Canonical Hedgehog signaling drives proangiogenic responses in endothelial cells

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Dissecting the role of INCENP-Aurora B in spindle assembly checkpoint function, chromosomal alignment and cytokinesis

Comment on: Becker M, et al. Cell Cycle 2010; 9:1360–72.

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Mitosis is a highly regulated process which involves centrosomes, the bipolar spindle and kinetochores to secure equal separation of chromosomes to both daughter cells. Kinases of the Aurora family, key regulators of this process and promising anticancer targets,[1] are functionally distinguished by their specific subcellular localization during mitosis.[2] Aurora A resides at spindle poles and functions in mitotic entry, centrosome maturation and spindle formation.[3] In contrast, Aurora B, part of the chromosomal passenger complex (CPC), localizes at centromeres/inner kinetochores, the spindle midzone/central spindle and finally the midbody during telophase and cytokinesis, reflecting crucial roles in the regulation of chromosome condensation, correct chromosome-microtubule attachment, mitotic spindle stability and cytokinesis.[4] Localization of Aurora B to centromeres/inner kinetochores requires regulatory subunits of the CPC, in particular INCENP (inner centromeric protein) (**Fig. 1**). One crucial function of Aurora B and the CPC before metaphase-anaphase transition is the correction of microtubule-kinetochore attachments which do not result in normal bipolar chromosomal alignment. For that, Aurora B phosphorylates various substrates at the microtubule-kinetochore interface, among them the microtubule-destabilizing kinesin MCAK and the kinetochore Ndc80 complex thus directly regulating the stability of microtubule plus ends and their affinity to kinetochores, respectively.[5] Lastly, centrosomal proteins, including TACC3, which interacts with the MCAK antagonist and microtubule stabilizing factor ch-TOG/CKAP5, may indirectly influence spindle-dependent localization and assembly of kinetochore proteins and spindle assembly checkpoint (SAC) components.[6]

During the early phases of mitosis when chromosomes exhibit incomplete or faulty attachments to spindle microtubules no tension across sister kinetochores can be generated. This scenario activates the SAC leading to the stabilization of cyclin B and securin, whose degradation by the anaphase-promoting complex is mandatory for metaphase-anaphase transition. Thus, activation of the SAC delays the onset of anaphase as long as all chromosomes are properly aligned on a metaphase plate. Essential components of the SAC are Bub1 and BubR1, whose recruitment to kinetochores is dependent on Aurora B.[7] Thus, the CPC may take over a direct role in SAC function as opposed to an indirect function by the correction of non-bipolar microtubule-kinetochore attachments. To which relative extent the CPC works in both scenarios to achieve efficient SAC function is currently unclear.

In their comprehensive and elegant study, Becker et al.[8] dissected the role and direct involvement of Aurora B kinase, selectively re-targeted to centromeres/inner kinetochores, in SAC function, chromosomal alignment and cytokinesis. The authors first treated mitotically arrested cells with Actinomycin D to specifically and efficiently displace endogenous Aurora B and other chromosomal passenger complex (CPC) subunits from centromeres/inner kinetochores without influencing Aurora B kinase activity. Concomitantly, this led to a premature loss of the checkpoint proteins BubR1 and Bub1 from kinetochores (**Fig. 1**, middle panel). This approach was specific since localization of Aurora A at spindle poles was not affected. The authors further show that Actinomycin D treatment causes chromosomal misalignment, very efficiently overrides SAC dependent mitotic arrest both in the presence of nocodazole or paclitaxel, and results in cytokinetic failure. Using this unique experimental scenario with mislocalized endogenous CPC and SAC components as starting point, Becker et al.[8] employed a CENP-B-INCENP[9] fusion protein to selectively re-target endogenous Aurora B to centromeres/inner kinetochores (**Fig. 1**, right panel). Remarkably, this approach led to a rescue of SAC function as indicated by Aurora B mediated phophorylation of CENP-A at Ser-7 and BubR1 recruitment to kinetochores indicating that centromeric/inner kinetochor localized INCENP-Aurora B is indeed sufficient for activation and maintainance of the SAC in the absence of microtubules. However, re-targeting INCENP-Aurora B to centromeres/inner kintochores failed to correct chromosomal misalignment and restore normal cytokinesis. Thus, the work by Becker et al.[8] clearly argues for a direct function of Aurora B in SAC activation (i.e., independent from the role of Aurora B/CPC in generating transiently unattachted kinetochores to resolve microtubule-kinetochore mal-attachments[10]) and establishes an unique experimental setting for further in-depth structure/function analysis of Aurora B and CPC components in relation to their subcellular localization during mitotic progression.

