SRC UTILIZES CAS TO BLOCK GAP JUNCTIONAL COMMUNICATION MEDIATED BY CONNEXIN43

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The Src tyrosine kinase phosphorylates Cas (Crk associated substrate) to confer anchorage independence and invasive growth potential to transformed cells. Gap junctional communication is often lower between aggressive tumor cells compared to normal or benign precursors. The gap junction protein Cx43 (connexin43) is a tumor suppressor that can inhibit tumor cell growth. Src can phosphorylate Cx43 to block gap junctional communication between transformed cells. However, mechanisms by which this event actually closes intercellular channels have not been clearly defined. Here, we report that Src and Cas associate with each other at intercellular junctions. In addition, Cas is required for Src to reduce dye transfer and electrical coupling between cells expressing Cx43. Thus, Src utilizes Cas to inhibit gap junctional communication mediated by Cx43. This finding introduces a novel role of the Cas focal adhesion linker protein in the gap junction complex. This observation may help explain how gap junctional communication can be suppressed between malignant and metastatic tumor cells.

Src is a membrane bound tyrosine kinase that triggers the transformation of normal cells into cancer cells (1). Src has been implicated in a variety of human cancers (2,3). As expected, metastatic cell growth is significantly reduced by agents that inhibit Src kinase activity (4,5).

Increased anchorage independent growth and migration distinguish most cancer cells from their nontransformed precursors (6,7). The Src kinase phosphorylates Cas (Crk associated substrate) to promote these fundamental hallmarks of tumor cell growth (8-10). Cas is an important component of the focal adhesion complex signaling network (11) that also includes FAK, Grb2, Shc, and paxillin (12,13). After phosphorylation by Src, Cas can bind to other proteins including Crk, PI-3-kinase, Nck, and PLCγ (14-16).

Src phosphorylates Cas on specific tyrosine residues to promote tumor cell invasion and metastasis. Src transformation of homozygous null Cas knockout (CasKo) cells does not fully promote their anchorage independence or ability to migrate. These transformed growth characteristics can be conferred to CasKo cells by transfection with wild type Cas (8,10,17,18).

Gap junctions form aqueous channels that connect the cytoplasm of adjacent cells. These channels are composed of integral membrane proteins called connexins. Connexins have evolved into a family of at least 20 mammalian members which are commonly named by their predicted molecular weights (19-21). Gap junctions allow adjacent cells to share intracellular signals and function in a coordinated fashion (22-24).

Connexins play an important role in cell growth control. Experiments have identified Cx43 as a tumor suppressor gene (25-27). In general, gap junctional communication is blocked in transformed cells (28,29). For example, Cx43 expression is robust in normal glial and mammary cells, but repressed in some glioma and mammary tumor cells. Moreover, restoration of Cx43 expression results in decreased cell growth and invasion. These findings support the concept that Cx43 may play a role in the control of cell growth and invasion.
expression can normalize the growth of glioma and mammary carcinoma cells (27,30,31).

Src phosphorylates Connexin43, but may require downstream events to block gap junctional communication. Like Cas, Cx43 is a functionally relevant Src substrate. Src phosphorylates Cx43 on critical tyrosine residues, and this event can lead to reduced intercellular communication (32-34). However, in addition to tyrosine phosphorylation, other factors may be required for Src to block gap junctional communication mediated by Cx43. For example, MAPK acts downstream of Src to phosphorylate Cx43 on serine residues and close gap junction channels (35). Other kinases activated by Src, such as protein kinase C also disrupt junctions formed by Cx43 (36). In addition, other components may be involved since potential SH3 binding domains on Cx43 are required for channel closure in Src transformed cells (35). Src and Cas both have SH3 domains (37).

As described above, Src utilizes Cas to promote tumor cell metastasis (8,10,17,18). Therefore, revealing functional relationships between Cas and Cx43 would aid in understanding how gap junctional communication may influence tumor cell growth. We hypothesized that Src utilizes Cas to block gap junctional communication mediated by Cx43. We report here that Cas associates with Cx43 at intercellular junctions and that Src requires Cas to suppress gap junctional communication mediated by Cx43.

