



# Mechanisms and consequences of entosis

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**Abstract** Multiple mechanisms have emerged where the engulfment of whole live cells, leading to the formation of what are called ‘cell-in-cell’ structures, induces cell death. Entosis is one such mechanism that drives cell-in-cell formation during carcinogenesis and development. Curiously, entotic cells participate actively in their own engulfment, by invading into their hosts, and are then killed non-cell-autonomously. Here we review the mechanisms of entosis and entotic cell death and the consequences of entosis on cell populations.

**Keywords** Entosis · Entotic cell death · Cannibalism · Cell-in-cell · Engulfment · Phagocytosis · Autophagy · LAP · Cell competition · Aneuploidy

## Introduction

Programmed cell death is required for proper tissue development and homeostasis and inhibits the development of diseases such as cancer. In metazoans, cell death hollows the amniotic cavity [6], establishes tissue patterns [52], eliminates developmental organs [28], and sculpts complex tissue structures like fingers [32], mammary gland [39], muscle [23], brain [31], and retina [19]. Cell death maintains immune function by eliminating auto-reactive

cells [50], and cell death maintains homeostasis by removing aged or damaged cells from adult tissues.

While physiologic cell death was once considered to occur only by apoptosis (Type I cell death), many ‘alternative’ forms of cell death have now also been identified that, like apoptosis, are controlled by genetic machinery and contribute to cell turnover [16]. For example, autophagic cell death (Type II cell death; e.g. autosis [35]) and programmed forms of necrosis (Type III cell death; e.g. necroptosis [8] and ferroptosis [9]) can eliminate cells during development [43] or carcinogenesis [24], and contribute to killing cells that are infected by viruses [40] or damaged by ischemia–reperfusion [33–35].

In addition to apoptotic, autophagic and necrotic forms of cell death, yet further mechanisms have emerged that may represent additional ways that cells can be eliminated. Among these, multiple mechanisms have been found where viable cells can become engulfed or cannibalized and then killed [46, 48, 63, 64]. These programs, termed entosis [12, 47], phagoptosis [3], suicidal emperipolesis [55], homotypic cell cannibalism [4], emperitosis [62], and cannibalism [38], appear to differ fundamentally from most classic forms of cell suicide, due to the requirement of engulfing cells for cell death execution. Like programmed forms of cell suicide, these cannibalistic programs may also contribute to cell turnover in normal or pathophysiologic contexts.

## Cannibalistic cell death

The term ‘cannibalism’ generally describes non-cell-autonomous killing activity that targets viable cells of the same or related species. Cannibalism is an ancient strategy that is used as a stress response by even some unicellular

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organisms. For example, the sporulating bacterium *Bacillus subtilis* can cannibalize neighboring bacteria in response to starvation, by secreting lytic factors that induce cell rupture and the release of nutrients into the environment [17]. Similarly, the cellular slime mold *Dictyostelium caveatum* cannibalizes neighboring amoeba by engulfing and degrading them in order to obtain nutrients when bacteria are scarce [44, 59]. *Dictyostelium* amoebae also cannibalize neighboring cells when entering into an alternative starvation response called the sexual life cycle, where zygote giant cells engulf up to hundreds of neighboring amoebae to form ‘macrocyts’, whose protective cell walls are constructed in part from recycled components of cannibalized cells [10, 45, 53].

Perhaps not unlike the cannibalization of individuals within ancestral single cell populations, the individual cells within the tissues of metazoan organisms can also cannibalize each other. While apoptotic cells are well known to be engulfed by phagocytic cells within such tissues (and in some cases phagocytosis is required for apoptosis execution [20, 51]), cannibalistic programs instead target viable cells, where engulfment does not respond to, but rather induces, cell death. Cell cannibalism in metazoan tissues can actually occur by a number of different molecular mechanisms. Some involve phagocytosis (see [3]), while others involve non-phagocytic mechanisms, such as suicidal emperipolesis [55], entosis [12], cell cannibalism [37], and others [63]. These mechanisms can be broadly characterized as heterotypic (occurring between different cell types) or homotypic (occurring between the same cell type), and lead to the formation of cell-in-cell structures [46, 63]. In this review we discuss the detailed molecular mechanism of one of these cell-in-cell processes, entosis, as well as the consequences of entosis on cell populations, and evidence that entosis occurs in vivo in normal and diseased contexts.