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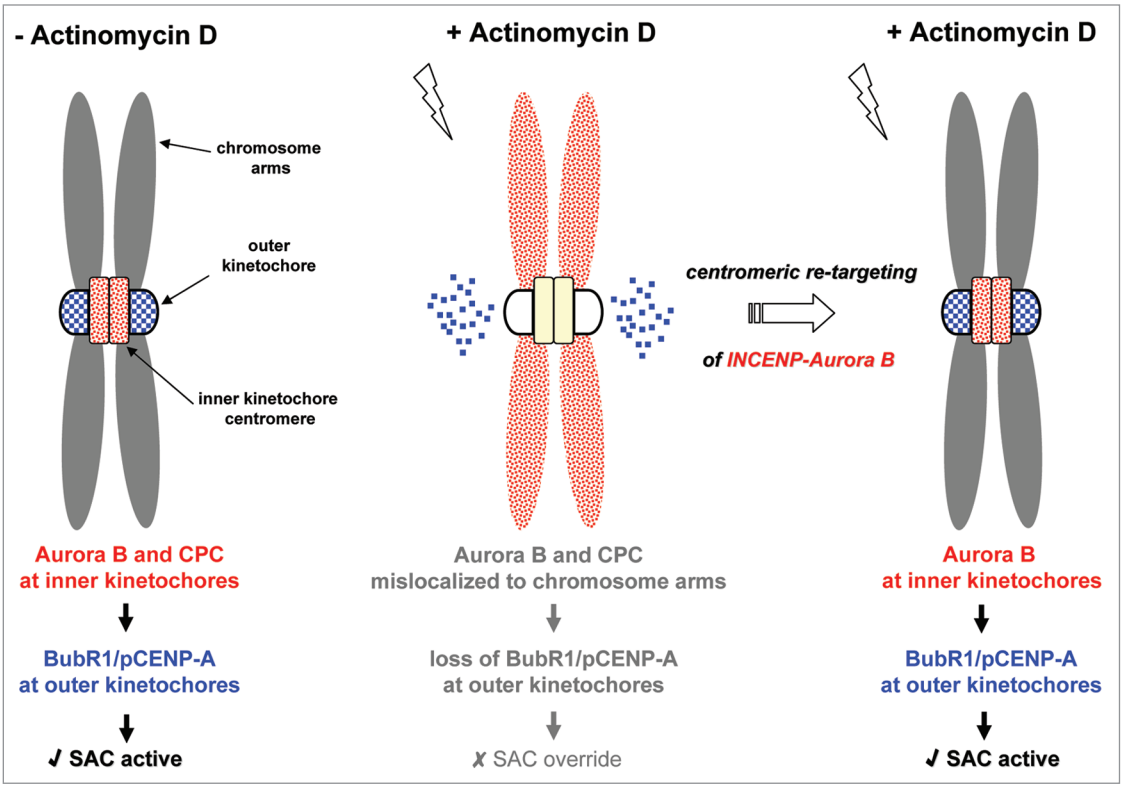

Figure 1. Model summarizing the work of Becker et al.[8] which argues for a *direct* role of Aurora B in SAC function in the presence of spindle damage. In particular, the authors used Actinomycin D to displace endogenous Aurora B/CPC from centromeres/inner kinetochores (middle panel) followed by selective re-targeting of INCENP-Aurora B to these structures (right panel). Using this approach the authors show that although centromeric/inner kinetochore localized Aurora B per se fails to correct chromosomal misalignment and restore normal cytokinesis it is very well sufficient to activate and maintain the SAC as indicated by Aurora B mediated phosphorylation of Cenp-A and relocalization of BubR1 to kinetochores.

Unraveling the relationship between n-myc and Focal Adhesion Kinase (FAK) in neuroblastoma?

Comment on: Beierle EA, et al. Cell Cycle 2010; 9:1005–15.