EXPERIMENTAL PROCEDURES

Cells and analysis of gene expression - Homozygous Cas knockout cells transfected with Cas (CasWt), v-Src (CasKoSrc), Cas and Src (CasWtSrc), or empty vector controls (CasKoHP) were maintained as previously described (10,38). Clones were not taken during the production of these cells in an effort to reduce concerns related to clonal variation. LA25 cells were maintained as described (39,40) and incubated at nonpermissive (39°C) or permissive temperature (34°C) for 14 to 18 hours before subsequent analysis. RNA extraction and microarray analysis of gene expression with 430A Mouse Expression Array gene chips (Affymetrix) was performed as previously described (38). Numerical results were normalized to a base value of 100. Duplicate independent experiments were done in all cases (n = 2).

siRNA transfection - Small interference RNA (siRNA) targeted to p130-Cas containing equal parts of the sequences GGUAUGCCUGCGCUUUUU, GGAGGCUAUGACUAGGUUU, GACCGACAUAUAAACUU, and GAAUUCUGGUUGCAUGUAUU (Dharmacon MQ09009600) or siCONTROL Non-targeting siRNA (Dharmacon D00120613) were transfected into cells at a final concentration of 100 nM with Lipofectamine 2000 (Invitrogen 11668019) for 24 hours as instructed by the manufacturers. After 24 hours, this solution was replaced with standard medium and cells were examined by preloading and western blot analysis after another 48 hours of growth.

Western blot analysis - Western blot analysis was done as described previously (38) with some modification. Briefly, the cells were lysed in 20mM Tris-HCl (pH 7.5) containing 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% TritonX-100, 1mM β-Glycerolphosphate, 50 mM NaF, 1mM Na3VO4, and 10ug/ml proteinase inhibitor cocktail (Sigma, P2714). Lysates were clarified by centrifugation. Bromophenol blue was added to 0.01% before protein (20ug/lane) was resolved on 10% gels and transferred to Immobilon-P membranes (Millipore, IPVH-00010). Rabbit polyclonal antiserum was used to detect Connexin 43 (Santa Cruz Biotechnology, sc-9059) and active Src kinase (Cell Signaling Technology, 2101L). Mouse monoclonal antiserum was used to detect viral Src (Upstate Biotechnology, 05-185), phosphotyrosine (Cell Signaling Technology, 9411), Cas (Transduction Laboratories, 610271), and β-actin (Sigma, A1978). Primary antiserum was recognized by appropriate secondary antiserum conjugated to horseradish peroxidase and detected using Enhanced Chemiluminescence Western Blotting Analysis Systems (Amersham Biosciences, RPN 2108). After Western blotting, membranes were stained with India ink to verify equal loading and transfer.

Immunofluorescence confocal microscopy - 200,000 cells were plated on 35-mm poly-D-lysine–coated glass-bottomed culture dishes (MatTek, Inc.). After 24 hours of growth, cells were fixed with cold 50% methanol-50% acetone solution, permeabilized with 0.2% Triton X-100 in
PBS for 10 minutes, and then washed thrice with 0.1% Tween 20 in PBS followed by 1% bovine serum albumin (BSA) in PBS for 30 minutes. The cells were incubated with Connexin 43 antibody at 4°C overnight, washed, and then labeled with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes, A11008). Likewise, monoclonal antiserum specific for Cas (Transduction Laboratories, 610271) was labeled with anti-mouse IgG conjugated to Alexa Fluor 595 (Molecular Probes, A11005). Cell nuclei were stained with Hoechst 33342 (Molecular Probes, H1399). Images of CasKo cells or LA25 cells were obtained with a Carl Zeiss Axiovert 200 Pascal UGB confocal microscope or LSM510 meta confocal microscope equipped with a Plan-Apochromat 63X objective. HeNe1 (ex: 543nm), argon (ex:488nm), and UV (ex:364nm) lasers were used with appropriate band pass filters (BP:560-615, BP:505-530, BP:420-480) to detect Alexa 594, Alexa 488, and Hoechst 33342 respectively.