## Mechanisms of Entosis

### Entotic cell engulfment

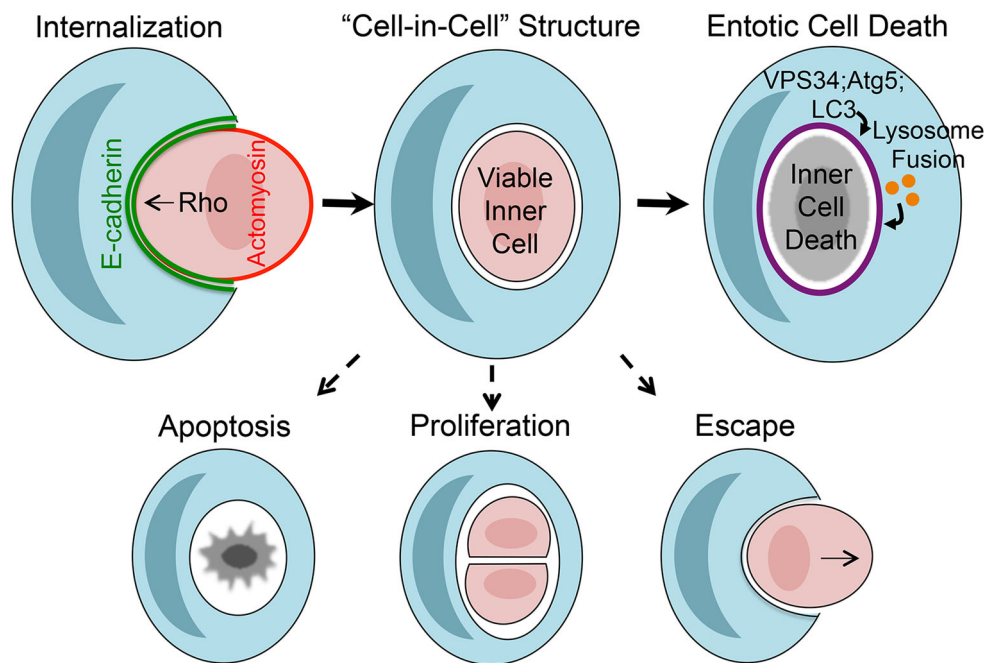
Entosis is triggered in culture by matrix detachment of adherent cells, similar to the apoptotic program of anoikis, although the processes are clearly distinct by functional studies [47]. The entosis engulfment mechanism, unlike phagocytosis, involves epithelial adherens junctions, composed of the cell–cell adhesion receptor E-cadherin and the adherens junction/cytoskeleton linker protein  $\alpha$ -catenin, which are necessary [47, 61], and sufficient [56, 61], to mediate entosis in breast tumor cell populations. Entotic cells, unlike cells engulfed by phagocytosis, also play an active role in controlling their own uptake through RhoA-

GTPase activity and the RhoA effector kinases Rho-kinases I and II (ROCKI/II) (Fig. 1) [47]. The overexpression of RhoA or ROCK I/II is sufficient to drive the uptake of epithelial cadherin-expressing cells [56], suggesting that entosis resembles more of a cell invasion activity leading to cell-in-cell formation rather than an engulfment per se [47]. Indeed internalizing entotic cells exhibit blebbing that is reminiscent of invading cells that utilize an amoeboid mode of motility [49]. This model is consistent with the localization patterns of actin and myosin during the entosis process, as actin and myosin heavy and light chains accumulate specifically within internalizing cells, at the cell cortex opposite the E-cadherin junctional interface, and do not accumulate within engulfing cells, as would occur during phagocytosis [49, 56, 60]. RhoA activity, measured with a FRET-based biosensor, and ROCK I and II, as well as the RhoA-regulated actin polymerizing formin mDia1, also accumulate specifically within internalizing entotic cells at the cell cortex colocalizing with actomyosin [49, 56]. The combined activities of a Rho-GTPase activating protein (GAP), p190-RhoGAP, which is recruited to E-cadherin-mediated cell–cell junctions and inhibits RhoA, and a Rho-guanine exchange factor (GEF) protein PDZ-RhoGEF, which recruits to the distal cortex of invading cells and activates RhoA, establish a zone of actomyosin contraction that is polarized in a manner to promote entotic cell uptake [49, 56]. Altogether, entosis appears to be a non-canonical engulfment mechanism, distinct from phagocytosis, that drives the ingestion of viable cells into their neighbors. While entosis leads to the formation of cannibalistic or cell-in-cell structures (in essence ‘engulfments’), the formation of such structures does not appear to involve classical ‘engulfing’ activity but is more reminiscent of a cell invasion (Fig. 1).

