	No
Mitogen inducible gene 6	mig6	16.7 +/- 42.5	No
Cellular FBJ osteosarcoma oncogene	c-fos	12.4 +/- 35.2	No
Cytochrome c oxidase subunit II	cox2	-3.8 +/- 10.2	No
Cysteine rich protein 61	cyr61	-8.4 +/- 25.8	No
Plasminogen activator inhibitor 1	PAI-1	-23 +/- 27.5	No
Interleukin 6	il6	-26.4 +/- 108.0	No
Inhibitor of DNA binding/differentiation 3	Id3	-60.8 +/- 38.3	No
Inhibitor of DNA Binding/Differentiation 1	Id1	-64.8 +/- 20.5	No
Zinc finger protein 36	zfp36	-75.9 +/- 154.1	No
Epiregulin	ereg	-203.4 +/- 168.8	No

Table S2

Mouse Genes	Gene Symbol	5' Primer	3' Primer
Cellular FBJ osteosarcoma oncogene (c-fos)	c-fos	AGCATGGGCTCTCCTGTCAAC	GCCACGGAGGAGACCAGAGT
Connective Tissue Growth Factor	CTGF	AACCGCAAGATTGGAGTGTGC	GGTATTTGCAGCTGCTTTGGAAGG
Cysteine Rich protein 61	cyr61	TCCACCGCTCTGAAAGGGATCT	TGGTGTTTACAGTTGGGCTGGAAG
Cytochrome c oxidase subunit II	cox2	GGCAGCAAATCCTTGCTGTTCCAA	TCACCATAGAATCCAGTCCGGGTA
Early growth response-1	egr1	AACAACCCTATGAGCACCTGACCA	ATAACTCGTCTCCACCATCGCCTT
Early growth response 2	egr2	TTTGACCAGATGAACGGAGTGGC	AGGTCTGGTTTCTAGGTGCAGAGA
Epiregulin	ereg	GTTTCTCATATAACCGCTGGA	GTCCGTAACCTTGATGGCACTG
Heparin binding EGF like growth factor	Hbegf	AAGAGAGACCCATGCCTCAGGAAA	ACTGGTAGAGTCAGCCCATGACA
Inhibitor of DNA binding/differentiation 1	Id1	AACGGCGAGATCAGTGCCTT	CCTCAGCGACACAAGATGCGAT
Inhibitor of DNA binding/differentiation	Id3	ACCTTCAGTGGTCTGGCA	AGCTCCTCTGTCTTGGAGATCA
Interleukin 6	il6	TCCAGTTGCCTCTTGGGACTGAT	AAGTCTCCTCTCCGACTTGTGAA
Leukemia inhibitory factor	LIF	TCAGCGACAAAGTTACTCCACCGT	AAGTGATGACAAAGCCCAACAGGC
Mitogen inducible gene 6	mig6	ACCATGGCCTACAATCTGAACTCC	TTGACCTTGGAGATGGACCACACT
Nocturnin	noc	TTCGCGTCATGCAGTGGAAACAT	TCAGGCACTTCTCTCTTCCATT
Nuclear hormone receptor 77	nur77	TCTGTGGTGACAATGCTTCGTGTC	TCAGGCACTTCTCTCTTCCATT
Plasminogen activator inhibitor	PAI	AACAAGAGCCAATCACAAGGCACC	TGAACCCTTTCCAGAGACCAGAA
Serum Response Factor	SRF	CAAGAGGAAGACGGGCATCA	GCAACAGCACCTGTGTCCCT
Thrombospondin	tbsp	ACTAGGCCTGTTCTGCTTCTCTCA	CGCTGGTTATGATTGGCAGCTGAT
Vinculin	vcl	GCCGGACCAACATCAGTGAT	GCGCAGAGTAAAGCCAGCAT
Zinc finger protein 36	zfp36	ATTCGCGCCACCATGGATCT	ACGGGATGGAGTCCGAGTTTATGT
18s ribosomal RNA	18s rRNA	TCGAGGCCCTGTAATTGGAAT	CCCTCCAATGGATCCTCGTTA
Human Genes	Gene Symbol	5' Primer	3' Primer
Inhibitor of DNA binding/differentiation 1	Id1	GTTCCATTTTCCGTATCTGCTTC	CCACTGGCGACTTTCATGAT