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Those of us who work with children understand the frustration in treating neuroblastoma. Neuroblastoma is the most common extracranial tumor of childhood. It accounts for approximately 15% of pediatric cancer deaths. Although sometimes this tumor presents as localized disease, quite often it presents with widely disseminated disease in children over 18 months of age and despite very aggressive therapies, including bone marrow transplantation, the long term survival remains around 20-35% in this group of patients.[1] There are many factors that are thought to play a role in this poor prognosis but by far the most important prognostic factor has been determined to be n-myc amplification. This transcription factor was found to be elevated in human neuroblastoma specimens in patients with advanced stage disease.[2] Since that time, much effort has been devoted to determining the role that n-myc plays in changing tumor behavior to make the tumors more aggressive. Despite this work, the exact function and gene targets of n-myc have yet to be determined. It has been determined that n-myc silencing via siRNA results in increased cleavage of caspase 3 which is important for apoptosis and decrease in the anti-apoptotic protein Bcl-xL.[3] Therefore, n-myc may work through anti-apoptotic mechanisms.

Focal Adhesion Kinase (FAK) has also been determined to play an important role in tumor growth and progression. The gene for FAK is located on chromosome 8q24. Under normal conditions, this protein controls a number of biological processes including cell proliferation and survival. Therefore, it was surmised that it may play a role in cancer progression. This was substantiated by the fact that FAK is overexpressed in many tumors including breast, colon, ovary, brain, etc. In addition, altering FAK signaling alters cell survival through anoikis (apoptosis due to lack of cell adhesion). In addition, increased FAK expression improves the tumor cell's ability to migrate, invade and metastasize.[4]

The overexpression of the active form of FAK ($p125^{FAK}$) has been demonstrated in a variety of human tumors and tumor cell lines. In a similar manner to n-myc, attenuation of FAK (via treatment with anti-sense nucleotides) in the human embryonal RD cell line resulted in increased apoptosis as demonstrated by flow cytometry, DNA laddering and electron

microscopy.[5] When HL-60 cells were transfected with FAK, they too became resistant to apoptosis and this resistance was through NFκβ activation.[6]

The work of Dr. Beierle and colleagues attempts to find a correlation between n-myc amplification and FAK expression. This is a very logical assumption and I believe her previous papers on the subject provide strong evidence for this assumption. In their first paper on the subject,[7] they were able to determine that n-myc amplified cell lines have increased expression of FAK. They determined this in both a non-isogenic (Sk-N-AS and IMR-32) and a isogenic (N-myc$^+$, Tet$^-$ and N-myc$^-$, tet$^+$) cellular comparison. They found both RNA expression and protein levels of FAK were increased in the cells with n-myc amplification. It was also determined that there was n-myc binding to the FAK promoter and the P-280 construct was important for this binding. This established a direct correlation between n-myc and FAK in neuroblastoma. They also found that this binding occurred in vivo. To further establish the relationship, the lab determined that downregulation of FAK with either siRNA or an inhibitor to FAK, decreased cellular viability in n-myc amplified cells. The laboratory of Dr. Beierle then continued to cement the importance of FAK expression in neuroblastoma. In their subsequent paper,[8] they performed testing on human neuroblastoma samples. They determined that FAK mRNA is present in human neuroblastomas and this correlated with p125FAK staining in the ganglion type cells seen on immunohistochemistry. They also determined that p125FAK staining in stage IV specimens was associated with amplification of the n-myc oncogene. This again provides evidence that there is a relationship between these two important genes.

In a recent study[9] published in *Cell Cycle*, Dr. Beierle's group provides evidence of the possible clinical importance of this association between n-myc and FAK in neuroblastoma. For these experiments they used an inhibitor to FAK previously shown to decrease tumor growth in the BT474 breast cancer cell line.[10] Using the isogenic cell line previously used in their laboratory (N-myc$^+$, Tet$^-$ and N-myc$^-$, tet$^+$), Dr. Beierle has shown that use of this inhibitor (Y15) causes dephosphorylation of FAK and this dephosphorylation was enhanced in the n-myc expressing cell line. In addition, with this treatment, the cells become detached and had decreased viability and increased apoptosis. To confirm this was a reproducible phenomenon, the same experiments were carried out using other neuroblastoma cell lines (SK-N-AS and SK-N-BE) and the results were confirmed. Most importantly, they showed that Y15 inhibited human neuroblastoma growth in vivo in those tumors with n-myc amplification.