### Entotic cell death

Cells engulfed by entosis (‘entotic’ cells) primarily die, although some can divide inside of their host cell vacuoles, and can also escape altogether, emerging unharmed and capable of subsequent rounds of cell division (Fig. 1) [47]. That some engulfed cells can be completely rescued from death suggests that entotic cells are not engaged in a cell-autonomous death program prior to their engulfment. Indeed, entotic cells do not expose the ‘eat-me’ signal phosphatidylserine, which is common in dying cells for activation of phagocyte receptors and clearance by phagocytosis [47].

While most entotic cells eventually die after they are engulfed [13, 47], cell death occurs in the absence of cleavage of caspase-3, a hallmark of apoptosis, and dying entotic cells do not exhibit morphological features of apoptosis such as nuclear condensation and fragmentation



**Fig. 1** Mechanisms of entosis and entotic cell death. *Top images:* Matrix detachment of adherent cells triggers entosis, involving formation of E-cadherin and  $\alpha$ -catenin-mediated cell junctions (green) between host (blue) and internalizing cells (red). RhoA and Rho kinase (ROCK) activity in internalizing cells leads to actomyosin accumulation at the cell cortex, which drives cell-in-cell formation, suggesting an active invasion-like process. Over time, most internalized cells undergo entotic cell death (*top right*), involving

lipitation of the autophagy protein LC3 onto the entotic vacuole (requiring VPS34 and LC3 lipitation machinery (e.g. Atg5)), followed by lysosome fusion and death of internalized cells. Alternatively, some entotic cells undergo apoptosis, particularly when inhibited for macroautophagy, and others are observed to divide inside of their hosts, or to escape and return to the culture (*bottom images*)

[47]. Entotic cell death is also not blocked by overexpression of the apoptosis inhibitor Bcl-2, altogether supporting a model where apoptosis does not play a significant role in entotic cell engulfment or death [13, 47]. Engulfed entotic cells do exhibit some features of Type II, or autophagic cell death, as they accumulate autophagosomes and have high rates of autophagy flux [13]. But autophagy in this context appears to function to support cell survival, as the inhibition of autophagy within internalized cells induces apoptosis. Because engulfed entotic cells are deprived of nutrients and growth factors from media in the host cell vacuole, it is likely that nutrient starvation induces autophagy, and in the absence of autophagy-dependent nutrient recycling, engulfed cells undergo apoptosis [13]. This is consistent also with the induction of apoptosis of engulfed entotic cells by treatment with the lysosome inhibitor concanamycin A, which inhibits nutrient recovery by the autophagy pathway [13, 47].

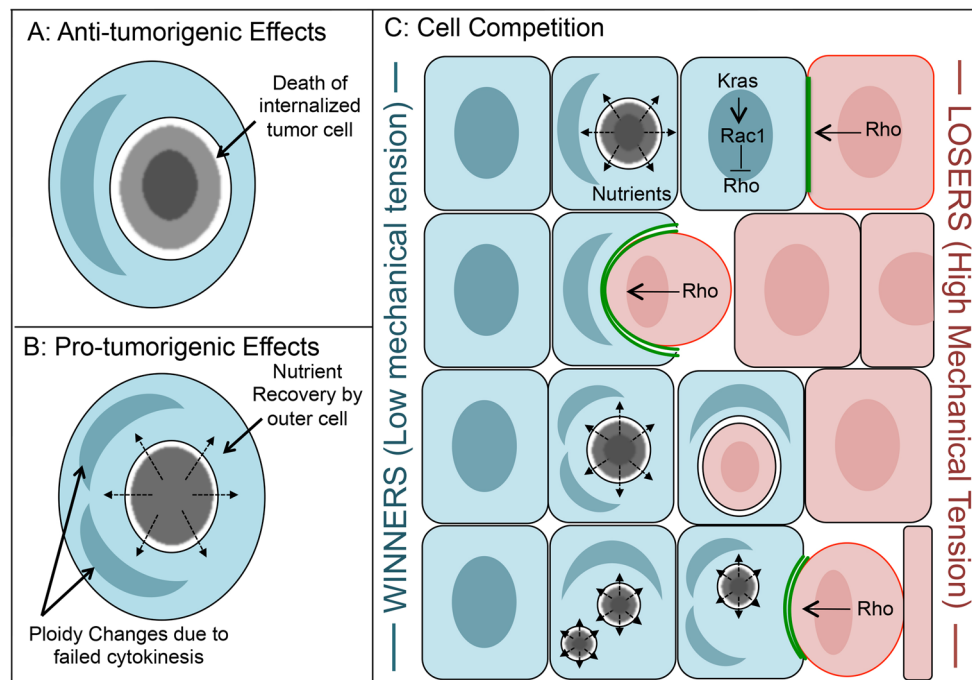
As autophagy activity is required for entotic cell survival, it was therefore a surprising finding that entotic cell death also requires autophagy pathway proteins [13]. But it is autophagy pathway proteins acting within the engulfing cells that are required, in a non-cell-autonomous manner, for the death of engulfed cells [13]. Autophagy proteins in this