Immunoprecipitation of Cx43 and Cas - Immunoprecipitation was performed as described (10,41) with some modifications. Briefly, confluent cells were washed with phosphate-buffered saline and lysed in 20mM Tris-HCl (pH 7.5) containing 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% TritonX-100, 1mM β-Glycerolphosphate, 50mM NaF, 1mM Na3VO4, and 10ug/ml proteinase inhibitor cocktail (Sigma, P2714). Lysates were clarified by centrifugation and incubated with Cx43 antibody (Santa Cruz Biotechnology, sc-9059). Immune complexes were precipitated with resin linked to protein G (Santa Cruz Biotechnology, sc-2002), washed with lysis buffer, and eluted in SDS-PAGE sample buffer.

For coimmunoprecipitation studies, cells were washed with phosphate-buffered saline and lysed in CSK buffer (100mM NaCl, 1.5mM MgCl2, 10mM pipes pH 6.8) containing 0.5% Triton-X-100, 1mM sodium vanadate (Sigma, S6508), 1mM PMSF (Sigma, P7626), 50mM NaF (Sigma, S7920) and 1% Protease inhibitor cocktail for mammalian cells (Sigma, P2417). Lysates were clarified by centrifugation and incubated with antiserum against Cx43 (Santa Cruz, SC9059) or Cas (Santa Cruz, SC860). Immune complexes were precipitated with Protein G beads (Santa Cruz), washed with CSK buffer, and eluted in SDS-PAGE sample buffer.

Evaluation of gap junction communication - Intercellular dye transfer was evaluated by a preloading assay as previously described (39,42). Basically, “Donor” cells were labeled with calcine and DiI before being plated with nonlabeled “Receiver” cells. Intercellular communication was visualized by the transfer of the small hydrophilic dye calcine (623 Da) to neighboring cells, while the lipophilic DiI does not transfer between cells. Cells were visualized on a Zeiss Axiovert 40 inverted microscope equipped with a Carl Zeiss AxioCam MRm CCD camera and Axiovision 4.3 analysis software. Gap junction communication was assayed as the number of unlabeled “Receiver” cells receiving Calcein (green) from a labeled “Donor” cell (green and red).

Gap junctional conductance was obtained from cell pairs using a double whole cell voltage clamp configuration as previously described (23) with some modifications. Briefly, cells on glass cover slips were transferred into a glass-bottom recording chamber under a Nikon TE2000 upright microscope 4-18 hours after plating. Access to the cytoplasm was achieved by using brief negative-pressure pulses after a giga-ohm seal was formed between polished glass micropipettes (4-7 MΩ) and cell membranes. Micropipettes were filled with patch solution (in mmol/L: 130 CsCl2; 2.0 CaCl2; 10 Hepes; 10 EGTA; pH 7.2). During recording, cells were kept at room temperature in cesium-containing solution (in mmol/L: 160 NaCl, 7 CsCl2, 2.0 CaCl2, 0.6 MgCl2, 10 Hepes, pH 7.4) to increase non junctional membrane resistance. Gap junctional conductance (gj) for cell pairs was determined as a result of dividing by 10mV the transjunctional current values (Ij) recorded while the transmembrane voltage of both cells was kept at 0.0 mV and a pulse of 10 mV-200ms duration was alternatively applied to each cell at 1Hz. Experimental stimulation, recording, series resistance compensation, and digitization were performed using a dual EPC-9 voltage clamp amplifier and Pulse and Pulsefit software (HEKA ELecltronik, Lambrecht/Pfalz, Germany).

**RESULTS**

Cx43 is the predominant connexin expressed by Src transformed CasKo cells. We utilized cells derived from homozygous null Cas knockout mice (CasKo cells) to examine the
effects of Cas and Src on Cx43 mediated gap junctional communication. These CasKo cells have been used to generate a well characterized system of cells that express neither Cas nor v-Src, Cas without v-Src, v-Src without Cas, or both v-Src and Cas. Previous work has demonstrated that Src utilizes Cas to promote anchorage independent growth and migration of these cells (10,38,43). Connexin expression in these cells was evaluated by microarray analysis. Probe sets were contained for Cx26, Cx29, Cx30, Cx30.3, Cx31, Cx31.1, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx47, Cx50, and Cx57. As shown in Figure 1, these data indicate that Cx43 was the most prominent connexin found at the mRNA level.

**Cas and Src do not suppress Cx43 expression.** Cx43 protein expression was examined in CasKo cells transfected with Cas, v-Src, both Cas and Src, or empty vectors. As shown in Figure 2a, western blot analysis indicates that Cx43 was expressed in these cells regardless of Cas or Src expression. The next best candidate based on mRNA analysis shown in Figure 1 was Cx45, and this protein was detected by western blot analysis (data not shown).