Neuroblastoma, especially those tumors that have poor prognostic indicators, one of which is n-myc have long frustrated clinicians as they are very resistant to treatment and about 60–70% of the children with these aggressive masses die from their disease. Any treatment modality that can reverse this outcome would be of great benefit to these unfortunate patients. We have known that n-myc is a poor prognostic indicator but as of yet, have not been able to use this information to improve outcome. The work by Dr. Beierle may be an important step in this process. She has determined a relationship between n-myc and FAK and found that FAK inhibition in the n-myc amplified tumors decreased tumor growth. If this work is confirmed in more animal experiments it could be a very important adjunct to traditional chemotherapies in patients with n-myc amplified tumors, the very group that most needs a better treatment regimen.

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The replisome: A nanomachine or a dynamic dance of protein partners?

Comment on: Wawrousek K, et al. Cell Cycle 2010; 9:1156–66.

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Exact duplication of the genome requires coordination of leading and lagging strand synthesis with parental DNA unwinding, as well as with DNA repair processes. A dynamic protein machine termed a replisome accomplishes this feat with high speed and remarkable fidelity. Eukaryotic replisomes assemble at sites marked by origin recognition complex through recruitment of a multiprotein complex containing the Mcm2-7 helicase in latent form. Upon activation of cell cycle-dependent kinases, Cdc45, Mcm2-7, GINS and additional factors generate an active CMG helicase and a functional replisome. The increasing number and variety of proteins associated with the replisome raise the question of whether the replisome is a nanomachine with a defined set of dedicated parts, or more like a troupe of versatile protein partners that jostle for space and time at the fork, changing with circumstances at the fork.

Recent progress toward resolving this question reveals And1/Ctf4 as a central node in eukaryotic replisomes[1] (**Fig. 1**). Human And1/Ctf4 associates with the CMG helicase, and, along with Mcm10 and its interaction partner RecQ4, is needed to initiate replication in vivo.[2,3] Importantly, Ctf4 and GINS directly bridge pol alpha with Mcm2-7 in yeast.[4,5] Purified human And1/Ctf4 forms a stable homodimer, interacts directly with replicative DNA polymerases (pol) alpha and epsilon, and more weakly, pol delta, while stimulating their activities in vitro.[6] Moreover, Costanzo and colleagues report that Xenopus Ctf4 associates directly with tipin to monitor fork progression and stabilize the fork when damage is encountered.[7] Altogether, these suggest Ctf4 may couple DNA unwinding, leading and lagging strand synthesis, sister cohesion and perhaps regulate replisome responses during replication stress.

In a previous issue of *Cell Cycle*, Campbell, Dunphy, and colleagues identify Dna2 at the intersection of DNA replication and repair. Yeast Dna2 is a 5′-3′ nuclease/helicase that cooperates with Fen1, another structure-specific 5′-3′ nuclease, in Okazaki fragment

processing. However, whether vertebrate Dna2 functions as a nuclear helicase, or instead is restricted to mitochondria, is controversial. In this work, Wawrousek et al. confirm that vertebrate Dna2 displays intrinsic helicase activity, localizes in nuclei, and associates with chromatin in a licensing-dependent but Mcm10-independent manner, concurrently with Cdc45. Notably, Xenopus Dna2 interacts with both Ctf4 and Mcm10 (**Fig. 1**). Definition of the interacting regions among these proteins, and the role of Dna2 helicase and nuclease in replisome function remain open questions.

Progressing replication forks frequently encounter damage before converging with an oncoming fork to terminate.[8] At some template lesions, the replisome adapts by shedding some proteins and loading others to bypass the damage, leaving a mispaired sequence or an RPA-associated gap in its wake. Alternatively, a double strand break (DSB) can result whose repair requires a different set of proteins, some of which are commonly associated with replicating chromatin, among them the MRN nuclease (MRX in yeast). Strand-specific 5′-3′ end resection by MRN/X and other nucleases including Dna2 is an early step in DSB repair.[9] Wawrousek et al. extend these studies to show that Dna2 interacts with ATM and Nbs1, yet it can bind to DNA ends that resemble DSBs independently of MRN and resect these ends to generate a 3′ overhang despite inhibition of MRN nuclease activity, albeit less efficiently. Since Dna2 can be recruited to DSB ends both in the absence of Mre11 nuclease activity and when Nbs1 is immunodepleted, the set of protein/DNA structures responsible for Dna2 recruitment to DSB ends remain to be defined. Other important questions to be explored include whether the Dna2 nuclease, helicase or both activities are involved in chromatin replication, whether association with Mcm10, pol α or Ctf4 affects Dna2 enzymatic activities, and how the end resection activity of Dna2 is related to its role(s) in the replisome.