context do not function in autophagosome formation, but instead to direct lipitation of the autophagy protein microtubule-associated protein 1A light chain 3 (LC3) onto the single-membrane vacuoles that harbor engulfed cells (Fig. 1) [13]. The core LC3 lipitation machinery that functions during autophagy (e.g. Atg5, Atg7), and the Beclin-Vps34 kinase, complex are required for LC3 lipitation in this context, but the autophagy pre-initiation kinase complex, composed of Ulk1/2 kinase and Fip200 and Atg13 adaptor proteins, is not required, genetically distinguishing entotic vacuole LC3 lipitation from canonical autophagy [13, 14]. The lipitation of LC3 onto entotic vacuoles appears to play a role to promote lysosome fusion, suggesting that entotic cell death is executed non-cell-autonomously by the autophagy pathway-dependent, lysosomal degradation of live engulfed cells (Fig. 1) [13, 14].

## Biological functions of entosis

### Entosis in cancer: pro- and anti-tumorigenic roles

Cell structures resembling those formed by entosis have been documented in a variety of human cancers, including



**Fig. 2** Consequences of entosis. **a** Potential anti-tumorigenic effects of entosis involve the killing of internalized tumor cells. **b** Potential pro-tumorigenic effects of entosis involve the scavenging of nutrients by host cells, as well as ploidy changes that result from failed cytokinesis. **c** Model of entosis promoting competition between neighboring cells. Differences in deformability between cells can induce entotic cell engulfment in a population, as cells with high mechanical tension (losers, *red*) internalize into cells with lower

mechanical tension (winners, *blue*) and undergo cell death. One pathway that can influence this competitive mechanism is the Kras pathway, where the activation of Rac1 by Kras reduces RhoA/ROCK-dependent actomyosin contractility and tension, rendering cells with Kras activation winners. Winner cells eliminate losers while becoming polyploid/aneuploid and also scavenging nutrients to support their own proliferation

breast, colon, liver, and pancreatic carcinoma, and others [21, 47, 54]. Similar to entotic structures in culture, engulfing cell structures from clinical breast tumor samples exhibit localization of the cell–cell adhesion protein  $\beta$ -catenin at the engulfing cell interface, and myosin light chain, phosphorylated at the ROCK I/II site (pMLC-S19), at the cortex of internalizing cells [47, 56]. And comparable to entotic cell structures, engulfed cell structures in human tumors sometimes involve complex, multiple cell relationships, such as ‘cell-in-cell-in-cell’ configurations [47]. Thus, while live tumor cell engulfments in human tumors have traditionally been referred to as ‘cell cannibalism’, and could form by several mechanisms, it is likely that at least in some tumors these cell structures form by entosis.

Entosis can limit the transformed growth of tumor cells cultured in soft agar, consistent with a potential role in tumor suppression. The treatment of entosis-competent breast tumor cells (MCF7) with an inhibitor of ROCKI/II (Y27632), significantly increases colony formation, and inhibits the appearance of entotic cell structures that are frequently observed under control conditions [47]. Remarkably, in soft agar entosis occurs between daughter

cells arising from the first divisions that attempt to establish colony growth [47, 56]. The inhibition of entotic cell death by autophagy protein knockdown also increases the transformed growth of cells exhibiting high rates of entosis, suggesting that entosis may reduce transformed growth in part by inducing cell death (Fig. 2a) [13]. For breast tumor cells that do not express E-cadherin, the enforced expression of epithelial E- or P-cadherin is sufficient to induce entosis and entotic cell death in soft agar, and to suppress colony formation [56]. The treatment of such tumor cells with Y27632 blocks entosis and restores transformed growth, again consistent with a potential anti-tumorigenic role [56]. Given that entosis is triggered by matrix detachment, which also triggers the apoptotic program of anoikis that is evaded during metastasis [18], it is likely entosis acts as another tumor suppressive mechanism to prevent anchorage-independent growth (Fig. 2a).