Chapter 3

Future directions

Our findings shed light on the serum regulation of the Ids. We found that Id1 is regulated by BMP in serum through the presence of BREs in its promoter. We also showed that the Src inhibitor, AZD0530 inhibits the serum/BMP2 activation by directly inhibiting the BMP responsive type 1 receptors, ALK1-3. There are still some unanswered questions. It would be interesting to find out whether other IEGs are regulated in a manner similar to the Ids.

One possible way to answer this question is to treat BMP2 induced NIH3T3 cells with AZD0530 and identify by microarray the IEGs whose expression changes. In addition, one could analyze available data to identify BMP induced IEGs. Further, one should identify which of the BMP2 induced genes are also induced by serum. This will ensure that the genes being studied are serum responsive. This can be accomplished by treatment of quiescent NIH3T3 cells with serum and then checking gene activation of the BMP2 induced IEGs by Q-PCR.

Interestingly *cyr61* was SRF independent even though it has known SREs [36]. *cyr61* is up regulated by BMP4 [207], therefore, it may also be regulated by a BMP pathway. AZD0530 may not block its serum induction because it may also be induced in a BMP independent manner. This kind of regulation will not be unprecedented as the RhoA pathway of *c-fos* regulation was only discovered after blocking the MAPK pathway [91]. One way to test this may be to treat shSRF-2 cells with AZD0530 and check serum/BMP induction of SRF independent IEGs such as *cyr16*.

AZD0530 directly inhibits ALKs, however, Smads may be activated by pathways other than the one involving BMP receptors, for example the JNK and p38 pathways [268]. Treating other BMP2 induced IEGs with AZD0530 will identify the IEGs that are activated in a manner similar to Ids. However, in order to identify genes regulated differently, one may treat cells with inhibitors of these alternative pathways such as p38 before induction of quiescent cells with BMP2. Another way to test this is to utilize BMP inhibitors such as noggin that prevent any BMP signaling. Microarray technology can be used to identify IEGs whose expression is inhibited by noggin.

Another interesting finding was that *c-fos*, the longest studied SRF dependent gene was found to be SRF independent. Understanding why this is so will shed light on how *c-fos* is regulated. We discussed the possibility that there is a threshold of SRF binding or that there is another pathway that regulates *c-fos*. In order to shed light on this the following experiments may be performed. Conditional knockout of SRF can be made in fibroblasts. Once the knockout has been confirmed the mRNA expression of *c-fos* in response to serum treatment should be assayed. Reporter assays in these cells will indicate whether the promoter activity of *c-fos* is affected by loss of SRF.

If the knockout does not completely abolish *c-fos* activation, this will indicate that there are additional regulatory elements in the *c-fos* promoter that are regulated independent of SRF. We unsuccessfully attempted to find additional serum regulatory elements in the *c-fos* promoter using reporter genes. This may be because we did not clone the region in which these elements are located. We can

use available databases to identify regions of conservation in the c-fos promoter.

The putative regions can be cloned in front of the c-fos minimal promoter, this would allow us to test whether these promoter regions are needed for serum induction of c-fos.

Finally we showed that that AZD0530 blocks Id1 expression in the HCT116 colon cancer cell line in which the BMP pathway is deregulated. Id1 overexpression /misregulation is present in many other cancers [269]. Inhibiting BMP pathway expression of Id1 presents a novel way to inhibit Id1 in cancer. Bio-informatics may be used to identify cancers in which Id1 is upregulated and/or the BMP pathway is perturbed. Candidate cell lines can be treated with BMP pathway inhibitors such as AZD0530, dorsomorphin or noggin to check whether this affects BMP signaling. The effect on Id1 expression can be assayed by Q-PCR and the effect on phospho-Smad1/5/8 by immunoblotting.

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