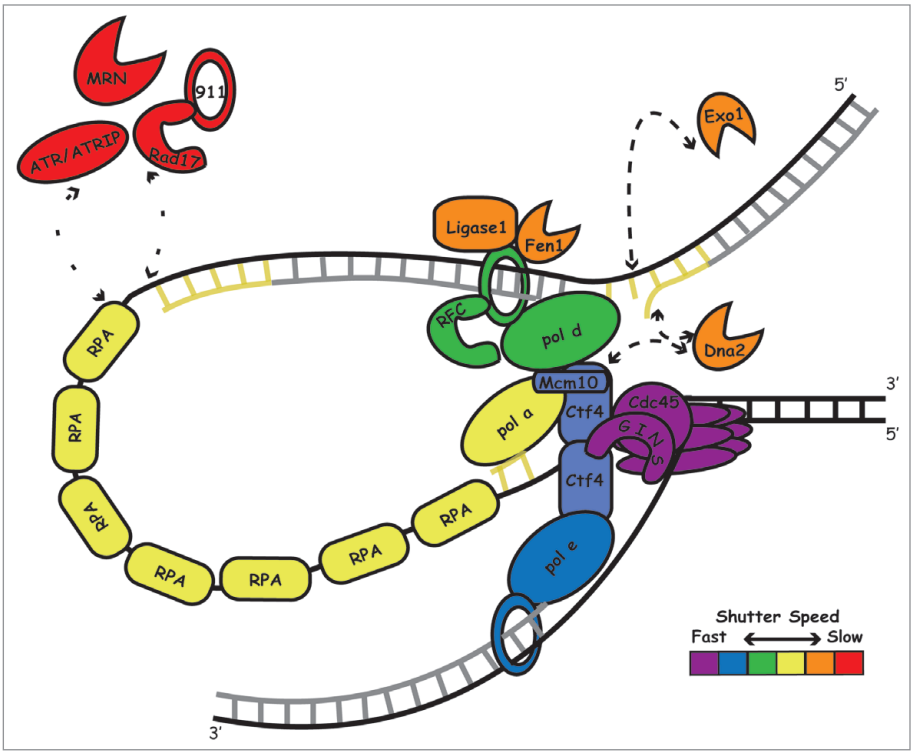

Figure 1. A hypothetical snapshot of the emerging replisome at different shutter speeds. During unperturbed replication, CMG helicase unwinds parental DNA as pol epsilon synthesizes the leading strand. The discontinuous nature of lagging strand synthesis requires the replisome to be remodeled as proteins synthesize or process the Okazaki fragments. Multiple weak interactions among the proteins in the replisome, of which only a few are depicted, enable replisome remodeling through exchange of interaction partners. This provides the plasticity crucial to adapt to different circumstances, e.g., replication through "slow zones" or damaged template, which lead to transient accumulation of specialized proteins, e.g., ATRIP/ATR and Rad911.

In summary, the plasticity of the eukaryotic fork is coming into view. Multi-tasking proteins like Dna2 and Ctf4 will continue to provide important clues into replisome dynamics. However, a deeper understanding of replisome operations will require detailed analysis of proteins competing for association with the complex, the role of overlapping binding sites, protein modifications, and protein turnover at the fork.

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Integrins open the way to epithelial-mesenchymal transitions

Comment on: Bianchi A, et al. Cell Cycle 2010; 9:1647–59.

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Epithelial-mesenchymal transitions (EMT) are important for early embryogenesis, organ development such as the heart and disease pathogenesis such as kidney and lung fibrosis or carcinoma invasiveness.[1] During EMT, an epithelial tissue gets disorganized because some cells change their intercellular junctions, their supporting actin and intermediate filament cytoskeleton and remodel their extracellular matrix so that the basement membrane lining of the tissue is destroyed and invasiveness of the mesenchymal cells is facilitated.[1] EMT is induced by extracellular signaling factors and cues provided by the microenvironment of the epithelial cell. Among such factors, transforming growth factor β (TGFβ) plays a major role in EMT.[2] In addition, integrin receptors that transmit signals from specific extracellular proteins such as laminin are known to cooperate with cytokines such as TGFβ in the establishment of the mesenchymal differentiation program.[1]