But contrary to tumor suppression, entosis has also been shown to lead to the induction of aneuploidy, which is known to promote tumor progression [25, 27]. Entotic cells disrupt the division of engulfing cells by blocking the cleavage furrow, leading to frequent cytokinesis failure [25]. The resulting binucleate cells propagate gross

aneuploidy [25], and are known to promote breast tumorigenesis [15], suggesting that while entosis leads to the death of engulfed cells, it may also promote the acquisition of tumorigenic properties by engulfing cells (Fig. 2b). Consistent with this, it was also recently reported that engulfing cells recover nutrients from digested entotic cells, and this form of nutrient scavenging can support cell proliferation and survival under conditions of starvation [26]. Therefore entosis could promote tumor progression by participating in multiple pro-tumor activities, by supporting the survival and proliferation of cells that also propagate gross aneuploidy (Fig. 2b). The reported correlations between homotypic cell-in-cell structures like those formed by entosis and high tumor grade [1, 25] as well as poor patient outcome [54] may indeed support the idea that this cell behavior promotes tumor progression.

### Entosis in cancer: cell competition

How can these opposing potential roles of entosis in cancer be reconciled? It is predicted that the opposing influences of entosis could promote competition between neighboring cells, where engulfed cells (losers), die at the expense of engulfing cells (winners), that benefit by acquiring nutrients (Fig. 2c). That entosis is mediated by E-cadherin uniquely poises this mechanism to allow neighboring cells to compete, as the junctions that neighboring cells form can directly mediate engulfment events that kill losers. Conceivably, a difference in Rho-pathway or actomyosin activity between neighbors could manifest as the engulfment and death of losers after the establishment of cell junctions (Fig. 2c).

Consistent with this hypothesis, we recently reported that entosis promotes competition between cells that have compatible cadherin expression and a mechanical differential, where winners have lower mechanical tension, or are more deformable (consistent with lower actomyosin), than losers [22, 57]. The co-culture of winner and loser cells, combined with repetitive entosis induction, progressively leads to the elimination of losers in a ROCK-dependent manner [57]. Winner cells also engulf losers in vivo in mixed xenograft tumors [57]. These findings suggest that human tumors with high cell-to-cell variability in mechanical tension are primed to engage in competition between individual cells by this mechanism. Intriguingly, the mechanical deformability of tumor cells is known to increase during tumor progression [65]. Entosis could contribute to this selection by promoting a form of cell competition.

Mechanical heterogeneity in tumors may be caused by genetic heterogeneity in pathways that control actomyosin. We have found that one pathway that controls whether cells are winners or losers during entosis is the Kras

pathway, which signals through the Rac1-GTPase to inhibit Rho activity and thereby reduce tension by lowering actomyosin contractility [57]. Activation of Kras by expression of a constitutive-active mutant, or activation of Rac1, is sufficient to render cells winners over controls [57]. Moreover, Rac1 expression is required for Kras-induced winner cell status, and the loser status of Rac1-knockdown cells can be reversed by knocking-down the expression of ROCK I/II, which reduces myosin contraction and inhibits entotic cell uptake [57]. These results have several important implications. First, they suggest that tumor cells, for example with activation of Kras, could engulf non-tumor cells, which, while not yet demonstrated in vivo, could play a role in tumor cell dissemination through a tissue. Second, these results demonstrate that Rac1-depleted cells can be rendered winners, or engulferers, by the simultaneous depletion of ROCK I/II. As Rac1 is a key mediator of phagocytosis by controlling actin dynamics in engulfing cells, these data further distinguish the entosis and phagocytosis mechanisms. Third, these findings highlight that oncogene expression can induce winner status during entosis by downregulating contractile myosin, which lowers the setpoint of mechanical tension. Therefore, while the entosis mechanism overall may resemble a cell invasion-like activity on the part of internalizing cells, the gain of function that can initiate this process can occur within engulfing cells, as a lower setpoint of actomyosin activity can induce the invasion of control cells with higher contractility. In this manner, oncogene-expressing cells can induce control cells to drive their own uptake (Fig. 2). Together these findings demonstrate that entosis can promote a form of cell competition, where genetically distinguished winner cells can engulf, kill, and benefit from the death of losers.