As shown in Figure 2a, Src transformation appeared to result in some increase in Cx43 production. This was also seen in LA25 cells, which express a temperature sensitive v-Src construct. As shown in Figure 2b, slightly more Cx43 was seen in LA25 cells cultured at permissive temperate than cells grown at nonpermissive temperature. These data are consistent with previous observations comparing Cx43 expression in Src transformed and nontransformed cells (41).

As shown in Figure 2c, siRNA was used to effectively block Cas production in LA25 cells. Interestingly, data from Figure 2 suggest that more Cx43 was seen in cells expressing Cas than cells without Cas. Moreover, this effect was independent of Src activity. Thus, Src and Cas did not decrease Cx43 production. Consistent with previous findings (10,38), data from Figure 2 also indicate that equivalent levels of v-Src and active Src kinase were expressed in Src transformed cells regardless of Cas expression.

**Cas and Cx43 colocalize at intercellular junctions between Src transformed cells.** Immunofluorescence microscopy was used to examine the effects of Src and Cas on Cx43 localization. As shown in Figure 3, Cas and Cx43 were both detected at regions of cell to cell contact between Src transformed cells. Thus, Src and Cas did not appear to suppress Cx43 protein expression or localization at intercellular junctions.

**Cx43 associates with Cas.** As shown in Figure 3, Cas and Cx43 colocalize when visualized by confocal microscopy. We hypothesized that Src utilizes Cas to close gap junction channels formed by Cx43. This hypothesis predicts that Cas associates with Cx43 in Src transformed cells. Immunoprecipitation experiments were performed to investigate whether or not Cas and Cx43 associate with each other. As shown in Figure 4, Cas antiserum precipitated Cx43 in addition to Cas and, conversely, Cx43 antiserum precipitated Cas in addition to Cx43. These data indicate that Cas and Cx43 associate with each other (either directly or nondirectly) in Src transformed cells.

**Src utilizes Cas to suppress gap junctional communication.** Gap junctional communication between CasKo cells transfected with Cas, Src, both Cas and Src, or empty vectors was visualized by intracellular dye transfer (22,39). Gap junctional communication was quantitated as the number of cells receiving dye from an individual preloaded cell (Figure 5a and 5b). As shown in Figure 5c, nontransformed CasKo cells, or CasKo cells containing Src but not Cas, transferred dye to an average of about 4 neighbors, while CasKo cells expressing both Cas and Src transferred dye to an average of only 1 neighbor. As shown in Figure 5d, LA25 cells grown at nonpermissive temperature transferred dye to over 30 neighbors. In contrast, these cells transferred dye to less than 10 cells at permissive temperature. However, transfection of LA25 cells with siRNA targeted to Cas increased dye transfer to over 30 neighbors at permissive temperature, while transfection with control siRNA has no effect (Figure 5d). Thus, cells expressing both Cas and active v-Src displayed significantly less gap junctional communication than cells expressing either Cas or Src alone (p<0.0001 by t-test). These data suggest that Cas decreased gap junctional communication between Src transformed cells by about approximately 3 to 4 fold.

In addition to dye transfer, effects of Src and Cas on gap junctional communication were evaluated by dual whole cell patch clamping. As
shown in Figure 6, CasKo cells transfected only with Cas exhibited a total conductance of about 15.8 nS. CasKo cells transfected only with Src displayed a similar total conductance of about 12.4 nS. In contrast, the total conductance of CasKo cells transfected with both Src and Cas was reduced to about 5.2 nS. Thus, as with dye transfer, cells expressing both Cas and Src displayed significantly less gap junctional communication than cells expressing either Cas or Src alone (p<0.02 by t-test). These data support the hypothesis that Src utilizes Cas to block gap junctional communication mediated by Cx43.