Recent work aimed at deciphering the signaling pathways and mediators of TGFβ that elicit the EMT response. Accordingly, TGFβ, via its two receptors, activates Smad proteins and cooperating mitogen-activated protein kinases such as p38, leading to transcriptional induction of several nuclear embryonic factors such as high mobility group A2, Snail1/2, Twist1 and ZEB1/ZEB2.[2] Many of these nuclear factors bind the Smads and repress epithelial genes such as E-cadherin and occludin, while inducing expression of mesenchymal genes such as fibronectin. TGFβ receptors also downregulate the epithelial tight junctions by phosphorylating the polarity complex protein Par6, which controls stability of the RhoA GTPase.[3] Furthermore, TGFβ causes a dramatic remodeling of the integrin receptors during the EMT response, via transciptional or translational mechanisms.[4-8] Interestingly, regulation of integrin expression by TGFβ was established even before the TGFβ receptors and Smads were identified.[6]

Remodeling of the integrin receptor repertoire in epithelial cells serves multiple purposes. Integrins provide sustained TGFβ ligand activation from the matrix-enriched microenvironment and coordinate signaling between matrix molecules such as laminin and TGFβ.[9] Integrin β1 or β3 ligation to matrix ligands controls the activation of critical effectors of the EMT process such as focal adhesion kinase, Src and p38 kinase downstream of TGFβ.[4,5] TGFβ also coordinates the internalization and downregulation of E-cadherin protein with the formation of integrin-enriched focal adhesions.[7] The process of E-cadherin internalization is also linked to the formation of an active complex between Smad2 and β-catenin.[8] Formation of the Smad2-β-catenin complex requires coordinated signaling by the TGFβ receptor and by the integrin α3β1 receptor in response to extracellular laminin.[8] Among the various integrins, TGFβ seems to regulate expression of α4/5/6 and β1/3/4/6 subunits in mesenchymal cells, such as fibroblasts or tumor-derived sarcoma cells, and during EMT, a process that contributes to the generation of mesenchymal cell types in fibrotic or tumor-associated stromal tissue. TGFβ therefore orchestrates a major integrin expression reprogramming which is tissue specific (e.g., for mammary or colon epithelium) and matrix ligand-specific (e.g., for laminin or fibronectin). This context-dependency often raises controversies as scientists attempt to identify the most critical regulatory events that govern the progression of a complex cellular response such as EMT. For example, certain reports favor more a role of integrin β1 while other reports favor more the role of integrin β6. This is why the paper presented by Bianchi et al. is of major importance, as it provides new molecular explanations about the role that specific integrin receptors play during TGFβ-induced EMT.

Using a well-established mammary cell model of EMT, the new evidence critically implicates integrin β5 in TGFβ-induced EMT. In this cell system, TGFβ, via the Smad pathway transcriptionally induces expression of the αV and β5 integrins, and silencing of either one perturbs the ability of the epithelial cell to undergo the mesenchymal transition. The role of the αVβ5 integrin complex appears to serve as a mediator of matrix-coupled signaling that regulates the remodeling of focal adhesions. Focal adhesion rearrangement plays critical roles during EMT of normal epithelial cells, but also critically affects the invasive properties of tumor cells that have metastatic capacities.

The elucidation of the signal transduction mechanisms that explain the intimate cross-talk between TGFβ and integrin receptor pathways promises a better understanding of the complex processes of tumor cell invasiveness and metastasis. Such mechanisms beautifully confirm the pioneering hypotheses made by E. D. Hay,[1] when she proposed that microenvironmental growth factors and matrix-dependent adhesion co-ordinately govern the fate and competence of the epithelial reprogramming.

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Canonical Hedgehog signaling drives proangiogenic responses in endothelial cells

Comment on: Chinchilla P, et al. Cell Cycle 2010; 9:570–9.

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Hedgehog (Hh) proteins constitute a highly conserved family of intercellular signaling molecules that are fundamental regulators of embryonic development as illustrated by dramatic embryonic malformations seen in humans and mice with perturbed Hh signal transduction.[1] The last decade it has become evident that the Hh pathway also remains active in the post-embryonic period and has for instance been found to play an essential role in maintaining tissue integrity and in tissue revascularization after ischemic stress.[2,3] The ligation of Hh ligand with its cellular receptor Patched (Ptch) alleviates the inhibitory action of Ptch on the associated receptor Smoothened (Smo) leading to the dissociation of a "Gli-inhibitor" complex. Subsequently, nuclear translocation of Gli transcription factors induces expression of, among others, angiogenic genes like vascular endothelial growth factor (VEGF) and the angiopoietins (Ang)-1 and 2.[2] Interestingly however, endothelial cells are considered to be unresponsive to Hh directly.[4,5] Rather, adjacent mesenchymal cells are thought to translate the presence of Hh ligand in signaling molecules that act on the endothelium.