Competition between individual cells is an ancient strategy that drives selection and fitness within cell populations. The concept of ‘cell competition’ originally derives from *Drosophila* genetics, where it was first discovered that mosaic tissues in development exhibit distinct patterns of cell death, where cells of ‘loser’ genotype (first noted for ribosome gene deletion [41]), undergo death at the expense of cells with ‘winner’ genotype (in this case wild-type cells). Cells overexpressing certain oncogenes (e.g. Myc) actually recognize wild-type cells as losers, and these ‘supercompetitor’ cells overpopulate tissues at the expense of wild-type cells that undergo cell death [7, 42]. The individual cells in mammalian tissues may similarly compete, as Myc-overexpressing cells out-compete wild-type cells in embryonic stem cell cultures and in vivo in the tissues of chimeric mice [5, 58]. Interestingly cell engulfment was reported to be required for winner cells to kill losers during cell competition in mosaic tissues in *Drosophila* [29], and some engulfments involving apparently viable target cells were



identified during cell competition in mammalian stem cell cultures [5]. But cell engulfment in these contexts is thought to primarily clear dead cells that have undergone apoptosis [36]. Whether an entosis-like mechanism, where engulfment causes cell death, could contribute to competition between cells within normal tissues as occurs in human tumors is an important topic for future study.

### Physiological role of entosis during embryo implantation

For implantation of the mouse embryo, epithelial cells in the lumen of the uterus have to be eliminated to allow the blastocyst to implant by contacting the stroma underneath. It was shown recently that uterine epithelial cells undergo entosis into trophoblast cells of the blastocyst, to allow for implantation to occur [30]. Cell-in-cell structures formed between uterine epithelial and trophoblast cells were imaged *in vivo* during implantation, and implantation failed in animals that were inhibited for ROCK activity. Moreover, entotic engulfments with uterine epithelial cells internalized into trophoblast cells occurred in mixed cultures, where immunostaining clearly localized the cell junction protein  $\beta$ -catenin at engulfment interfaces [30]. Interestingly the death of the internalized uterine epithelial cells in this context also did not involve the cleavage of caspase-3, suggesting that a non-apoptotic death mechanism resembling entotic cell death could also be occurring in this context. Together these data provide the first evidence that entosis may control a critical stage of mammalian development, embryo implantation.

### Conclusion

Here we have discussed the molecular mechanisms controlling cannibalistic cell behavior through one particular program, entosis. We emphasize that while entosis is one mechanism whereby viable cells can find themselves being cannibalized, it is not the only one. It is important to keep in mind in this field that the term ‘cannibalism’, while used to describe a specific molecular mechanism in some reports, is more commonly used to indicate simply the appearance of viable engulfed cells in tissues or in culture. While the term ‘cannibalism’ itself is suggestive of an active engulfment, several distinct mechanisms like entosis and suicidal emperipolesis [2], where internalizing cells exert major control over their own uptake, also result in the formation of cell structures with identical appearance. Therefore mechanistic studies in contexts where viable cell engulfments are observed are needed before conclusions can be made about how such cells become cannibalized. For entosis, while we [47] and others [49] have described

this process as a cell invasion-like activity, this form of cannibalism can also be controlled by engulfing cells with altered setpoints of mechanical tension. One clear example discussed herein is cells expressing oncogenic Kras or activated Rac1 that preferentially cannibalize neighboring cells with higher setpoints of mechanical tension, through entosis [57]. While internalizing cells may do a large part of the ‘work’ associated with these entotic events, clearly oncogene expression within host cells can directly influence the ability of these cells to act as the ‘engulfers’, or ‘cannibals’, by this mechanism.

In future studies of entosis it will be important to identify additional inducers of this mechanism. To date, the only clear inducer of entosis in cell populations is detachment of cells from extracellular matrix, which, while likely relevant to induction of this mechanism in some cancers [47], may not be the only inducer of this process. Imbalances in mechanical tension [60], or perhaps differences in cell size [30], between neighboring cells may also be involved in inducing entosis. Whether other stresses to cell populations in addition to matrix detachment could contribute to the manifestation of such heterogeneities within a population is an important topic for future studies. We note here that several examples of ancestral cannibalistic cell behavior share nutrient starvation as a common inducer, as also pointed out by others [11]. As one clear consequence of entosis is the scavenging of nutrients by engulfing cells [26], it will be of importance to determine if forms of nutrient deprivation can also induce this process.

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