Cas is not required for Src to induce tyrosine phosphorylation of Cx43. Phosphorylation of Cx43 on tyrosine has been implicated in suppression of gap junctional communication between Src transformed cells (32). However, results also indicate that additional events are required to actually close channels formed by Cx43 (35). Taken together, data shown here indicate that Src activity was not sufficient to block gap junctional communication mediated by Cx43. This led us to examine whether or not Cas influenced the ability of Src to phosphorylate Cx43. As shown in Figure 7, Cx43 was phosphorylated on tyrosine in Src transformed cells regardless of Cas expression. These data suggest that phosphorylation of Cx43 on tyrosine was not sufficient to block gap junctional communication between tumor cells; Cas was also required.

DISCUSSION

It has been well established that the Src kinase can block gap junctional communication mediated by Cx43 (32,44). Src phosphorylates Cx43 on two tyrosine residues, Y247 and Y265. Phosphorylation of these residues has been associated with inhibition of gap junctional communication between Src transformed cells (33,34). However, other events, such as phosphorylation of Cx43 on serine residues is required for Src to block gap junctional communication mediated by Cx43 (35). It should be noted that, in addition to tyrosine, a relatively large amount of serine phosphorylation is detected on Cx43 in Src transformed cells (34,41). Zhou et al have reported that Src utilized MAPK to block gap junctional communication mediated by Cx43 (35). However, we found that Cas did not augment MAPK activity in the Src transformed CasKo cells or LA25 cells used for these studies (10) (and unpublished data).

Taken together, reports thus far suggest that while phosphorylation of Cx43 on tyrosine is involved in blocking gap junctional communication, it is not sufficient. Events in addition to tyrosine phosphorylation are required for Src to close channels formed by Cx43 between cells (34,35). For example, Lin et al have recently found that modification of tyrosine residues 247 and 265 to glutamate did not appear to affect channel function (45). Our data are consistent with this scenario. Without Cas, gap junctional communication persisted in Src transformed cells while Cx43 was phosphorylated on tyrosine. These data indicate that proteins are recruited by tyrosine phosphorylation to block gap junctional communication between tumor cells.

Involvement of ancillary proteins suggest a more complex regulation of gap junctions than simple phosphorylation events closing connexin channels. Several Cx43 binding partners have been identified. For example, Src (46,47), zona occludens proteins (ZO-1 and ZO-2) (48,49), and CCN3 (NOV) (50) all bind to the carboxyl tail of Cx43. In particular, the SH3 domain of Src can associate with Cx43 (47,51). Thus, it may be suspected that the SH3 domain of Cas may also bind to Cx43. However, it should be noted that while the colocalization and immunoprecipitation studies presented here indicate that Cas and Cx43 associate with each other, this association may not be direct. Instead, associations between Cas and Cx43 may be mediated by a mutual partner.

Our data suggest that Cas does not appear to act as an adaptor protein required for Src to phosphorylate Cx43. Cx43 was still phosphorylated in Cas deficient cells. However, such a scenario should not be ruled out at this point. For example, a Cas homolog, such as Hexf (11), may have mediated such an interaction in the absence of Cas.

A functional role of Cas in blocking gap junctional communication between tumor cells presents some clear biological significance. Cas is required for Src to promote metastatic cell growth, and Cx43 is a tumor suppressor. Src utilizes Cas to confer tumor cells with the most fundamentally important properties of metastatic growth
including anchorage independence and increased motility (8,10,17). Cx43 is a tumor suppressor gene that can inhibit tumor cell growth (27,28,52). Thus, it stands to reason that mechanisms promoting tumor cell growth should effectively silence Cx43 activity. Indeed, as outlined in Figure 8, our data indicate that Src utilizes Cas to block gap junctional communication in addition to promoting hallmarks of tumor cell growth.

Cas links integrins to other components in the focal adhesion complex (37). The present studies introduce a novel role of Cas in the gap junction complex. A double role for junctional adaptor proteins is not unprecedented. For example, β-catenin acts a structural adaptor protein linking cadherins to the cytoskeleton and as a nuclear transcription factor involved in the Wnt signaling pathway (53,54). It should be interesting to elucidate mechanisms by which Cas acts as a Src effector for both focal adhesions and gap junctions.
References


FOOTNOTES

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FIGURE LEGENDS

**Figure 1:** Cx43 was the prominent connexin expressed by CasKo cells. Microarray expression analysis was performed on CasKo cells transfected with Src alone or both Src and Cas as indicated. Levels of mRNA encoding individual connexins are presented as mean+SEM of all probe sets representing indicated connexins. Cx43 was the predominant connexin expressed by these cells.