In a recent paper published in *Cell Cycle*, Chinchilla and coworkers elegantly show that endothelial cells do respond to Hh although not by inducing the canonical Gli-dependent pathway.[6] Instead, Hh induces Smo-dependent actin stress fiber formation in a Gi-protein and Rac1-dependent manner without inducing a transcriptional response. This non-canonical Hh pathway, previously shown to induce lamellipodia formation and consequent chemotaxis towards Hh in fibroblasts[7] and to induce the projection of neurites in neuralized embryonic stem cells,[8] induced tubulogenesis of endothelial cells suggesting that Hh controls angiogenesis by targeting endothelial cells to prime the initial steps of angiogenesis and mesenchymal cells to subsequent vessel maturation. Noteworthy, Chinchilla and coworkers strongly suggest that tubulogenesis of endothelial cells is Gli-independent although they do not directly target Gli to prove or refute this hypothesis.

Interestingly, we have recently obtained data that strongly suggest a Gli-dependence for tubulogenesis in response to Hh. We studied the effect on tubulogenesis in endothelial cells treated with GANT61, an inhibitor that interferes with Gli1 DNA binding and subsequently tumor cell proliferation in both in vitro as well as in vivo xenograft models.[9] Both human umbilical vein endothelial cells (HUVECs) as well as immortalized 2H11 murine endothelial cells form proper endothelial tube like structures in response to Sonic Hedgehog (Figure 1 for HUVECs). Importantly, the addition of 5 μM GANT61 largely diminished Shh-induced tube formation showing that Gli1 plays an important role in Shh-induced tubulogenesis of endothelial cells. The role of the canonical Hh pathway, or at least that of the Gli family of transcription factors, in tubulogenesis and angiogenesis is therefore even more complex as anticipated. It seems however that, as already suggested by Dr. Kanda and coworkers in 2003,[10] Hh induces tubulogenesis of endothelial cells through a combination of rapid Gi-dependent signaling and Gli1 mediated transcriptionally regulated pathways.

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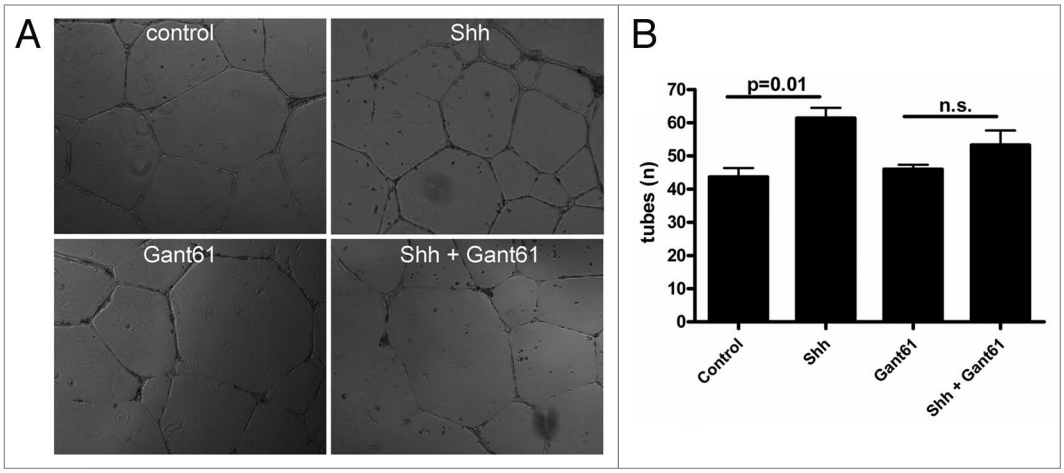

Figure 1. Gli1 inhibition by Gant61 reduces Shh-induced endothelial tubulogenesis. HUVECs (4.5x10e4 cells per well) were plated on growth factor reduced matrigel in a 24-well tissue culture plate in the absence (control) or presence of Shh, Gant61 or Shh/Gant61. After 24 hours stimulation, the number of tubes was measured in five independent fields at a 4x magnification. (A) Representative pictures of the different conditions. (B) Quantitative representation of the results depicted in (A). Shown is the fold increase in number of tubes [mean+/-SEM (n=3)]. n.s., not significant.