**Figure 2:** Cas and Src did not suppress Cx43 expression.
Equal amounts of protein (20 μg per lane) from (a) CasKo cells transfected with empty vectors, Cas, Src, or both Cas and Src, (b) LA25 cells incubated at permissive (Src+) or nonpermissive (Src-) temperatures for Src activation, or (c) LA25 cells transfected with siRNA directed against Cas (Cas-) or nontargeting control siRNA (Cas+) before incubation at permissive or nonpermissive temperature were evaluated for Cx43, Cas, active Src, and β-actin by Western blot analysis as indicated. Cx43 was detected in cells regardless of Cas or Src expression.

**Figure 3:** Cas and Cx43 localized to intercellular junctions between Src transformed cells.
Cx43 and Cas were visualized by immunofluorescence microscopy of (a) CasKo cells transfected with Src alone or Cas and Src or (b) LA25 cells incubated at permissive temperature as indicated. DIC images were also acquired or Hoechst was used to stain nuclei (bars = 20 microns). Colocalization of Cas and Cx43 was further investigated by (c) orthogonal imaging in cut out view and (d) an intensity plot profile over distance in one focal plane of an area observed in LA25 cells. Cas and Cx43 were both detected between at regions of cell to cell contact (indicated by arrows in merged panels).

**Figure 4:** Cx43 associated with Cas.
Protein from CasKo cells transfected with Cas, Src, both Cas and Src, or empty vector controls, or LA25 cells transfected with siRNA directed against Cas or nontargeting control siRNA before incubation at permissive or nonpermissive temperature cells, was immunoprecipitated with Cas antiserum or Cx43 antiserum as indicated. Cx43 and Cas were then analyzed by Western blotting. Cas immunoprecipitated with Cx43, and Cx43 immunoprecipitated with Cas when both proteins are present in cells. Thus, Cas and Cx43 associated with each other in Src transformed cells.

**Figure 5:** Src utilized Cas to suppress gap junctional communication.
Gap junctional communication between cells was visualized between (a) CasKo cells transfected with empty vectors, Cas, Src, or both Cas and Src, or (b) LA25 cells transfected with siRNA directed against Cas or nontargeting control siRNA before incubation at permissive or nonpermissive temperatures. Communication was detected by calcein dye transfer from a preloaded cell, labeled with calcein and DiI, to nonlabeled neighboring cells. The hydrophilic calcein traveled through gap junction channels to adjacent cells, while the lipophilic DiI remained in the preloaded cell (bar = 25 μm in panel a and 50 μm in panel b). Gap junctional communication was suppressed in cells that expressed both Cas and Src. Intercellular dye transfer between (c) CasKo cells or (d) LA25 cells was quantitated as the number of cells receiving dye from an individual preloaded cell. Data is shown as mean+SEM (n=30). Cells expressing both Cas and high Src kinase activity displayed less dye transfer than cells expressing either Cas or high Src kinase activity alone (p<0.0001 by t-test).

**Figure 6:** Src utilized Cas to suppress total gap junctional conductance.
Total conductance between cells was evaluated by dual whole cell patch clamping. Data is shown as mean+SEM (n>8). Cells expressing both Cas and Src displayed less total conductance than cells expressing either Cas or Src alone (p<0.02 by t-test).
**Figure 7:** Cas was not required for Src to induce tyrosine phosphorylation of Cx43.
Protein from cells expressing Cas, Src, both Cas and Src, or null controls were immunoprecipitated with Cx43 antiserum as indicated. Immunoprecipitated protein was then analyzed by Western blotting with antiserum specific for Cx43 or phosphotyrosine as indicated. Only Cx43 from cells expressing Src alone, or both Src and Cas, was recognized by antiserum specific for phosphotyrosine. Thus, Cas was not required for Cx43 to be phosphorylated on tyrosine in Src transformed cells.

**Figure 8:** Src utilizes Cas to promote hallmarks of metastatic cell growth and block gap junctional communication mediated by Cx43.
Communication was evaluated between cell expressing neither Cas nor v-Src, Cas alone, v-Src alone, or both Cas and v-Src. Previous work has demonstrated that Src utilizes Cas to promote anchorage independent cell growth and migration (see text).
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